ELSEVIER

Biochimica et Biophysica Acta 1594 (2002) 1–5

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Rapid report

Human stefin B readily forms amyloid fibrils in vitro

Eva Žerovnik [a,*], Maruša Pompe-Novak [b], Miha Škarabot [c], Maja Ravnikar [b], Igor Muševič [c], Vito Turk [a]

[a] *Department of Biochemistry and Molecular Biology, Institute Jožef Stefan, Jamova 39, 1000 Ljubljana, Slovenia*
[b] *Department of Plant Physiology and Biotechnology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia*
[c] *Department of Condensed Matter Physics, Institute Jožef Stefan, Jamova 39, 1000 Ljubljana, Slovenia*

Received 21 August 2001; received in revised form 2 October 2001; accepted 4 October 2001

Abstract

Human stefin B (cystatin B) is an intracellular cysteine proteinase inhibitor broadly distributed in different tissues. Here, we show that recombinant human stefin B readily forms amyloid fibrils in vitro. It dimerises and further oligomerises, starting from the native-like acid intermediate, I_N, populated at pH 5. On standing at room temperature it produces regular (over 4 μm long) fibrils over a period of several months. These have been visualised by transmission electron microscopy and atomic force microscopy. Their cross-sectional diameter is about 14 nm and blocks of 27 nm repeat longitudinally. The fibrils are smooth, of unbranched surface, consistent with findings of other amyloid fibrils. Thioflavin T fluorescence spectra as a function of time were recorded and Congo red dye binding to the fibrils was demonstrated. Adding 10% (v/v) trifluoroethanol resulted in an increased rate of fibrillation with a typical lag phase. The finding that human stefin B, in contrast to the homologue stefin A, forms amyloid fibrils rather easily should promote further studies of the protein's behaviour in vivo, and/or as a model system for fibrillogenesis.

Keywords: Amyloid fibril; Cystatin; Stefin B; Epilepsy; Atomic force microscopy

Amyloid fibril formation of proteins which cause human disease is currently the focus of biochemical and biophysical research [1–3]. It is closely related to cellular, neurologic and medical studies [4–7]. Amyloidoses are diseases of slow onset and usually degenerative [8]. There is a strong causal link between fibril formation and the onset of pathological signs. This link is supported by genetic evidence, namely that mutations found in early onset Alzheimer's disease or familial Parkinson's disease [1,6,9] lead to polypeptides which are more amyloidogenic.

Not only proteins connected to human disease can form amyloid fibrils. Increasing evidence has been obtained to support the view [10–12,5] that fibrillation is a generic property of proteins, and that only modification in solvent conditions is sufficient to produce fibrils from structurally different proteins or polypeptides. The ordered fibrillar aggregates have been studied by a range of techniques: TEM (transmission electron microscopy), AFM (atomic force microscopy) [13], X-ray fibril diffraction, CD, IR spectroscopies, and characteristic dye binding. The amyloid fibrils appear straight, 50–130 Å in diameter. Polypeptide chains are oriented in such a way

* Corresponding author. Fax: +386-1-257-35-94.
E-mail address: eva.zerovnik@ijs.si (E. Žerovnik).

0167-4838/02/$ – see front matter
PII: S0167-4838(01)00295-3

that β-strands run perpendicular to the long axis of the fibril, allowing β-sheets to propagate in the direction of the fibril [8,14].

Human stefins are globular α/β-proteins of 98 amino acid residues without disulphide bonds [15]. Their three-dimensional fold consists of a five-stranded β-sheet overlaid by an α-helix. The crystal structure of human stefin B in complex with papain and the solution structure of a homologous protein, human stefin A, have been determined [16,17]. Dimers of stefin B are highly populated at room temperature [18], whereas stefin A dimer can only be accessed at pre-denaturation temperatures over 80°C [19], due to a high kinetic barrier. The crystal structure of human cystatin C dimer [20] has been determined and shows a domain-swapping mechanism.

Both stefins, in their monomeric form, act as intracellular cysteine proteinase inhibitors. Their distribution differs, with stefin A localised primarily in skin and leucocytes and stefin B equally distributed in all tissues. It has recently been shown that human stefins A and B are important in regulating specific cellular events, such as apoptosis [21,22]. Lack of human stefin B is closely related to signs of epilepsy [23] and myoclonic epilepsy and cerebellar apoptosis appear in stefin B-deficient mice [22]. Animal studies have shown that this protein gets overexpressed following an epileptic seizure [24]. It is of interest that stefin B localisation is not limited to cytosol where it co-localises with cathepsins; it also was detected in nuclear fractions with as yet unknown function [25].

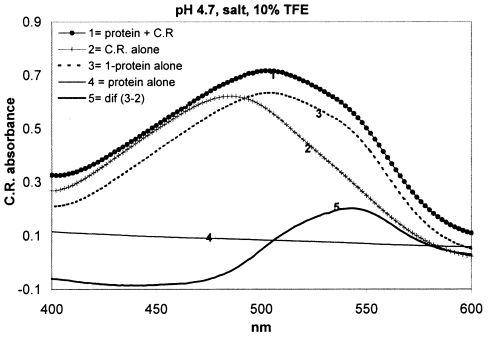

Fig. 1. Absorbance spectra of Congo red (C.R.). Protein concentration was 8.2 μM (diluted from fibrils making a solution of 45.5 μM) and Congo red concentration was 12.9 μM. 1, protein bound C.R.; 2, C.R. dye alone; 4, protein alone; 5, difference between corrected C.R./protein spectrum (3) and spectrum of C.R. dye alone (2). In the corrected spectrum (3) the scattering due to the protein alone was subtracted, i.e. spectrum (1)−spectrum (4).

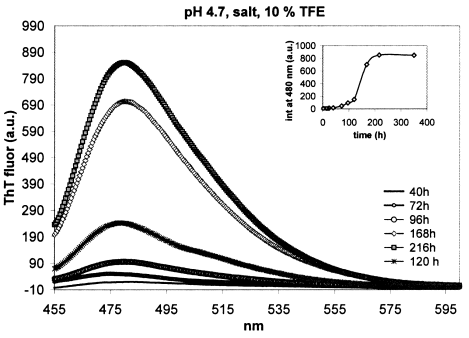

Fig. 2. Fluorescence emission spectra of Thioflavin T (ThT) dye in the presence of stefin B. Excitation was at 440 nm and spectra were recorded from 455 to 600 nm. The protein solution, usually of 34 μM, was dissolved in a Thioflavin T buffer (pH 8.5, 50 mM glycine/NaOH buffer, 15 μM ThT) just before the measurement. The final protein concentration was 6.8 μM. Times after dilution in the solvent pH 4.7, 10% TFE are indicated in the figure. (Insert) Intensity at 480 nm as a function of time.

In this short report we demonstrate for the first time that human stefin B can be made to form amyloid fibrils in relatively mild solvent conditions, such as pH 5 buffer. The fibrils exhibit characteristics common to those observed for other protein fibrils: binding of Congo red [26] and Thioflavin T dyes [27], overall shape, and kinetics of growth. They are smooth, of unbranched surface. Many structural details could be observed by TEM and AFM which shed further light on protein fibrillation.

The starting conformation of human stefin B at pH around 5 is only slightly different from the native one, as revealed by CD spectra [28]. It has been shown previously that the protein forms several equilibrium intermediates, including a molten globule state [18,28,29], in contrast to the homologue stefin A. The finding that fibrillation of stefin B proceeds from a native-like acid intermediate shows strong similarity to transthyretin [2]. This protein also undergoes acid denaturation through a native-like intermediate at pH 4.5 and a molten globule state at pH 2.0 [30,2]. The solution NMR and hydrogen exchange measurements of the amyloidogenic intermediate [31] have shown selective destabilisation of one half of the β-sandwich structure of transthyretin at pH 4.5.

Congo red dye binding to amyloid fibrils is a fibril specific probe. A change in colour from orange–red

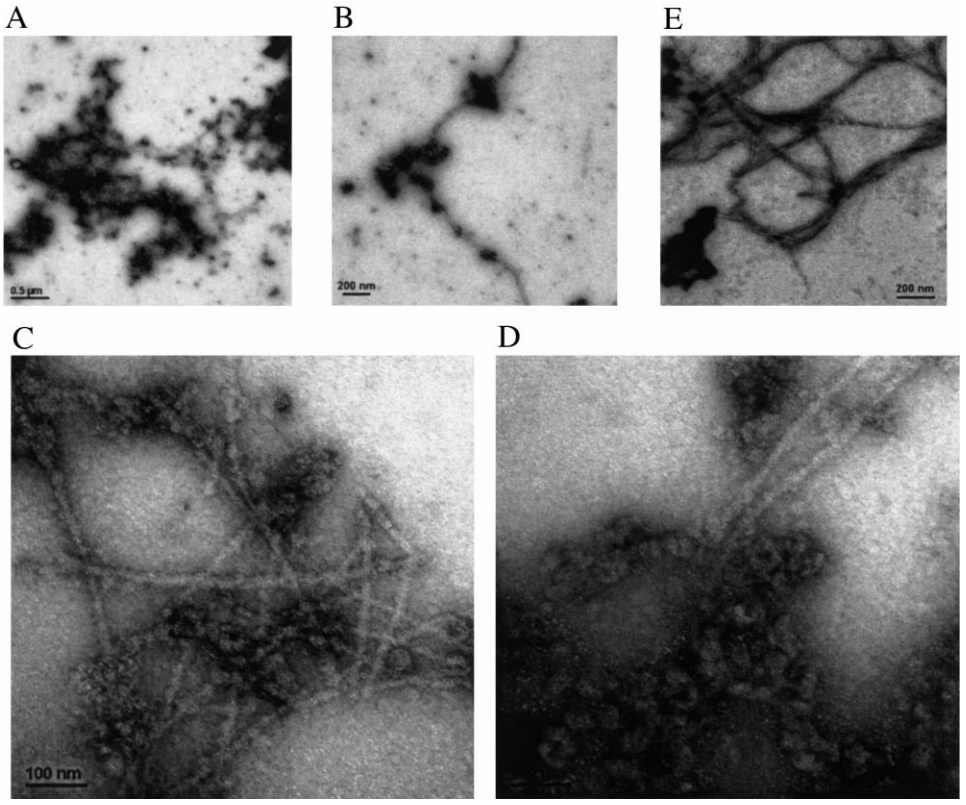

Fig. 3. TEM. Protein samples (20 μl of 34 or 45 μM protein solution) were applied to a Formvar and carbon coated grid. After 5 min the sample was soaked away and stained with 1% uranyl acetate. Samples were observed with a Philips CM 100 transmission electron microscope at 80 kV. TEM images show the growth of human recombinant stefin B fibrils at pH 4.7 (A–D) and pH 4.7, 10% TFE (E). (A) Fibrils and granular aggregates. (B) A single fibril with bunches of granular aggregates. (C) Magnification of a part from A, showing that fibrils originate from granular aggregates. (D) Magnification of another part from A, showing two fibrils originating from pre-fibrillar aggregates (oligomers), forming circular arrangements. (E) Tangles of the fibrils grown at pH 4.7, 10% TFE.

to rose is induced that corresponds to the characteristic shift in the Congo red absorbance spectrum [26]. Congo red alone has an absorbance spectrum with a maximum at 487 nm. When fibrils are present the intensity increases and the absorption maximum shifts to 505 nm. In Fig. 1, the spectral change on adding stefin B fibrils to Congo red solution is shown. The difference spectrum (bold line in Fig. 1) exhibited a characteristic shift in the maximum of absorbance to 540 nm.

Thioflavin T dye also undergoes a characteristic spectral change when bound to regular fibrils [27]. The maximum of fluorescence emission shifts from 435 to 482 nm. In Fig. 2, the fluorescence emission spectra of Thioflavin T dye in the presence of stefin B are shown, gathered at times after diluting stefin B in pH 4.7, 10% TFE (2,2,2-trifluoroethanol) solvent in which fibril growth was observed. The kinetics of the change in intensity at 480 nm was read from the spectra in Fig. 2 (shown as insert to Fig. 2). The half-time of growth of the fibrils at room temperature is about 60 h. A lag phase is clearly observed, lasting up to 90 h, and confirmed by several independent experiments. The lag phase is characteristic for such processes [32]. The kinetics of fibrillation which are very slow at pH 4.7 (months at room temperature) could be accelerated to days by adding 10% TFE. This concentration of TFE is non-denaturing [33], preceding the transition to an all α-helical state. Similarly to some other organic solvents, TFE is known to stabilise hydrogen bonding in local secondary structures, either α-helical or β-sheet [34,35]. It has been shown that TFE also affects the kinetics of folding [36,37]. Other authors have used

TFE for producing fibrillar aggregates [11]. In contrast to our case, they have either used stronger acidic conditions [38,10] or added TFE [11], but not a combination of the two.

Imaging of the amyloid fibrils was done using TEM and AFM. TEM was applied at various stages of fibril growth. In Fig. 3A–D, intermediate stages of fibril growth (1.5 months) in solutions of stefin B at pH 4.7 are shown. Regular, smooth fibrils originate from granular aggregates (Fig. 3A). In Fig. 3B, one fibril with attached nuclei of granular aggregates is shown. In Fig. 3C,D, in which two parts from Fig. 3A have been magnified, circular formations are observed. The average diameter of the fibrils as read from many different preparations (different grids) and different positions on a single grid is 14.5 ± 2.5 nm. In Fig. 3E, the fibrils, which grew at pH 4.7, 10% TFE in 3 weeks are shown. These are single chains with a diameter of 7 ± 1.5 nm and entangle in arrays, 2–4, twisted together (Fig. 3E).

AFM was used to complement the TEM data and gave good resolution images of the fibrils' structure. In Fig. 4A, a collection of many intertwined fibrils, grown over 3 months at pH 4.7 (without TFE) is shown. The length of the fibrils varied from 50 nm up to 5 μm, as shown in Fig. 4A. Fig. 4B is taken under higher magnification where the structure and dimensions of fibrils can be traced. Measurements on many fibrils give an average height of the fibril of 3.4 ± 0.3 nm and an average cross-section diameter of 13 ± 3 nm. Here it should be noted that fibrils were dried on the grid (mica); therefore, the height and the diameter may be different from those in solution since fibrils are subject to flattening and adhesion effects. We have also observed that fibrils show a periodicity of approx. 27 ± 2 nm along the fibril axis. Periodicity was determined from the cross-sectional analysis (Fig. 4C).

Some features of stefin B fibrils resemble those of amyloid Aβ peptide fibrils [14]. The 27 nm longitudinal repeat (β-helical twist) in the structure of stefin B fibril is in complete agreement with data on Aβ 40, specifically fibrils of type 2, which are 12 nm thick and show a periodicity of 25 nm [13]. The stefin B fibril diameter is around 14 nm, according to TEM. There also exist type 1 fibrils which do not show the β-helical twist periodicity and are thinner. We believe these may correspond to fibrils in 10% TFE, pH 4.7, in our case each 7.0 nm in diameter (according to TEM, Fig. 3E).

It is not possible to claim at present that fibrillation of this physiologically important protein has relevance to any pathological condition. Nevertheless, it can be expected that such mildly destabilising conditions would occur in the cellular environment (at membranes, endosomes), especially under stressful conditions for the cell. It is of note that stefin B gets overexpressed following myoclonus seizures [24],

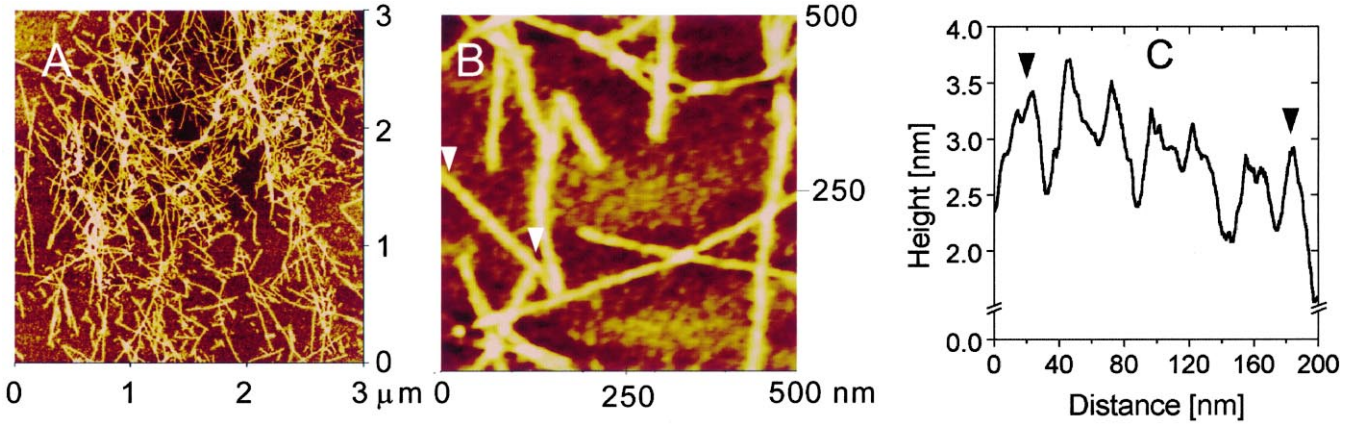

Fig. 4. AFM. 20 μl of stefin B sample (45 μM protein concentration) were spread across a freshly cleaved mica surface (1 cm^2), incubated for 5 min and gently washed with pH 4.7 solvent and deionized water. Excess water was removed with a stream of nitrogen. Images were obtained with a Nanoscope III Multimode scanning probe microscope (Digital Instruments) operating in contact mode. AFM images represent height variation. (A) A collection of intertwined fibrils, deposited from pH 4.7 solution of human stefin B (as for Fig. 3A–D) on a freshly cleaved mica surface. (B) Selected part of the sample showing isolated fibrils. (C) Height profile along the length of the fibril. In between the two markers one can trace six regular repeats. The periodicity is 27 nm.

and that a closely related protein human cystatin C is a well-known amyloidogenic protein [39,40].

Even though protein fibrillation is thought to be a generic property of proteins, it is of note that the homologous protein human stefin A does not form fibrils under similar conditions. The model system is very suitable to study details of structural and kinetic determinants for fibrillation which still await conclusive answers.

We thank Prof. Roger H. Pain (IJS, Ljubljana) for carefully reading the manuscript and editing the English. Thanks go to Drs. Jonathan P. Waltho (University of Sheffield), Roman Jerala (National Institute of Chemistry, Ljubljana) and Magnus Abrahamson (University of Lund) for useful discussions. This study has been supported by the Ministry of Education, Science and Sport of the Republic of Slovenia.

References

[1] P.T. Lansbury Jr., Proc. Natl. Acad. Sci. USA 96 (1999) 3342–3344.
[2] J.W. Kelly, Curr. Opin. Struct. Biol. 8 (1998) 101–106.
[3] J.W. Kelly, Nat. Struct. Biol. 7 (2000) 824–826.
[4] R.W. Carrel, B. Gooptu, Curr. Opin. Struct. Biol. 8 (1998) 799–809.
[5] C.M. Dobson, Trends Biochem. Sci. 24 (1999) 329–332.
[6] M. Goedert, M.G. Spillantini, S.W. Davies, Curr. Opin. Neurobiol. 8 (1998) 619–632.
[7] A. Alves-Rodrigues, L. Gregori, M.E. Figueiredo-Pereira, Trends Neurosci. 21 (1998) 516–520.
[8] J.C. Rochet, P.T. Lansbury Jr., Curr. Opin. Struct. Biol. 10 (2000) 60–68.
[9] K.A. Conway, J.D. Harper, P.T. Lansbury, Nat. Med. 4 (1998) 1318–1320.
[10] J.I. Guijarro, M. Sunde, J.A. Jones, I.D. Campbell, C.M. Dobson, Proc. Natl. Acad. Sci. USA 95 (1998) 4224–4228.
[11] F. Chiti, P. Webster, N. Taddei, A. Clark, M. Stefani, G. Ramponi, C.M. Dobson, Proc. Natl. Acad. Sci. USA 96 (1999) 3590–3594.
[12] M. Fandrich, M.A. Fletcher, C.M. Dobson, Nature 410 (2001) 165–166.
[13] T.T. Ding, J.D. Harper, Methods Enzymol. 309 (1999) 510–525.
[14] L.C. Serpell, Biochim. Biophys. Acta 1502 (2000) 16–30.
[15] V. Turk, W. Bode, FEBS Lett. 285 (1991) 213–219.
[16] M.T. Stubbs, B. Laber, W. Bode, R. Huber, R. Jerala, B. Lenarèiè, V. Turk, EMBO J. 9 (1990) 1939–1947.
[17] J.R. Martin, C.J. Craven, R. Jerala, L. Kroon-Žitko, E. Žerovnik, V. Turk, J.P. Waltho, J. Mol. Biol. 146 (1995) 331–343.
[18] E. Žerovnik, R. Jerala, N. Poklar, L. Kroon-Žitko, V. Turk, Biochim. Biophys. Acta 1209 (1994) 140–143.
[19] R. Jerala, E. Žerovnik, J. Mol. Biol. 291 (1999) 1079–1089.
[20] R. Janowski, M. Kozak, E. Jankowska, Z. Grzonka, A. Grubb, M. Abrahamson, M. Jaskolski, Nat. Struct. Biol. 8 (2001) 316–320.
[21] B. Jones, P.J. Roberts, W.A. Faubion, E. Kominami, G.J. Gores, Am. J. Physiol. 275 (1998) G723–G723.
[22] L.A. Pennacchio, D.M. Bouley, K.M. Higgins, M.P. Scott, J.L. Noebels, R.M. Myers, Nat. Genet. 20 (1998) 251–258.
[23] L.A. Pennacchio, A.E. Lehesjoki, N.E. Stone, V.L. Willour, K. Virtaneva, J. Miao, E. D'Amato, L. Raminez, M. Faham, M. Koskiniemi, J.A. Warrington, R. Norio, A. de la Chapelle, D.R. Cox, R.M. Myers, Science 271 (1996) 1731–1734.
[24] E. D'Amato, Z. Kokaia, A. Nanobashvili, M. Reeben, A.E. Lehesjoki, M. Saarma, O. Lindvall, Eur. J. Neurosci. 12 (2000) 1687–1695.
[25] M. Riccio, R. Di Giaimo, S. Pianetti, P.P. Palmieri, M. Melli, S. Santi, Exp. Cell Res. 262 (2001) 84–94.
[26] W.E. Klunk, R.F. Jacob, R.P. Mason, Methods Enzymol. 309 (1999) 285–305.
[27] H. Le Vine, Methods Enzymol. 309 (1999) 274–284.
[28] E. Žerovnik, R. Jerala, L. Kroon-Žitko, V. Turk, K. Lohner, Eur. J. Biochem. 245 (1997) 364–372.
[29] E. Žerovnik, R. Jerala, L. Kroon-Žitko, R.H. Pain, V. Turk, J. Biol. Chem. 267 (1992) 9041–9046.
[30] Z. Lai, W. Colon, J.W. Kelly, Biochemistry 35 (1996) 6470–6482.
[31] K. Liu, H.S. Cho, H.A. Lashuel, J.W. Kelly, D.E. Wemmer, Nat. Struct. Biol. 7 (2000) 754–757.
[32] T.R. Serio, A.G. Cashikar, A.S. Kowal, G.J. Sawicki, J.J. Moslehi, L. Serpell, M.F. Arnsdorf, S.L. Lindquist, Science 289 (2000) 1317–1321.
[33] E. Žerovnik, R. Virden, R. Jerala, L. Kroon-Žitko, V. Turk, J.P. Waltho, Proteins 36 (1999) 205–216.
[34] M. Buck, Q. Rev. Biophys. 31 (1998) 297–355.
[35] K. Shiraki, K. Nishikawa, Y. Goto, J. Mol. Biol. 245 (1995) 180–194.
[36] F. Chiti, N. Taddei, P. Webster, D. Hamada, T. Fiaschi, G. Ramponi, C.M. Dobson, Nat. Struct. Biol. 6 (1999) 380–387.
[37] D. Hamada, F. Chiti, J.I. Guijarro, M. Kataoka, N. Taddei, C.M. Dobson, Nat. Struct. Biol. 7 (2000) 58–61.
[38] T. Konno, K. Murata, K. Nagayama, FEBS Lett. 454 (1999) 122–126.
[39] O. Jensson, A. Palsdottir, L. Thorsteinsson, A. Arnason, M. Abrahamson, I. Olafsson, A. Grubb, Biol. Chem. Hoppe Seyler 371 (Suppl.) (1990) 229–232.
[40] M. Abrahamson, Scand. J. Clin. Lab. Invest. 226 (Suppl.) (1996) 47–56.