FEBS Letters 582 (2008) 203–209

Similar toxicity of the oligomeric molten globule state and the prefibrillar oligomers

Slavko Čeru, Eva Žerovnik*

Department of Biochemistry, Molecular and Structural Biology, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

Received 25 October 2007; revised 28 November 2007; accepted 3 December 2007

Available online 17 December 2007

Edited by Jesus Avila

Abstract **We report that a mutant of human stefin B is in a molten globule conformation. It has all the spectroscopic characteristics for such a state. We also demonstrate that the molten globule is oligomeric, eluting on SEC within a similar MW range than the higher order oligomers of the wild type protein, which is confirmed by DLS and AFM. Both, the higher oligomers and the molten globule state bind ANS, implying a high degree of hydrophobic patches exposure and partial opening of the structure. Finally, we demonstrate that the oligomeric molten globule is as toxic as the prefibrillar aggregates obtained at acid pH or the higher order oligomers prepared at neutral pH.**

Keywords: Amyloid fibril; Toxic oligomer; Molten globule; Cystatin B; Stefin B; Prefibrillar oligomer; Protein folding; Protein aggregation

1. Introduction

Human stefin B (also termed cystatin B) is a member of the family of cystatins, the cysteine protease inhibitors [1], a family in the IH clan of proteases as classified by MEROPS [2]. Human cystatin C is a well-known amyloidogenic protein, causing amyloid pathology with mutant L68Q observed in hereditary cystatin C amyloid angiopathy (HCCAA) [3]. Mutations in the human cystatin B gene cause EPM1, also known as Unverricht Lundborg disease [4]. On the basis of their in vitro behaviour [5], a hypothesis was formulated that some of the EPM1 mutants, those affecting exonic parts of the gene, may aggregate in the cell and thus cause some of the symptoms [6]. However, the present study is not meant to clarify possible physiological function(s) of stefin B oligomers. It rather aims to contribute to understanding of the common properties of amyloidogenic proteins.

Protein folding to an alternative, amyloid precursor state leads to protein aggregation [7–9]. Amyloid aggregates are observed in various amyloidoses, among them a prominent class of neurodegenerative diseases [10]. In Alzheimer's disease (AD) amyloid plaques, made predominantly of the cleavage product amyloid-β (A-β), deposit in the brain, whereas the paired helical filaments made of hyper-phosphorylated protein Tau accumulate intraneuronally [11]. In Huntington's disease, huntingtin's aggregates cause slowing of the axonal transport along microtubules [12,13] and in Parkinson's disease Lewy bodies are found in the affected neurons [11]. Familial cases and animal models demonstrate that certain protein mutants which lead to more heavy aggregation in vitro also lead to early outburst of the disease [14,15], therefore, they are likely the primary cause [11].

More and more evidence is being gathered that prefibrillar oligomers/aggregates appearing with amyloidogenic proteins in vitro [16,17] and in vivo [18] are cytotoxic. Toxicity is not restricted to pathological proteins, it rather stems from some common structural (conformational) characteristics of the prefibrillar oligomers [19,20]. An anti-amyloid antibody raised against one prefibrillar oligomer was shown to bind oligomers of a number of other amyloidogenic proteins [21]. Therefore, even a non-pathological protein, such as stefin B, makes a good model system to study toxicity. Human stefin B is prone to form amyloid fibrils under mildly acidic conditions and it has served as an appropriate model protein in our in vitro studies of amyloid fibril formation [22–26].

In the era of the "protein folding problem" in the 1990s the molten globule state was proposed as a general folding intermediate [27,28]. The main characteristics of the molten globule are high amount of secondary structure (not necessarily native) and less rigid tertiary structure. This reflects in absent or low near UV CD, low dispersion and broader peaks in NMR and high propensity to bind ANS [28,29]. It seems likely that this intermediate state, which has broken tertiary structure contacts and exposed hydrophobic surfaces, would play a role at the cross-road between folding and amyloid-fibril formation.

It has been shown that proline 74 in the P79S mutant of the stefin B iso-form harbouring a Y at site 31 and adopting a tetrameric state, is in a *cis* conformation [30]. We also know from previous studies that mutation P74S on the backbone of the Y31 iso-form (from now on P74S(Y31) mutant) leads to a molten globule state [31]. For this work, we characterised further this mutant by using CD, ANS fluorescence and 1D NMR, confirming its molten globule characteristics. In order to determine its oligomeric state, dimensions and morphology, SEC, DLS and AFM were used.

In our previous studies, using our model system: human stefin B, we characterised the morphology, toxicity and membrane interaction of prefibrillar oligomers/aggregates obtained at pH values of 3 and 5 [22–26,32]. In this study, we compare conformation of the higher order oligomers obtained at pH 7, which were shown of similar toxicity than the prefibrillar

*Corresponding author. Fax: +386 1 477 3984.
E-mail address: eva.zerovnik@ijs.si (E. Žerovnik).

Abbreviations: AFM, atomic force microscopy; DLS, dynamic light scattering; SEC, size-exclusion chromatography

0014-5793/$32.00
doi:10.1016/j.febslet.2007.12.002

oligomers/aggregates of pH 3 and 5 [32], to that of the oligomeric molten globule state, which also proves toxic.

2. Materials and methods

2.1. Chemicals

CellTiter 96® AQueous One Solution Cell Proliferation Assay (a) was from Promega. This is a reagent for colorimetric determination of the number of viable cells in proliferation or cytotoxicity assays. It contains tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and electron coupling reagent (phenazine ethosulfate; PES). Fluorogenic substrate Ac-DEVD-AFC for determining caspase-3-like activity was from Bachem. Bradford assay was from Bio-Rad 1, anilino-naphthalene 8-sulfonate (ANS) was purchased at SERVA, Heidelberg, GE. Thioflavin T (ThT) was from Aldrich, Milwaukee, WI, USA, and 2,2,2-trifluoroethanol (TFE), >99% pure was from Fluka, Buchs, CH. All other chemicals were of analytical grade. Double distilled water was used and solvents were filtered through 0.22 μm filters.

2.2. Protein isolation

The recombinant stefin B, the usual iso-form with tyrosine at site 31 was prepared as described [33,34]. Purification steps consisted of CM-papain Sepharose affinity chromatography and gel-filtration on Sephacryl-200 (Amersham-Pharmacia, Uppsalla, Sweden). The P74S(Y31) mutant, which is inactive, was prepared by two-gel filtration steps. Protein concentration was evaluated using an extinction coefficient of 0.48 ml mg^{-1} at 280 nm.

2.3. Preparation of the prefibrillar aggregates at pH 3

Preparation procedures and buffers were exactly the same as those described [24,26]. Briefly, proteins were incubated in in 0.015 M glycine of pH 3.3 (0.26 M sodium sulfate) for 24–48 h, at room temperature.

2.4. Size exclusion chromatography (SEC)

The higher order oligomers StBpH7 and the molten globule state P74S(Y31) were analyzed by the size exclusion chromatography (SEC) using Superdex 75 and Superdex 200 (Amersham-Pharmacia Biotech) columns on an AKTA FPLC system (Amersham-Pharmacia Biotech). Before injection, all samples were filtered through a 0.2 μm filter (Sartorius Minisart). The mobile phase was 0.01 M phosphate/0.15 M NaCl at pH 7, the flow rate was set at 0.5 ml/min, and the elution peaks were detected by absorbance at 280 nm.

2.5. MTS reduction assay

Cytotoxicity was determined by the MTS reduction assay. SH-SY5Y cells were plated onto 96-well plates at a density of 10 000 cells per well in 100 μl fresh medium. To probe cellular toxicity, 20 μl of concentrated (120 μM) prefibrillar aggregates of pH 3 (and neutralized), oligomers separated at pH 7 and the molten globule P74S(Y31) at pH 7 were added to 96-well plates. Buffer alone, non-amyloidogenic stefin A, and staurosporine were added to separate wells as controls. After an overnight incubation at 37 °C in a 5% CO_2 humidified environment, 20 μl of MTS was added to each well and the plates were incubated for an additional 2–3 h. After solubilization, the absorbance of the formazan product was measured at 490 nm using an automatic plate reader.

2.6. Caspase-3-like activity

Caspase-3-like activity was measured by the DEVD (Bachem) assay, in which Ac-DEVD-AFC is a fluorogenic substrate cleaved by caspase-3 but also by several other caspases. SH-SY5Y cells were plated onto 12-well plates and incubated to 80–90% confluent density. Two hundred microliter of concentrated prefibrillar aggregates of pH 3 (StBpH3) or P74S(Y31) mutant was added to 800 μl of complete cell medium. After treatment, aliquots of cell lysates (50 μg of protein as determined by Bradford assay) of untreated and treated cells were used to determine the caspase activity.

2.7. CD spectroscopy

CD spectra were measured using an Aviv model 62ADS CD spectropolarimeter equipped with a thermoelectric sample holder for temperature control in the cell. To record far- and near-UV CD spectra, bandwidths were set at 1 nm and 0.5 nm for 0.1 cm and 1 cm cells, respectively. Data in the far-UV were collected every 1 nm and in the near-UV every 0.5 nm. Averaging times were 5 s and 3 s for the far- and near-UV, respectively. Temperature was maintained at 25 °C throughout. Protein concentration of P74S(Y31) was 1.6 mg/ml (145 μM) for the near UV and 0.33 mg/ml (30 μM) for the far UV. Concentrations of other variants were the same in molar terms.

2.8. ANS fluorescence measurement

Fluorescence was measured using a Perkin–Elmer model LS 50 B luminescence spectrometer. Excitation was at 370 nm, emission spectra were recorded from 400 to 600 nm using a 0.5 cm cell. ANS concentration in the buffer for dilution (0.01 M phosphate, 0.15 M NaCl, pH 7) was 1.5 mM. Proteins: P74S(Y31) stefin B (the oligomeric molten globule state) and the isolated oligomers of the wild type, initially at 57 μM and 75 μM, respectively, were diluted 1:3, to the final ANS concentration of 1 mM. Resulting protein concentrations were: 19 μM for the P74S(Y31) and 25 μM for the w.t. oligomers. Protein concentration of P74S(Y31) was also varied from 12 μM to 25 μM at the same final ANS concentration, which represented ratios ANS:protein from 80 to 42.

2.9. Atomic force microscopy (AFM)

The P74S(Y31) mutant was unfrozen from a batch at 120 μM and filtered through 0.2 μm filter (Sartorius Minisart). After a 10-fold dilution, a 20 μl of protein sample was mounted onto a freshly cleaved mica surface (1 cm^2) incubated for 5 min and gently washed with the solvent or deionized water. Excess solvent was removed with a stream of nitrogen. Images were obtained with a Nanoscope III Multimode scanning probe microscope (Digital Instruments) operated in tapping mode.

In more detail: scan rate was 1 Hz and tip oscillation rate was 170–180 kHz. Silicon doped cantilevers were from nanosensors (nodel NCLR). Pixel resolution was 512 × 512. Scanner E was from Digital Instruments (12 × 12 μm) and approximate tip diameter was 10–15 nm. Polynomial line flattening was used after obtaining raw image data.

2.10. Dynamic light scattering (DLS)

DLS measurements were performed at room temperature using PDDLS/BatchPlus System (Precision Detectors). All buffers used were filtered using Centriprep YM 50 filters (Amicon). The sample of the oligomeric molten globule P74S(Y31) was taken from the same batch as for AFM and was filtered through 0.2 μm filter (Sartorius Minisart). Higher order oligomers were collected from SEC and used as such. Before measurements all samples were briefly centrifuged. A 400 μl sample was inserted in a quartz cuvette. A run time of 1 s and sampling time of 5 μs were set. Typically, at least 50 accumulated correlation functions were used to to calculate the diameter of scattering particles using the PrecisionDeconvolve software provided by the manufacturer. Scattering peaks of <0.1 nm radius and more than 1000 nm were ignored.

3. Results

3.1. Oligomeric state, size and toxicity of the molten globule

By using size-exclusion chromatography (SEC) Superdex 75 column we isolated higher order oligomers of stefin B wild type, higher than the tetramers. When these were applied to Superdex 200 column they eluted as a broad peak covering 66–350 kDa, corresponding to 6-mers to 32-mers, with predominant peak apparently at the 12-mers (Fig. 1A). When the molten globule P74S(Y31) was applied on the same column, the elution was similar to the higher oligomers of the wild type (Fig. 1A).

To characterise the oligomers further dynamic light scattering (DLS) was used. In a previous DLS study, hydrodynamic radii of the isolated dimer and tetramer were determined as 2.9 nm and 3.5 nm, respectively [30]. In Fig. 1B, DLS measure-

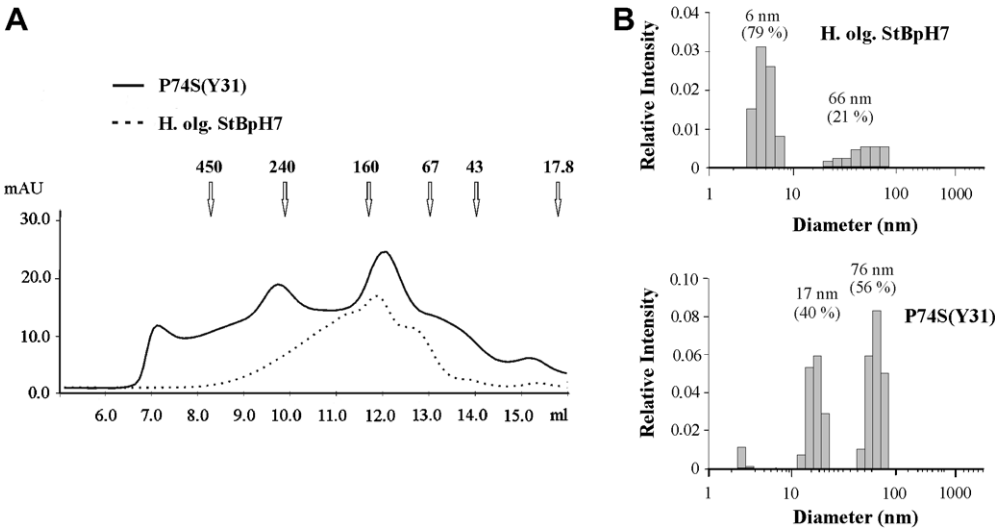

Fig. 1. (A) Size exclusion chromatography – SEC on Superdex 200 column of the higher order oligomers of the wild type stefin B (StBpH7) and of the molten globule mutant P74S(Y31). The mobile phase was phosphate 0.01 M sodium phosphate buffer, 0.15 M NaCl, pH 7. (B) Dynamic light scatteing – DLS. Top – SEC isolated higher oligomers of the wild type stefin B (StBpH7) showing bimodal distribution of particle diameters. Bottom – the oligomeric molten globule P74S(Y31); similarly showing a bimodal distribution.

ment of the molten globule sample (lower panel), in comparison to the isolated higher oligomers (higher panel) is shown. DLS measurement of the higher oligomers resulted in a bimodal distribution with approximately 79% of oligomers displaying average diameters ranging from 4.8 to 8.3 nm, while the average diameters of the other 21% ranged from 30 to 88 nm (see, H.olg.StBpH7 in Fig. 1B, top). Slightly different distribution was observed for the molten globule sample with approximately 40% of oligomers displaying average diameters ranging from 14 to 24 nm, while the average diameters of the other 56% ranged from 50 to 88 nm (see, P74S(Y31) in Fig. 1B, bottom). There was also a trace of particles smaller than 5 nm.

The dimensions obtained by DLS for the oligomeric molten globule P74S(Y31) are consistent with the dimensions determined for the pH 3 prefibrillar aggregate [45], which initially also is in a molten globule conformation [24,35].

By AFM (Fig. 2B and unshown data) the molten globule P74S(Y31) appeared as globular (elliptoid) oligomers with

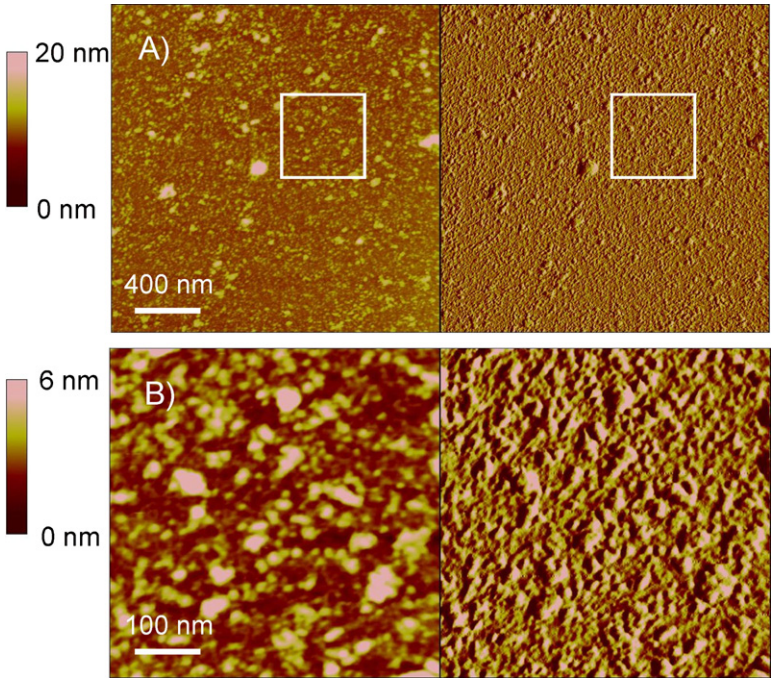

Fig. 2. Atomic force microscopy – AFM images of the molten globule sample. The sample of the P74S(Y31) mutant was unfrozen from a batch isolated by gel filtration on Sephacryl-200. Dilution to around 12 μM concentration was performed by using double distilled Millipore water. The images were taken in tapping mode with a Nanoscope III Multimode scanning probe microscope. For each image: height – left, amplitude – right. (A) A lower magnification to 2 μm. (B) An insert at higher magnification.

lateral dimensions from 10 to 15 nm and a height of 1–2 nm, similarly to those of the prefibrillar aggregate of pH 3, where an average value of 12.5 was determined before [26]. This fits well to the diameter observed by DLS for the major part of oligomers (Fig. 1B, bottom). However, in addition to these, one can detect particles of up to 100 nm lateral dimensions and heights of 20 nm by AFM (Fig. 2A), again consistent with a range of diameters seen by DLS (Fig. 1B, bottom).

In Fig. 3A and B are shown the results of the MTS and caspase-3-like activity assays. To measure cell viability an MTS assay was performed after incubation of SH-SY5Y cells for 16 h at 37 °C with either acid-induced prefibrillar aggregates or SEC separated higher-order oligomers diluted into the medium of pH 7.3. There was no significant difference in cytotoxicity between acid-induced prefibrillar aggregates (neutralized from pH 3 before dissolving into the medium), isolated higher order oligomers, and the P74S(Y31) mutant, this latter adopting a molten globule conformation. The effect observed with 44 μM oligomer or aggregate concentration was modest in all cases, with 20–30% lower formazan production (Fig. 3A). The pH 3 prefibrillar aggregates also caused a significant enhancement of DEVD activity, a measure for caspase-3-like activity. Similar effect was observed for the molten globule P74S(Y31) oligomers (Fig. 3B).

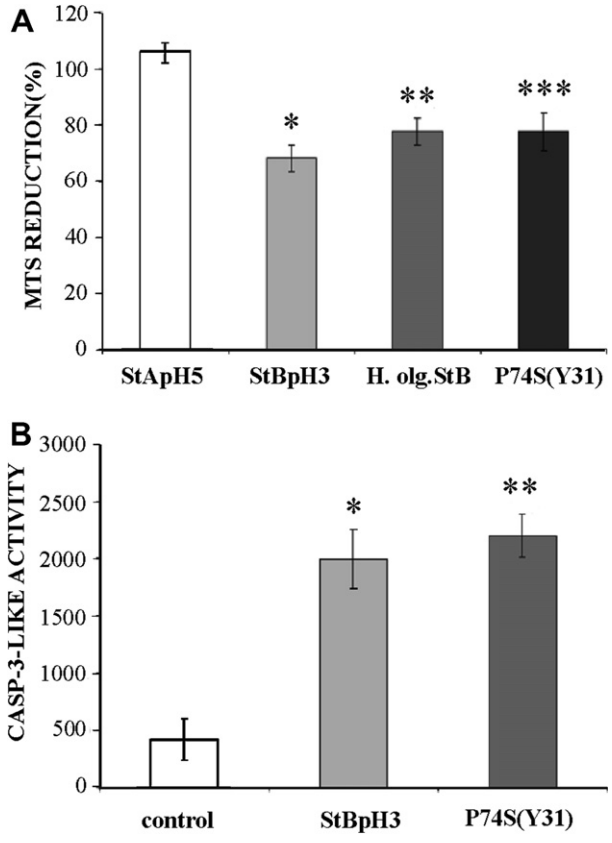

Fig. 3. Cell viability assays. (A) MTS reduction assay was performed after cells were incubated in the cell medium with dissolved prefibrillar aggregates of pH 3 (first neutralized), SEC isolated higher order oligomers and the molten globule mutant P74S(Y31). (B) DEVD assay, which measures caspase-3-like activity, was performed for the pH 3 prefibrillar aggregates (after being neutralized) and for the oligomeric molten globule P74S(Y31).

3.2. Structural/conformational characteristics of the toxic oligomers

It is very difficult to obtain structural data (using crystallography or heteronucelar NMR) of the prefibrillar oligomers and assemblies, due to inhomogeneity and mobility of such samples. Stefin B tetramer structure in crystal and in solution has been determined by Jenko Kokalj et al. [30] and the structures of the domain-swapped dimers of cystatins C and stefin A (i.e., cystatin A) by Jaskolski et al., Staniforth et al. [36,37], respectively. However, the lower oligomers including the tetramer, do not show cytotoxicity [45].

To probe general structural characteristics, such as the secondary structure and surface exposure, CD and ANS fluorescence emission spectra of the higher-order oligomers of the wt and the oligomeric molten globule P74S(Y31) were determined. CD spectra of the prefibrillar aggregates accumulating in the lag phase at pH 5 and 3 were reported before [24] and were reminiscent of a native-like state and "structured molten globule", respectively [35].

CD spectra of the P74S(Y31) mutant in the far UV (Fig. 4A) and near UV (Fig. 4B) are first compared to the corresponding CD spectra of the parent stefin B (stB-Y31) and its P79S(Y31) mutant, the latter forming a tetramer [30].

In Fig. 4C are shown the far UV CD spectra of separated oligomers: monomers, tetramers and the higher oligomers, as well as of the P74S(Y31) mutant, which shares a similar oligomeric state as the higher oligomers (Fig. 1A and B). In Fig. 4D are shown ANS fluorescence spectra upon binding of the dye to the separated monomers, dimers, tetramers, higher oligomers and the oligomeric molten globule P74S(Y31).

ANS dye was used to determine exposed hydrophobic patches [29]. Molten globule state is known to bind ANS extensively [28,29]. As seen from Fig. 4D, the isolated monomer and dimer of the wild type protein do not bind ANS, tetramer binds a little, whereas the higher oligomers bind quite an amount. The oligomeric molten globule shows the highest ANS binding (Fig. 4D). It is of note that the far UV CD spectra of the higher oligomers of the wild-type do not resemble the spectrum of the molten globule [35], they can be described as "native-like". The peak at 225 nm, which increases with oligomerization may be characteristic of the tyrosine contribution towards the far UV CD [38]. It is not surprising that tyrosine environment would change upon oligomerization.

1D NMR spectra of the P74S(Y31) mutant were also recorded (Fig. 4E) and are consistent with its molten globule character, i.e., low dispersion of chemical shifts and broader peaks than for completely denatured proteins, also characteristic of the 1D NMR of stefin B at pH 3.

4. Discussion

It seems logical and was in certain cases shown experimentally, that partially unfolded states and folding intermediates lye at the cross-road between folding and amyloid-fibrillation [7–9]. We are using human stefin B (cystatin B) as a model amyloidogenic protein for quite some time [5,22–26,30,32], and have shown that it shares most properties with other amyloidogenic systems. We recently have encountered on a mutation P74S(Y31), which causes the protein to adopt a "structured" molten globule state already at neutral pH. In

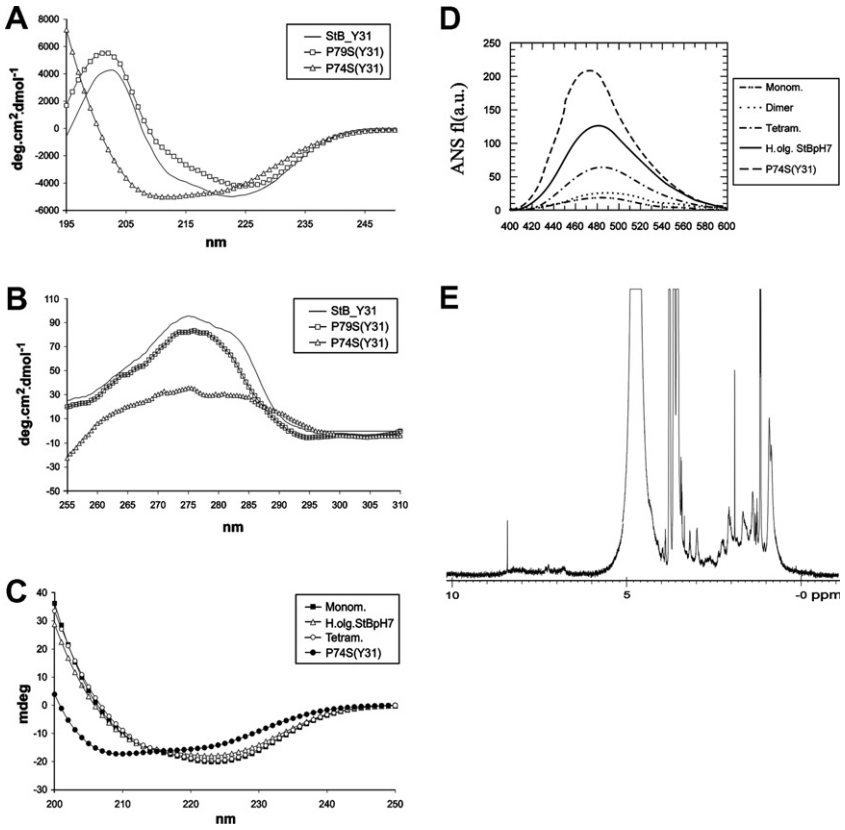

Fig. 4. Conformational characterization of the molten globule. (A) Far UV CD spectra and (B) near UV CD spectra of the parent stefin B (stB-Y31), the proline mutant P79S(Y31), which is tetrameric, and the P74S(Y31) mutant, which is molten globule and oligomeric. (C) Far UV CD spectra: comparison of the spectrum of isolated monomer, dimer, tetramer and higher order oligomers of the wild type stefin B (stB-wt) in comparison to the oligomeric molten globule P74S(Y31). (D) ANS binding to isolated monomers and oligomers of the wild type stefin B in comparison to the mutant P74S(Y31), which is oligomeric molten globule. (E) ^{1}H NMR spectrum of the oligomeric molten globule P74S(Y31). Protein concentration was around 1 mM, buffer was 0.005 M sodium phosphate, 0.06 M NaCl, pH 6.3. The spectrum was taken using Brucker AMX-500 machine.

this work, we show that the molten globule is oligomeric and, what is very interesting by our opinion, toxic. To obtain an answer to the question: ''What is the conformation of toxic oligomers of amyloidogenic proteins?'', we measured general spectroscopic properties of the oligomeric molten globule state in comparison to the higher oligomers (higher than the tetramer) of the wild type protein.

In our studies of amyloid fibril formation of stefin B we have observed that the protein can start fibrillation directly from the molten globule state at pH 3 (after a lag phase of 48 h at r.t.), or it goes transiently through such a state in the early growth phase at pH 5, 10% TFE [22,24]. We now came across an observation that not only external denaturation can induce a molten globule state but this also can happen upon suitable mutation.

The induction of the molten globule intermediate at equilibrium is dependant on the protein's stability. The 31Y iso-form of stefin B (our usual variant used in most folding studies so far) is prone to undergo such a transition rather easily during denaturation by chemical denaturants [39], acidic pH and temperature [35]. The molten globule state is also attained by the P74S mutant of the Y31 iso-form of the protein (termed P74S(Y31)), already at neutral pH. Interestingly, the mutation P74S in the wild type protein does not change conformation of the folded protein (E.Ž., unpublished).

In this work, we compare conformational characteristics of the molten globule state to the isolated higher oligomers of the wild type protein. We also show that both are toxic to cells. However, to our surprise the molten globule mutant P74S(Y31) was at least equally toxic than the higher oligomers of the wild type (Fig. 3A). This became more understandable when the mutant was applied to SEC. The elution of the molten globular P74S(Y31) mutant on SEC was even broader than the higher order oligomers (Fig 1A), implying its oligomeric nature.

One needs to be cautious when comparing native and molten globule states on the basis of their elution by SEC. It is known that molten globular state can show up to 20% increased Stokes radius on gel filtration [27]. It may sometimes appear as a dimer, even though it could be a somewhat open monomer. Therefore, it is important to use an alternative method. We have chosen DLS (Fig. 1B) and confirmed that the molten globule is indeed oligomeric and prone to aggregate.

By AFM (Fig. 2B) the lateral dimension (diameter $\geqslant$ 12 nm) of the basic oligomeric unit in the molten globule P74S(Y31) sample falls in between that of the isolated higher oligomers and of the prefibrillar aggregates at pH 3 [26,45]. However, there also are some bigger particles and aggregates present (Fig. 2A), which explains the spread in the diameters obtained by DLS.

From all the collected results, we can safely conclude that the molten globule of P74S(Y31) is oligomeric and that it is higher than the tetramer, which has a hydrodynamic radius of 3.5 nm [30]. It also can be concluded that the oligomeric

molten globule is highly prone to aggregate with no evidence for protofibrils or fibrils. DLS measurement (lower panel in Fig. 1B) and AFM (Fig. 2A) show particles with diameters of up to 100 nm. These do not seem as chains of ''globular oligomers'' but rather as an aggregate with heights of up to 20 nm as determined by AFM (Fig. 2A). In contrast, it was observed before that the ''globular oligomers'' making the pH 3 aggregate were prone to chain up and start growing into protofibrils, both, by AFM and DLS [45].

Even though of similar toxicity, there are differences in conformation between the oligomeric molten globule P74S(Y31) and the separated higher order oligomers of the wild type. This is reflected in the CD spectra and ANS binding. The CD spectra (Fig. 4C) of the higher order oligomers are still ''native-like'' whereas the ANS binding is increased (Fig. 4D). The molten globule state P74S(Y31), on the other side, has a characteristic far UV CD and very low near UV CD (see, Fig. 4A–C), binds ANS to the highest level (Fig. 4D) and shows broad resonances in 1D NMR with little dispersion (Fig. 4E).

At first glance, the toxicity of the separated higher oligomers and the molten globule state (Fig. 3A and B) seem to correlate with the ANS binding (Fig. 4D). On the basis of high ANS binding (Fig. 4D), which is the highest for P74S(Y31), one would expect even higher toxicity for the molten globule state. This is not observed within experimental errors (Fig. 3A). However, one cannot say with certainty if membrane components themselves do not influence the initial ''native-like'' conformation of higher oligomers to change into molten globular prior to binding. Role of the molten globule conformation as the membrane binding conformation was suggested by Ptitsyn and co-workers [40]. The questions of how ''amyloid toxins'' cross lipid-bilayers and if that resembles PFT (pore forming toxins) remain to be seen.

It is to be mentioned that molten globule conformation recently was found in prion oligomerization [41]. Our study suggests that this could be a more common and even toxic conformation of amyloidogenic proteins.

5. Conclusions

1. We believe that our finding that a mutant of stefin B, which adopts an oligomeric molten globule state, is toxic, and that its toxicity is similar to that of the isolated higher-order oligomers, is significant. It sheds some light on the question: ''What is the conformation of toxic oligomers of amyloidogenic proteins?'' Part of the answer may be: ''A more open conformation, at least partially unfolded or molten globule.''
2. The oligomeric molten globule accumulates in the form of prefibrillar oligomers already at neutral pH and does not proceed into amyloid fibrils. Thus, alternative folding from the molten globule might be needed for fibril formation.
3. In view of the hypothesis that toxicity of amyloidogenic proteins occurs via membrane permeation [42], our results indicate that conformational change to a molten globule state is sufficient and very likely a prerequisite for membrane interactions and toxicity.
4. The same conformational change may happen to native-like oligomers when they encounter membranes or, at the onset of fibril formation. We have observed a temporarily increased CD at 222 and 208 nm (more α-helix) under fibril promoting conditions [24]. Temporarily increased helicity was reported by some others [43] and by theoretical calculations [44].

Acknowledgements: The authors thank Miha Škarabot (JSI, Ljubljana) for performing AFM imaging on Nanoscope III Multimode scanning probe microscope (Digital Instruments) and Simona Golič Grdadolnik (NIC, Ljubljana) for recording the 1D NMR spectra. We acknowledge discussions with Saša Jenko Kokalj (JSI, Ljubljana). This work was funded by Grant OB14P04SK from the Ministry of Higher Education, Science and Technology of the Republic Slovenia by the Slovenian Research Agency (ARRS).

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