In vitro study of stability and amyloid-fibril formation of two mutants of human stefin B (cystatin B) occurring in patients with EPM1

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Abstract

Myoclonus epilepsy of type 1 (EPM1) is a rare monogenic progressive and degenerative epilepsy, also known under the name Unverricht-Lundborg disease. With the aim of comparing their behavior in vitro, wild-type (wt) human stefin B (cystatin B) and the G4R and the R68X mutants observed in EPM1 were expressed and isolated from the *Escherichia coli* lysate. The R68X mutant (Arg68Stop) is a peptide of 67 amino acids from the N terminus of stefin B. CD spectra have shown that the R68X peptide is not folded, in contrast to the G4R mutant, which folds like wild type. The wild type and the G4R mutant were unfolded by urea and by trifluoroethanol (TFE). It has been shown that both proteins have closely similar stability and that at pH 4.8, where a native-like intermediate was demonstrated, TFE induces unfolding intermediates prior to the major transition to the all- α -helical state. Kinetics of fibril formation were followed by Thioflavin T fluorescence while the accompanying changes of morphology were followed by the transmission electron microscopy (TEM). For the two folded proteins the optimal concentration of TFE producing extensive lag phases and high fibril yields was predenaturational, 9% (v/v). The unfolded R68X peptide, which is highly prone to aggregate, formed amyloid fibrils in aqueous solution and in predenaturing 3% TFE. The G4R mutant exhibited a much longer lag phase than the wild type, with the accumulation of prefibrillar aggregates. Implications for pathology in view of the higher toxicity of prefibrillar aggregates to cells are discussed.

Keywords: amyloid; cystatin B; epilepsy; folding intermediates; lag phase; prefibrillar aggregates; protein stability

Human cysteine cathepsins (papain family of the CA clan of cysteine proteases) are lysosomal enzymes. Cathepsin knockouts have demonstrated that cathepsins have specif-

ic and individual functions, which are very important for the normal functioning of the organism (Turk et al. 2001). Among others, they degrade the extracellular matrix, of importance in tumor progression and invasion (Jedeszko and Sloane 2004). Accidentally escaped cathepsins from lysosomes are trapped by cystatins, the endogenous protein inhibitors. Cystatins are divided into stefins, cystatins, and kininogens, among which only stefins are intracellular.

Human stefin B (family I25 of cystatins by MEROPS classification), also termed cystatin B, is a cysteine proteinase inhibitor (Turk and Bode 1991; Turk et al. 2001). It is a protein of molecular mass 11 kDa, containing no disulphide bonds. It was first purified from a human source (Brzin et al. 1983), then cloned and expressed in

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Abbreviations: CD, circular dichroism; EPM1, progressive myoclonus epilepsy of Unverricht-Lundborg type; IPTG, isopropyl- β -D-thiogalactopyranoside; PCR, polymerase chain reaction; PEI, polyethyleneimine; SEC, size-exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TFE, 2,2,2 Trifluoroethanol; ThT, Thioflavin T; UV, ultraviolet.

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Escherichia coli in high yield (Jerala et al. 1988). The 3D structures of stefin B in complex with papain (Stubbs et al. 1990) and of stefin A in complex with cathepsin H (Jenko et al. 2003) have been determined by X-ray diffraction. The solution structure of free stefin A has also been determined by heteronuclear NMR (Martin et al. 1995). In the homologous inhibitors stefin A and cystatin C domain-swapped dimers have been demonstrated (Jerala and Žerovnik 1999; Janowski et al. 2001; Staniforth et al. 2001) that may have a role in amyloid fibril formation of this family of proteins (Staniforth et al. 2001).

Human stefins inhibit several papain-family cysteine proteases, the lysosomal cathepsins. They are ubiquitously expressed in human tissue. In cells stefin B is located in the lysosomes and the cytoplasm, but also in the nucleus (Riccio et al. 2001). In studies of stefin B-deficient mice, the lack of this inhibitor is associated with signs of cerebellar granular cell apoptosis, ataxia, and myoclonus (Pennacchio et al. 1998). Further, genes involved in activation of glial cells are overexpressed (Lieuallen et al. 2001). In double knockout mice of stefin B and cathepsin B genes (Houseweart et al. 2003) apoptosis was markedly reduced, whereas ataxia and myoclonus remained, suggesting that stefin B may have other functions beside proteinase inhibition. This seems in accordance with findings that stefin B is part of a multi-protein complex of unknown function, specific to the cerebellum (Di Giamo et al. 2002). In the complex, it is bound to proteins, none of which is a protease. These proteins are the protein kinase C receptor (RACK-1), the brain β -spectrin, the neurofilament light chain (NF-L), and two unknown proteins, one of which belongs to a myotubularin family.

An inherited progressive myoclonus epilepsy of the Unverricht-Lundborg type has been shown to result from mutations in stefin B gene (Pennacchio et al. 1996). The most common change reported is the dodecamer repeat expansions in the promoter region of the stefin B (cystatin B) gene, which leads to reduced mRNA and protein levels. Four coding-region mutants have been reported in EPM1 (Alakurtti et al. 2004). They are nonsense (g.2388C > T, p.Arg68X), frameshift (g.2400_2402delTC, p.Lys73fsX2), and two missense (g.426G > C, p.Gly4Arg and g.2398A > C, p.Glu71Pro) mutations. All four mutants have been transfected and expressed in BHK-21 cells, and intracellular targeting was observed (Alakurtti et al. 2004). In contrast to wild-type (wt) stefin B, which was localized in the cytoplasm, nucleus, and lysosomes, none of the mutant proteins studied was associated with lysosomes. Both missense mutants and prematurely truncated mutant Lys73fsX2 were distributed diffusely in the cytoplasm and the nucleus, while the R68X (Arg68X) mutant

showed diffuse cellular staining, but was detected only in the presence of a proteosomal inhibitor lactacystin, indicating rapid degradation of the newly synthesized protein.

In our in vitro studies we have shown that human stefin B, its mutants, and iso-forms are prone to form amyloid fibrils (Žerovnik et al. 2002, 2005; Kenig et al. 2004). It has been shown that human stefin B readily forms amyloid fibrils under mildly denaturing conditions, in contrast to its homolog stefin A (Žerovnik 2002; Jenko et al. 2004). By following the kinetics of fibril formation, conditions were defined where the protein exists in the form of prefibrillar oligomers/aggregates, which persist during the lag phase. The prefibrillar forms were shown to be cytotoxic and to interact with acidic phospholipids (Anderluh et al. 2005).

Here, an in vitro study of the stability, folding, and amyloid fibril formation of the G4R mutant and the fragment of 67 amino acid residues from the N terminus of stefin B R68X is presented. We have measured CD spectra in the far- and near-UV regions, performed denaturation by urea and titration with trifluoroethanol (TFE) at several pH values, all in comparison to the wild-type protein. Further, amyloid fibril formation was measured. It was observed that stefin B and the G4R mutant exhibited nucleation and growth reactions (Lomakin et al. 1997), similar to many other amyloidogenic proteins, while the fragment underwent transition to fibrils without any measurable lag phase. Unusual aggregation of the two mutants occurring in EPM1 is discussed in view of possible implications for pathology.

Results

CD spectra

Far- and near-UV CD spectra of the proteins dissolved at pH 7.0 were recorded at 25°C. Far-UV CD spectra (Fig. 1A) for the wt stefin B and the G4R mutant are very similar; both proteins give characteristic CD spectra for α/β secondary structure with minima at 222 nm, while the R68X mutant gives a CD spectrum characteristic of unfolded proteins with a minimum at 200 nm and low ellipticity in the 210–230 nm region. This fragmented protein remains unfolded at pH 7 but also at pH 5 or 3 (spectra not shown). The near-UV CD spectra for the wt stefin B and the G4R mutant were measured in addition and were very similar to each other (Fig. 1B). The G4R mutant is concluded to have the same tertiary structure as the wt stefin B and, therefore, is correctly folded.

Molecular parameters, needed for calculations of the ellipticity units, are given in Table 1.

Figure 1. CD spectra in the far- and near-UV regions. (A) Far-UV CD spectra of all three proteins (*inset*). A 0.1-cm light-path cell was used. Protein concentrations were such that $A_{280} = 0.1$. (B) Near-UV CD spectra of wt stefin B and G4R mutant were measured from 320 nm to 250 nm, using a 1-cm light-path cell. Protein concentrations were such that $A_{280} = 1.2$. All measurements were carried out at 25°C in 10 mM sodium phosphate buffer (pH = 7.0).

Urea denaturation

The stability of wt stefin B and the G4R mutant was determined by urea denaturation. In Figure 2, CD data at 222 nm are plotted as a function of urea concentration, together with curves fitted to the data assuming a two-state approximation for the transition (Santoro and Bolen 1988); the thermodynamic parameters of unfolding are given in Table 2. The stability of the G4R mutant is closely similar to that of the wt stefin B—the proteins unfold at almost the same concentration of urea (Table 2).

Urea denaturation was performed under standard conditions of pH 7 and 25°C (Fig. 2). Salt was added to 0.2 M to shield electrostatic interactions, which makes the data more comparable to those in GuHCl (Žerovnik et al. 1992; Kenig et al. 2001). The free energies of unfolding of -44 ± 5 kJ/mol obtained for both the G4R mutant and the wild-type protein (Table 2) show that stefin B and the G4R mutant are stable proteins. However, this value is substantially higher than that reported (Žerovnik et al. 2005) for the recombinant stefin B, which is an iso-form of the protein based on amino acid sequence of stefin B from human liver (Ritonja et al. 1985; Jerala et al. 1988).

Denaturation by TFE

The effect of TFE on conformation was followed by far-UV CD, at pH 4.8 and 7.0 (Figs. 3A,C,E,G, 4A). With increasing concentration of TFE the α/β secondary structure of the wt stefin B and the G4R mutant first changes to an all- β -structure and then to an all- α -helical conformation with two characteristic spectral minima, at both pH values (Fig. 3A,C,E,G). For the R68X mutant at pH 4.8 (Fig. 4A), a transition is observed from an unfolded state to a β -structure, and, at highest TFE concentrations, to an all- α -helical conformation.

Ellipticity at the spectral minima was plotted as a function of TFE concentration (Figs. 3B,D,F,H, 4B). For the wt stefin B and the G4R mutant the denaturation curves, at both 220 and 208 nm, are very similar but differ from the R68X mutant. With the latter, a cross-over point between unfolded and β -structure is observed at 208 nm; hence, 220 nm was the wavelength of choice to observe the changes in the secondary structure for this mutant.

The wt stefin B and the G4R mutant start to unfold at lower concentrations of TFE at pH 4.8 than at pH 7.0. At pH 4.8 (Fig. 3B,F), there are significant transitions in the secondary structure before the major unfolding transition. These intermediates of unfolding appear from around 13% to 17% of TFE for both proteins. In the case of the R68X mutant, the secondary structure is modified at lower concentrations (5%) of TFE, again pointing to folding intermediates (Fig. 4B).

Choosing an optimal TFE concentration to induce amyloid fibril growth

All protein solutions were prepared under slightly acidic conditions (pH 4.8) that caused destabilization of the protein structure. It had been previously reported that native-like and molten globule intermediates exist in stefin B acid denaturation (Žerovnik et al. 1997). It also was observed that amyloid fibril formation could be initiated from either of the acid-induced intermediates, and that TFE accelerated the reaction (Žerovnik et al. 2002).

Table 1. Molecular parameters calculated from the protein sequences

Protein	<i>M</i> (Da)	<i>M</i> _{MRW} (Da)	<i>E</i> ₂₈₀ (mL/μm)
WT stefin B	11,123.5	113.5	0.35
G4R mutant	11,222.6	114.5	0.34
R68X mutant	7437.4	111	0.17

Values of molecular mass (*M*), mean residue molecular mass (*M*_{MRW}) and extinction coefficients (*E*₂₈₀) for the WT stefin B, the G4R and the R68X mutants.

Figure 2. Urea denaturation curves of the wt stefin B and the G4R mutant. Changes in ellipticity were followed at 222 nm (far-UV CD) at 25°C and pH 7 after equilibration for at least 16 h. A 0.1-cm light-path cuvette was used. Protein concentration in each sample was such that $A_{280} = 0.3$. The curves represent calculated values according to Santoro and Bolen (1988).

Optimal TFE concentrations for promoting amyloid fibril formation were chosen, based on the denaturation curves (Figs. 3B,F, 4B). Nine percent of TFE is predenaturational, for both the wt stefin B and G4R mutant (Fig. 3B,F). Seventeen percent TFE is the concentration after the transition to an unfolding intermediate in both cases, which starts at 13% TFE. For the R68X mutant, 0% and 3% of TFE are predenaturational (Fig. 4B), whereas 5% TFE is at the midpoint of the transition to a folding intermediate and 9% TFE is the concentration after this transition.

Amyloid fibril formation

Amyloid fibril formation was studied by measuring ThT fluorescence. ThT exhibits a characteristic fluorescence spectrum change when bound to fibrils: The emission maximum shifts from 435 nm to 482 nm (LeVine 1999). The propensity to fibrillate was obtained by measuring the kinetics of change of ThT fluorescence. The intensity of ThT fluorescence at the maximum of 482 nm was read and plotted against time. The observed lag phases are greatest at 9% TFE (Fig. 5A,B), while they are not always observed at higher concentrations (Fig. 5C–F). In the presence of 9% of TFE the lag phase for the wt

stefin B is at 120 ± 20 h (Fig. 5A), and for the G4R mutant at 430 ± 20 h (Fig. 5B), indicating that wt stefin B starts to fibrillate much earlier than the G4R mutant. Thus, the prefibrillar aggregates accumulate for a longer time for this mutant. The final yield of fibrils (judged by ThT fluorescence intensity) is also slightly lower for the G4R mutant than for the wt stefin B.

At 13% TFE the observed difference in the lag phases (i.e., the propensity to fibrillate) for these two proteins is not so marked (Fig. 5C,D). At 17% TFE fibrillation starts without a distinct lag phase (Fig. 5E,F), but with a final yield of fibrils much lower than at 9% TFE. It should be noted that, at these two concentrations of TFE, some intermediates of unfolding are present (see previous section).

For the R68X mutant, in the absence of TFE and in 3% TFE, fibril formation was very much faster (Fig. 6). No distinct lag phase could be observed, and in a few hours the whole reaction reached the plateau phase. At 9% TFE (not shown), fibrillation was immediate, but with very low final yields compared to the other two proteins.

TEM images

Fibril formation was followed in parallel by TEM. Samples of wt stefin B and the G4R mutant were taken for TEM analysis in the lag phase on the third day (Fig. 7A,B), a sample of the wt stefin B in the plateau phase on the 14th day (Fig. 7C), and a sample of the G4R mutant in the plateau phase on the 28th day (Fig. 7D). Only prefibrillar aggregates were observed in the lag phase in the case of G4R mutant (Fig. 7A), whereas in the case of wt stefin B, some fibrils had already grown (Fig. 7B). The difference between these two proteins was also observed in the plateau phase where only mature fibrils were present in wt stefin B (Fig. 7C), whereas in the G4R mutant sample, some aggregates remained in addition to short fibrils (Fig. 7D). This mutant gave smaller amounts of mature fibrils than wt stefin B (as we can judge qualitatively by observing TEM data).

The R68X mutant showed a very high tendency to fibrillate (Fig. 8). Fibrils formed in 1 h without the addition

Table 2. Thermodynamic parameters of unfolding by urea denaturation experiment

Protein	ΔG°_{N-U} (kJ/mol)	m (kJ/Mmol)	c_m (M)
WT stefin B	-44.4 ± 5.1	8.5 ± 1.0	5.23 ± 0.04
G4R mutant	-43.4 ± 7.2	8.4 ± 1.4	5.18 ± 0.11

ΔG°_{N-U} = the free energy of unfolding, c_m = the concentration of denaturant at which half of the molecules are unfolded, m = the cooperativity that represents the difference in the surface of protein exposed to the solvent in the folded and unfolded states. The values are the average of three independent measurements.

Figure 3. Effect of TFE on the wt stefin B and the G4R mutant conformation. Far-UV CD spectra of the wt stefin B at pH 4.8 (A) and at pH 7.0 (C) and the G4R mutant at pH 4.8 (E) and at pH 7.0 (G) as a function of TFE concentration. TFE denaturation curves at wavelengths of 208 and 220 nm of the wt stefin B at pH 4.8 (B) and at pH 7.0 (D) and of the G4R mutant at pH 4.8 (F) and at pH 7.0 (H). Proteins were dissolved in TFE (0%–60%) 2 h before recording the far-UV CD spectra. A 0.1-cm light-path cuvette was used, and A_{280} of the protein solutions was 0.22.

of TFE (Fig. 8A1,A2). However, these fibrils were shorter from those grown in the presence of TFE, as judged from TEM images (Fig. 8B1,B2,C).

Discussion

Unfolded proteins or fragments, such as α -synuclein or amyloid- β (A- β), can form amyloid fibrils on partial folding. In this work, we demonstrate for the first time that fragmentation of stefin B at residue 68 leads to an unfolded state. Further, we show that this pep-

tide, which is present in some EPM1 patients, transforms into amyloid-like fibrils very easily. We also show that, despite their equal stability, wt stefin B and missense G4R mutant undergo amyloid fibrillation with very different lag phases, indicating accumulation of prefibrillar aggregates in the case of the EPM1 mutant.

The fact that TFE, a hydrogen bond-promoting solvent, has an accelerating effect on amyloid fibrillation is well known. However, the concentration dependence of the TFE effects have been studied in detail in only a few

Figure 4. Effect of TFE on the conformation of the R68X mutant. (A) Far-UV CD spectra as a function of TFE concentration at pH 4.8. (B) TFE denaturation curve at a wavelength of 220 nm.

cases (Chiti et al. 1999; Munishkina et al. 2003). In this study, amyloid fibril growth was accelerated by adding different concentrations of TFE to solutions of stefin B at pH 4.8, where the protein was shown partially unfolded (Žerovnik et al. 1997).

TFE-induced denaturation of the wt stefin B and the G4R mutant (Fig. 3) was accompanied by transformation of the initial α/β secondary structure to a partially unfolded conformation, then through a β -sheet-enriched conformation, to a significantly ordered all- α -helical state. The unfolded R68X mutant folded in a multiphase manner (Fig. 4), similar to that found for natively unfolded α -synuclein (Munishkina et al. 2003). In the absence of TFE the spectrum of the R68X mutant was typical of an unfolded polypeptide chain. Increasing TFE concentration initially induced formation of a partially folded intermediate, then β -structure-enriched species were formed, shown by the pronounced minimum near 218 nm, typical of an extensive β -structure. As observed with other proteins, a predominantly α -helical conformation was observed at high TFE concentrations (Hamada and Goto 1997).

The propensity to form amyloid fibrils in the presence of different concentrations of TFE was observed for all three proteins (Fig. 5A–F). TFE affected the kinetics of fibrillation in a concentration-dependent manner. The final yield of fibrils, in the case of wt stefin B and the G4R mutant, was substantially decreased in the presence of 13% TFE (Fig. 5C,D) and 17% TFE (Fig. 5E,F), at which concentrations partially unfolded intermediates

are present. These intermediates readily self-associate to form β -structure-enriched oligomers but do not form amyloid fibrils. This can explain the lower ThT fluorescence at the plateau of the fibrillation reactions in the presence of 13% and 17% of TFE. At a predenaturation concentration of 9% TFE, where the two proteins are in the initial, native-like intermediate state, the yields of the mature fibrils are much higher and the lag phases much longer. It is thus confirmed that partially folded predenaturation intermediates appear to be critical species in the fibrillation process.

Although the G4R mutant and the wild-type protein are almost equally stable, they have very different propensities for fibrillation. The G4R mutant (at pH 4.8, 9% TFE) has a much more prolonged lag phase than the wild-type protein (almost four times) (Fig. 5A,B), with accumulation of prefibrillar aggregates (Fig. 7A). This mutant also shows a lower yield of the final fibrils than wt stefin B, possibly due to different partitioning between mature fibrils and aggregates (Chiti et al. 2002). The R68X mutant started to fibrillate immediately under all conditions (0%, 1%, 3%, and 9% of TFE) with no distinct lag phases (Fig. 6). Fibrils that formed without TFE were shorter, as seen by TEM (Fig. 8A1), than those growing in the presence of TFE (Fig. 8B1,B2,C). The fragmented protein clearly has a high tendency to aggregate and fibrillate.

We believe that our *in vitro* studies may have some implications for pathology. As described in the Introduction, in EPM1 disease several missense and stop codon mutants were observed, in addition to the prevalent dodecamer repeat expansion; among them, the two studied here. Our finding of very different aggregation and amyloid fibrillation behavior of the G4R mutant and the R68X fragment compared to the wild-type protein, may have implications for pathology. Namely, the prefibrillar aggregates are reported to be more toxic to cells than the mature fibrils (Walsh et al. 1999, 2002). Also, for some other amyloidogenic proteins involved in conformational diseases, such as α -synuclein in Parkinson's disease, it was observed that pathogenic mutations lead to a prolonged lag phase and accumulation of prefibrillar oligomers (Conway et al. 2000). These oligomeric species can make pores in membranes that can cause leakage, and are of similar structure to pores made by bacterial toxins. Increased permeability of the membranes can be the cause of nonfunctionality of cells or even their death (Lashuel et al. 2002).

Our hypothesis states (Čeru et al. 2005) that, at least in a subset of EPM1 patients, intracellular aggregation of the mutated stefin B protein may take place, producing "gain in toxic function," in addition to the loss of normal function. On the basis of the present *in vitro* study, accumulation of toxic prefibrillar aggregates is

Figure 5. ThT fluorescence emission intensity at 482 nm plotted as a function of time. Fibrillation reaction of the wt stefin B at 9% (A), 13% (C), and 17% of TFE (E) and of the G4R mutant at 9% (B), 13% (D), and 17% of TFE (F). Circles, diamonds, and triangles (A,B) or circles and diamonds (C–F) represent raw ThT data from independent experiments performed at the same conditions. In all cases protein concentration was 34 μ M. Fibrils were grown in the pH 4.8 buffer in the presence of different concentrations of TFE and were diluted into ThT buffer (pH 7.5) at different times of fibril growth. Then, ThT fluorescence spectra were recorded, from which an intensity at 482 nm was read.

expected only with the G4R mutant while the fragmented protein, which fibrillizes faster, could be caught in cytoplasmic inclusions or degraded by proteasome. If any of the above two mechanisms are taking place in the real disease remains to be seen on patients' samples and, if this is too difficult to obtain, on transgenic animal models. For example, one could look for proteasome dysfunction and/or cellular inclusions by using anti-ubiquitin antibodies.

In a somewhat broader context than EPM1 it may be of importance that it has been shown in rat studies that stefin B gets overexpressed after epileptic seizures (D'Amato et al. 2000), implying its neuroprotective role. When proteins get overexpressed and at the same time cells are perturbed by some additional stress, protein aggregation can become a problem (Marx et al. 2003).

Materials and methods

Restriction enzymes and *Taq* DNA ligase were from New England Biolabs; vectors, from Novagen; T4 DNA ligase, from Boehringer-Mannheim; *Taq* DNA polymerase, from MBI Fermentas; 2,2,2 Trifluoroethanol (TFE), from Fluka; and Thioflavin T (ThT), from Aldrich. Other chemicals were from Sigma, Carlo Erba, Serva, and Merck.

Cloning

The starting protein used for mutagenesis was recombinant human stefin B, cloned in 1988 (Jerala et al. 1988), based on the amino acid sequence of stefin B isolated from human liver (Ritonja et al. 1985). This recombinant protein has Glu 31 replaced by Tyr, and Ser 3 replaced by Cys. The "wild-type"-like stefin B (the polymorphic form reported in the gene bank) was prepared using primer Y31E (5'-GAAGAGAAAGAGAAC AAGAAATTC-3') previously phosphorylated by T4 poly-

Figure 6. ThT fluorescence emission intensity at 482 nm of the R68X mutant plotted as a function of time. Protein concentrations were 34 μ M. Fibrils were grown in the pH 4.8 buffer, without (open circles) and with (filled circles) 3% of TFE in solutions that were diluted in ThT buffer (pH 7.5) at different times of fibril growth, and ThT fluorescence intensity was measured.

nucleotide kinase, stefin B forward primer (5'-ATCGGGATCC TAGAAGTAGGTCAGCTCGTCG-3'), and stefin B backward primer (5'-ATCGCATATGATGTCTGGTGCTCCGTC-3'). Mutagenesis was performed by PCR on a Perkin-Elmer Gene Amp 2400 PCR System using *Taq* DNA polymerase and *Taq* DNA ligase. A chemically synthesized gene for human stefin B was used as a template (Jerala et al. 1988). For the G4R (Gly4Arg) mutant the mutagen primer (5'-ATCGCATAT GATGTCTCGTGCTCCGTC-3') and stefin B forward primer were used and for the R68X (Arg68Stop) mutant the mutagen primer (5'-ACTTCTGAAACAAGTGGACATCTAGGGCT A-3') and the stefin B backward primer were used. For both stefin B mutants the gene for the "wild-type"-like stefin B was used as a template, so the Cys3 replacement by Ser remained.

DNA fragments of 200 and 300 bp were demonstrated by electrophoresis in 1.5% agarose gels, then purified using QIAquick gel extraction protocol (Qiagen) and ligated into the pGEM T-easy vector (Promega). DNA sequence was confirmed using the Sanger method on a ABI Prism 310 Genetic Analyser (Applied Biosystems).

Expression in *E. coli*

DNA fragments for the wt stefin B, G4R, and R68X mutants were digested with *Nde*I and *Bam*HI and ligated into the pET11a expression vector, previously digested with the same enzymes. These DNA constructs were transformed into the BL21(DE3)pLysS strain of *E. coli*. Ten milliliters of the overnight culture was inoculated into 500 mL LB medium containing 100 μ L/mL of ampicillin (Sigma) and 25 μ L/mL of chloramphenicol (Sigma). The culture was incubated at 37°C up to $OD_{600} = 0.6$ to 1, and IPTG then added to a final concentration of 1 mM. Three hours after induction cells were separated from the medium and lysed. Efficiency of protein expression was confirmed by SDS-PAGE. After centrifugation, supernatant was collected in the case of the wt stefin B and the G4R mutant, but for R68X mutant the pellet with inclusion bodies was used for further isolation.

Purification procedure

Cell lysates for the wt stefin B and the G4R mutant were additionally purified with repeated steps of adding 4% of PEI

and centrifugation to remove contaminants such as nucleic acids and most bacterial (predominantly acidic) proteins. The wt stefin B was isolated from purified cell lysate by affinity chromatography on carboxymethylated (CM) papain-Sepharose. Nonspecifically bound material was eluted with 0.01 M Tris-HCl containing 0.5 M NaCl at pH 8.0. The wt stefin B was eluted with 0.02 M TEA buffer at pH 10.5. Additional purification was done using SEC on Sephacryl S-200 (Amersham Pharmacia Biotech) equilibrated with 0.01 M phosphate buffer, containing 0.12 M NaCl at pH 6.1. This chromatography was also the first step in the purification procedure for the Gly4Arg (G4R) mutant. The second step was performed by cation exchange chromatography with SP Sepharose Fast Flow (Amersham Pharmacia Biotech) using 0.01 M phosphate buffer at pH 6.05. The mutant protein was eluted with a linear gradient of NaCl from 0 to 0.8 M in the same buffer. Both proteins were stored at -20°C in 0.01 M phosphate buffer containing 0.06 M NaCl at pH 6.

The R68X mutant was isolated from the inclusion bodies, which were separated from cell pellet after washing first with 0.1% Triton X-100 in TE buffer (50 mM Tris, 2 mM EDTA) at pH 8.0 and then with 2 M urea in TE buffer. Inclusion bodies were dissolved in 6 M GuHCl, then diluted sixfold in 0.1% TFA before purification by hydrophobic chromatography on an HPLC system. This mutant was eluted with a gradient of acetonitrile from 0% to 90% in 0.1% TFA and was stored at -20°C in 0.1% TFA at pH 2.

The purity of the isolated proteins in all cases was confirmed by 15% SDS-PAGE. Inhibitory activity against papain for the wt stefin B was determined in the presence of synthetic substrate BANA. N-terminal sequences of proteins were confirmed using the amino acid sequencer 475A (Applied Biosystems).

Figure 7. TEM images in the lag and plateau phases of the fibrillation reaction. The G4R mutant (A) and the wt stefin B (B) in the presence of 9% of TFE on the third day of fibrillation. The wt stefin B (C) on the 14th day, and the G4R mutant (D) on the 28th day of fibrillation in the presence of 9% of TFE.

Figure 8. TEM images of amyloid fibril formation of the R68X mutant. After 1 h without TFE (A1,A2), with 3% of TFE (B1,B2) and after 3 days of fibrillation with 1% of TFE added to the solution (C).

CD spectroscopy

CD spectra were measured using an Aviv model 62 ADS CD spectropolarimeter equipped with a thermoelectric sample holder for temperature control in the cell; 0.1 cm and 1 cm cells at bandwidths of 1 nm and 0.5 nm were used to record far- and near-UV CD spectra, respectively. Temperature was maintained at 25°C throughout. Data in the far-UV were collected every 1 nm and in the near-UV, every 0.5 nm. Protein concentrations were such that $A_{280} = 0.1$ for the far-UV CD and 1.2 for the near-UV CD.

For urea denaturation, proteins were dissolved in different concentrations of urea (0–7.5 M) in 0.02 M phosphate buffer, containing 0.2 M NaCl at pH 7.0, and were equilibrated overnight. The changes in secondary structure were followed at 222 nm, where the largest differences between the native and the unfolded states were observed. A cuvette of 0.1 cm light path was used. Ellipticity values at 252 nm and 222 nm were obtained by averaging the signal for 30 sec at each wavelength. The urea stock solution concentration was determined by refractive index measurement and the solution adjusted to pH 7.0. Protein samples with different urea concentrations were prepared volumetrically. Protein concentration in each sample was such that A_{280} was 0.3.

For TFE denaturation, proteins were dissolved in different concentrations of TFE (0%–60%) in 0.01 M phosphate buffer, containing 0.1 M NaCl at pH 7.0, or in 0.015 M acetate buffer,

containing 0.15 M NaCl at pH 4.8, 2 h before the measurement of the far-UV CD spectra. An 0.1 cm light-path cuvette was used and the absorbance A_{280} of proteins was 0.22.

CD spectra were expressed in the usual units. The measured values of ellipticity were transformed to mean residue ellipticity $[\Theta]_{MRW}$ (degcm²/dmol) based on the equation

$$[\Theta]_{MRW} = \frac{\Theta \times M_{MRW}}{10 \times c \times l}$$

where M_{MRW} is the mean residue molecular mass (g/mol), c is the concentration of the protein (mg/mL), and l is the path length of the ray through the cell (cm). Data for molecular masses (M), mean residue molecular masses (M_{MRW}), and extinction coefficients (E_{280}) for the wt stefin B, the G4R mutant, and the R68X mutant are shown in Table 1.

ThT fluorescence measurement

ThT dye was used to determine the presence of amyloid-like fibrils. Fluorescence was measured using a Perkin-Elmer model LS 50 B luminescence spectrometer. For ThT emission, excitation was at 440 nm and spectra were recorded from 455 nm to 600 nm. ThT was dissolved in phosphate buffer (25 mM, 0.1 M NaCl at pH 7.5) at 15 μ M ($A_{416} = 0.6$). Fibrils were grown under mild conditions at pH slightly < 5 (0.015 M acetate buffer, 0.15 M NaCl at pH 4.8); protein concentrations were 34 μ M. Fibril formation was accelerated by adding low concentrations of TFE. Eighty microliters of the protein solution in which fibrils were growing were added to 575 μ L of the ThT buffer just before the measurement. ThT fluorescence was measured using 0.5 cm cuvette at 25°C. Excitation and emission slits were set at 5 nm and 7 nm, respectively. Data were collected every 0.5 nm.

TEM

Protein samples (15 μ L of 34 μ M protein solution) were applied on a Formvar and carbon-coated grid. After 10 min the sample was soaked away and stained with 1% uranyl acetate. Samples were observed with a Philips CM 100 transmission electron microscope operating at 80 kV. Images were recorded by Bioscan CCD camera Gatan, using Digital Micrograph software.

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