Amyloid-forming peptides selected proteolytically from phage display library

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(RECEIVED October 10, 2002; FINAL REVISION January 8, 2003; ACCEPTED May 21, 2003)

Abstract

We demonstrated that amyloid-forming peptides could be selected from phage-displayed library via proteolysis-based selection protocol. The library of 28-residue peptides based on a sequence of the second zinc finger domain of Zif268, and computationally designed $\beta\beta\alpha$ peptide, FSD-1, was presented monovalently on the surface of M13 phage. The library coupled the infectivity of phage particles to proteolytic stability of a peptide introduced into the coat protein III linker. It was designed to include variants with a strong potential to fold into $\beta\beta\alpha$ motif of zinc finger domains, as expected from secondary structure propensities, but with no structure stabilization via zinc ion coordination. As our primary goal was to find novel monomeric $\beta\beta\alpha$ peptides, the library was selected for stable domains with the assumption that folded proteins are resistant to proteolysis. After less than four rounds of proteolytic selection with trypsin, chymotrypsin, or proteinase K, we obtained a number of proteolysis-resistant phage clones containing several potential sites for proteolytic attack with the proteinases. Eight peptides showing the highest proteolysis resistance were expressed and purified in a phage-free form. When characterized, the peptides possessed proteolytic resistance largely exceeding that of the second zinc finger domain of Zif268 and FSD-1. Six of the characterized peptides formed fibrils when solubilized at high concentrations. Three of them assembled into amyloids as determined through CD measurements, Congo red and thioflavin T binding, and transmission electron microscopy.

Keywords: Amyloid; phage display; zinc finger; proteolytic selection; Zif268; FSD-1

Amyloids are assemblies of misfolded proteins or peptides that are often associated with tissue damage and degeneration, and occur in pathological states such as Alzheimer's, Parkinson's, Huntington's diseases, type 2 diabetes, and spongiform encephalopathy (Tan and Pepys 1994; Kelly 1996; Dobson 1999). To date, there are ~20 proteins associated with different amyloidoses. These proteins show no sequential or structural similarities, including sequence length. In fact, the ability to form amyloid fibrils is not limited to a small group of proteins involved in pathological

proteins, but appears to be a more common phenomenon found in many unrelated proteins not linked to any pathological state known (Guijarro et al. 1998). Contrary to the observed conformational diversity of soluble proteins that show the propensity to form amyloids, the fibrils they form share several surprisingly common features. Amyloid fibrils are organized in a cross β -structure with a helical array of β -sheets, in which the long axis of the fibril is parallel to the long axis of the helix and is perpendicular to the β -strands (Blake and Serpell 1996). The fibrils are long and unbranched filaments, and they are able to bind diagnostic Congo red (CR; Klunk et al. 1999) and thioflavin T (ThT; LeVine III 1999). Finally, amyloid deposits are strongly resistant to proteolytic degradation.

In vitro experiments show that amyloids can be formed from globular proteins under a variety of partially denaturing conditions (e.g., low pH, high temperature, or moderate

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0236103>.

concentrations of fluoro-alcohols), and there is a body of evidence indicating that amyloids grow from partially folded protein conformation (Chiti et al. 2000; Ramirez-Alvarado et al. 2000). Indeed, mutations that destabilize native protein conformation of transthyretin and lysozyme facilitate amyloid formation through promotion of partially folded intermediate(s) (McCutchen et al. 1993; Hurle et al. 1994). However, there is a limited knowledge about detailed molecular and sequential requirements to develop amyloid.

A model protein studied in this paper is the second zinc finger domain of the DNA binding protein Zif268 (Zif268.2). This domain belongs to Cys₂His₂ zinc finger family and represents the most common DNA-binding motif (>20,000 identified domains in databases) and the most frequently encoded domain in the human genome (Venter et al. 2001). No amyloid formation has been shown for this protein family. The domain contains a $\beta\beta\alpha$ motif of 28 amino acid residues stabilized by zinc ion. Dahiyat and Mayo (1997) showed that this fold could be formed in the absence of metal ions. Their article describes the first fully automated design of a novel sequence (called FSD-1) through extremely extensive computer search of $\sim 10^{27}$ possible sequences. Here, we applied biological approach of

proteolytic monovalent phage display selection to search for foldable sequences of a $\beta\beta\alpha$ motif. Surprisingly, we found that although no stable, well-ordered, monomeric structures could be identified, some of the selected peptides easily formed amyloid fibrils. Because we failed to demonstrate amyloid formation both for Zif268.2 and FSD-1 peptides, the properties of amyloidogenic sequences are compared with those of both template peptides.

Results

Library design

We designed a library (called $\beta\beta\alpha$) on a template sequence of the second zinc finger domain of murine transcription factor Zif268 (Zif268.2), which is built of 28 amino acid residues. The library comprised $1.4 \cdot 10^7$ sequences, which were intended to provide stable $\beta\beta\alpha$ domains without zinc ion coordination (Fig. 1). To allow stable fold formation, hydrophobic residues were introduced at positions that are significantly buried in the crystal structure of Zif268.2 domain (Elrod-Erickson et al. 1996). We also introduced amino acids of high propensity for respective secondary

Figure 1. $\beta\beta\alpha$ Library design. (A) The ribbon drawing of $\beta\beta\alpha$ motif of Zif268.2 with zinc ion coordination site shown. (B) Randomization sites of $\beta\beta\alpha$ library (labeled as dark regions on the ribbon drawing). (C) Comparison of sequences of Zif268.2 and designed $\beta\beta\alpha$ library. Zinc ion coordination site of Zif268.2 and randomized positions of $\beta\beta\alpha$ library are shown in bold. Unintentionally introduced amino acids resulting from restrictions of genetic code are shown in brackets. The structure ribbons were prepared by using the MOLMOL program (Koradi et al. 1996).

structure segments to increase probability that polypeptide chains adopt a desired conformation. The DNA-binding region of Zif268.2 (Elrod-Erickson et al. 1996) was generally remained unchanged (position 10, segment 13–17, partially allowed at positions 20 and 22). As biological methods impose limits on a library size, we applied only partial randomization. In total, 11 of 28 amino acid residues present in Zif268.2 were randomized with two to nine different amino acid residues introduced (Fig. 1). The following reasoning was applied in the design of the $\beta\beta\alpha$ library. Four positions defining zinc ion coordination site (Cys5, Cys8, His21, and His25) were eliminated. Exclusively alanine was allowed at the only fully buried position 5, in agreement with the computer design of Dahiyat and Mayo (1997). Cys8, located in the exposed turn, was mutated to nine polar amino acids. Histidines 21 and 25 as potential core forming residues were mutated to hydrophobic amino acids. Other randomization sites included positions 3, 4, and 11 of the β -strand region, position 7, and positions 19, 20, 23, and 24, forming a part of the α -helix. Only Tyr and Phe were allowed at position 3 in agreement with consensus observed in 500 sequences of zinc finger domains homologous to Zif268.2 (data not shown). In addition, Tyr and Phe, together with Thr, Ile, and Leu introduced at positions 4 and 11, are of high β -strand forming propensity. Partially buried position 7 was restricted to hydrophobic residues. As the helical region in Zif268.2 shows low propensity to form α -helix in the absence of zinc ion, we randomized positions 19, 20, 23, and 24 to allow amino acids of high helical propensity. In addition, His17 was mutated to Glu, as it was found that both neutral and charged histidine highly destabilized FSD-1 structure (K. Koscielska-Kasprzak and J. Otlewski, unpubl.). Glu17 is present in the first and third zinc finger domains of Zif268 and in case of those domains specific DNA binding is provided by Arg20 instead of residue 17. Because Arg was one of the residues allowed at position 20, we expected that DNA binding property should not be excluded by this substitution. Met8 was mutated to glycine, because Gly is 70% conserved in 500 zinc finger domains analyzed and it is present in FSD-1 sequence.

Library construction and selection

Our selection approach was based on the assumption that folded domains should be resistant to proteolysis. We used the infectivity-linked approach developed by Kristensen and Winter (1998). The $\beta\beta\alpha$ library was inserted into the N₂-C linker of pProtSel phagemid encoded M13 coat protein III, and separated domains responsible for phage infectivity from a phage body. The library contained $>10^8$ independent transformants, and thus, it was considered to be representative. As the native protein III is highly proteolysis resistant, the procedure involved the use of KM13 helper phage, which protein III contained a modified N₂-C linker se-

quence of high proteolytic sensitivity (Kristensen and Winter 1998). Similar sequence was present in the initial pProtSel phagemid. Thus, in the presence of a protease, the infectivity was retained only for phages presenting the library peptides that could not be digested with the enzyme. This approach should enrich the pool in folded sequences, because contrary to unfolded peptides, structured domains are known to be highly protease resistant (Fontana et al. 1986; Buczek et al. 2002). In a control experiment, we showed that both Zif268.2- and FSD-1-presenting phages could be enriched over control (pProtSel-based) phages through applying BTP digestion. Thus, despite their small size and many potential sites for proteinase action, both $\beta\beta\alpha$ domains, when folded, were resistant to proteolysis.

The selection of $\beta\beta\alpha$ library was performed as 3-h digestions of the phage pool in various conditions of buffer, temperature, and proteinase concentration, using three proteinases: bovine trypsin (BTP), bovine chymotrypsin (BCh), and proteinase K (Fig. 2). We observed that after three to four rounds of enrichment, the pool acquired significant proteolytic resistance. The library pools that gained the highest resistance to proteolysis (labeled with gray background on Fig. 2) were subjected to single clone analysis. Several clones from those pools were picked up, sequenced, and analyzed for their proteolytic resistance. From all selection schemes applied, we obtained 45 different peptide sequences (data not shown) with no consensus present. Actually, any selected peptide differed from all other sequenced at 3 to 11 positions. Most of the peptides were characterized to possess a proteolytic resistance largely exceeding that of Zif268.2 and FSD-1. We chose nine peptides (Table 1) showing the highest resistance to proteolysis for further characterization in a phage-free state.

Peptide expression and characterization

We expressed eight peptides in *Escherichia coli* in the form of inclusion bodies with a hydrophobic leader sequence. A single peptide (IVc.5) could not be expressed due to its toxic effect on bacteria cell growth. After purification in a leader sequence-free form, all peptides formed soluble or insoluble aggregates, depending on a drying procedure and buffer conditions. The peptides showed a broad range of different CD spectra, with two (IIIb.3 and IIIc.7) most closely resembling that of a $\beta\beta\alpha$ fold (Fig. 3). For three of the peptides (IIIc.4, IIIc.9, and IVe.2), after lyophilized samples were dissolved in water or 10 mM sodium acetate (pH 5.5), we easily noticed a slow decrease of CD signal at 217 nm, indicating a high content of β -structure (Fig. 4). As the observation indicated a possibility that the peptides formed amyloid fibrils, we performed diagnostic tests for amyloid presence by using CD spectroscopy, CR and ThT binding, and transmission electron microscopy (TEM).

Figure 2. Selection conditions applied to $\beta\beta\alpha$ library. Each round of digestion (denoted with roman number and letter) was performed for 3 h under conditions shown on the scheme. Proteolytic resistance relative to control (pProtSel phages), which characterizes given selection round, is shown as a number in brackets next to the round denotation. Library phage pools after different selection stages are shown in boxes; those that were subject to single clone analysis have a gray background, while those double-framed were the source of the peptides characterized. ^aSelection conditions included high bacterial cell to phage ratio (1000 : 1) during infection after proteolytic digestion; in other cases, standard 100 : 1 ratio was maintained.

IIIc.4, IIIc.9, and IVe.2

The spectral and microscopic analysis revealed that those peptides formed typical amyloid fibrils. They bound CR with red shift of the maximum light absorption with the largest difference observed around $\lambda_{\text{max}} = 545$ nm (Fig. 5A), and IIIc.4 showed clear birefringence after CR staining of its aggregates in polarized light (Fig. 5B). The peptides also bound ThT and increased its fluorescence emission at ~ 485 nm after excitation at 440 nm (Fig. 6). All three peptides in aggregated state showed CD spectra with λ_{min} at ~ 217 nm, typical for β -structure-rich peptides (Fig. 7) resembling those reported for amyloids (Fezoui et al. 2000; Wilkins et al. 2000). Their conformations in a monomeric state fairly showed any similarities among themselves, as judged from different far-UV CD spectra (Fig. 3). IIIc.4 and IIIc.9 also in a monomeric state had CD spectra indicative of high content of β -structure.

The fibrils formed by all three peptides could be disaggregated upon heating (Fig. 8). The thermal transition mea-

surements were performed on a 200-fold diluted sample and monitored by CD. The cooperativity and transition temperature (T_{dis}) depended on fibril age: For 20-d-old IIIc.4 and IIIc.9, the cooperativity was high and T_{dis} values were 44°C and 65°C , respectively. IVe.2 transition was much less cooperative, but T_{dis} was in a similar range ($T_{\text{dis}} = 49^\circ\text{C}$). Thermal transition was reversible only for IIIc.9, and immediately after cooling, we observed $\sim 20\%$ of the initial ellipticity. After the same incubation time, the sample showed $\sim 80\%$ of the initial CD spectrum observed before recording of the first transition. The sample could be repeatedly disaggregated, albeit with lower transition temperature and lower cooperativity, which was probably due to 200-fold lower peptide concentration and different buffer conditions compared with initial ones. This clearly reveals that IIIc.9 peptide shows a very strong propensity to form amyloid fibrils. No reversibility could be demonstrated for IIIc.4 and IVe.2, even after 1 week of incubation at 4°C of 200-fold diluted samples. Finally, all three peptides formed fibrillar structure visible by TEM (Fig. 9).

Figure 3. Far-UV CD spectra of HFIP-treated peptides. Spectra were measured in 1-mm cuvette in 10 mM sodium acetate (pH 5.5), at 50 μ M peptide concentration, directly after >100-fold dilution of HFIP-solubilized peptides. (A) Reference spectra of $\beta\beta\alpha$ peptides: FSD-1 (squares), unfolded Zif268.2 with no zinc ion coordinated (solid circles), and folded Zif268.2 domain (empty circles). (B) CD spectra of the amyloid-forming peptides: IIIc.4 (squares), IIIc.9 (solid circles), and IVe.2 (diamonds). (C) CD spectra of the other $\beta\beta\alpha$ library peptides: IIIa.1 (asterisks), IIIb.1 (crosses), IIIb.3 (squares), IIIc.7 (solid circles), and IVe.4 (diamonds).

IIIc.7, IIIa.1, and IVe.2

We classified properties of these peptides as fibril-forming, although no clear indication of amyloid structure formation could be observed. After 1 month of incubation, all three were visible as fibrils in TEM (Fig. 9). However, with

the exception of weak CR (with no shift in absorption spectrum) and ThT binding (Fig. 6), other hallmarks of amyloid formation were absent. No characteristic CD spectra, indicative of a presence of β -structure, were observed (Fig. 7). In agreement, we found only monotonic changes rather than cooperative transition of CD signal at 215 nm upon increase of temperature. However, the CD spectrum of IIIc.7 in the monomeric state showed a clear α -helix signature and the closest resemblance to the spectrum of $\beta\beta\alpha$ folded control peptides (Fig. 3). The strongest α -helix signature was also observed for its aggregated state spectrum.

IIIb.1 and IIIb.3

These two peptides when left for a prolonged period of time produced only amorphous aggregates. No CR or ThT binding could be demonstrated for these peptides. Their CD spectra showed no indication of β -structure content even after a prolonged incubation (Fig. 7). However, in case of IIIb.3, CD spectra both in monomeric and aggregated state showed some α -helix content. For both peptides, no thermal transitions monitored by CD signal could be observed. In TEM experiments, they were visible as amorphous aggregates (Fig. 9).

Discussion

In this article, we found that proteolytic selection of the designed $\beta\beta\alpha$ library comprising $>10^7$ amino acid sequences presented in M13 coat protein III-fused form led to enrichment in clones that are strongly resistant to trypsin, chymotrypsin, and/or proteinase K digestion compared with folded proteins (Zif268.2 and FSD-1) presented on the

Figure 4. Far-UV CD spectra of IIIc.4 acquired during aggregation process. Freshly lyophilized peptide was solubilized in water at 1 mM and incubated at 4°C. After certain incubation times—no incubation (empty diamonds), 24 h (light gray diamonds), 52 h (dark gray diamonds), and 76 h (solid diamonds)—the peptide sample was diluted to 50 μ M in 10 mM sodium acetate (pH 5.5), and CD spectrum was measured. The CD spectrum of the HFIP-treated peptide is shown for comparison (solid line).

Figure 5. CR binding to the peptides. (A) Differential absorption spectra of CR binding to the selected peptides. The absorption spectra were measured for 10 μ M CR and 6 μ M peptide in 10 mM sodium phosphate (pH 7.5) and 150 mM NaCl after 30-min incubation at room temperature, and they are presented after subtraction of dye spectrum. Data are shown for all selected peptides: amyloid-forming IIIc.4 (solid squares), IIIc.9 (solid circles), and IVe.2 (solid diamonds); fibrillar IIIa.1 (empty squares), IIIc.7 (empty circles), and IVe.4 (gray diamonds); amorphous IIIb.1 (empty triangles) and IIIb.3 (gray squares); and FSD-1 (crosses) and Zif268.2 (asterisks) as a control. (B) Optical microscope image showing the birefringence of CR-stained IIIc.4 peptide in polarized light compared to the bright field image of the same sample.

phage surface. The proteolytic selection schemes applied (Fig. 2) worked nicely leading to hundred- and thousand-fold increase in proteolytic resistance of the phage pool. Eight most-resistant sequences, when purified in a free form, retained their proteolytic resistance, most probably due to aggregation. Again, this resistance to proteolysis exceeded those of Zif268.2 and FSD-1 when compared as free protein forms (data not shown).

Although proteolysis-based phage display did not allow us to select folded monomeric sequences, the method appeared to be very efficient to find fibril/amyloid-forming sequences. Neither Zif268.2 nor FSD-1 formed any aggregates under conditions applied. Unintentionally, when applying proteolysis as a selection pressure, we enriched the $\beta\beta\alpha$ library by using a method that corresponds with one of amyloid fibril properties. However, it should be stressed

that, most probably, the proteinase(s) selection did not act on aggregated form of peptide due to its monovalent presentation. Therefore, the molecular mechanism selecting fibril-forming peptides cannot be explained at this stage of research. In our opinion, the most probable mechanism of proteolysis resistance relies on interaction of presented peptide with hydrophobic and β -structure-rich surface of the N_1 and N_2 domains of coat protein III (Lubkowski et al. 1998). Such peptide-coat protein domains association might effectively prevent proteolytic degradation of protein III-fused peptide. To test this hypothesis, we are currently preparing several protein samples for crystal structure determination containing selected peptides fused to the N_1 and N_2 domains in the same orientation as in pProtSel vector.

We would like to stress that some properties of the selected peptides are in agreement with this reasoning. The

Figure 6. Differential emission fluorescence spectra of ThT in the presence of the peptides. The fluorescence emission spectra of 10 μ M ThT were measured in 20 mM sodium phosphate (pH 7.5) alone or in the presence of 2 or 1 μ M (IIIc.9) peptide after excitation at 440 nm. Peptides were incubated at 1 mM in water or 10 mM sodium acetate (pH 5.5; IVe.2) for 5 (IIIc.6 and IIIc.9) or 20 d. Amorphously aggregating peptides (IIIa.1 and IIIb.3) and Zif268.2 resulted in no difference in ThT spectrum. (A) Amyloid forming peptides: IIIc.4 (solid circles), IIIc.9 (dark gray circles), and IVe.2 (light gray circles). (B) Other fibrillar aggregates forming peptides: IIIa.1 (solid circles), IIIc.7 (dark gray circles), and IVe.4 (light gray circles).

overall hydrophobicity of these peptides is significantly higher than that of Zif268.2 and FSD-1 (Table 1). Second, although segment 19–25 was designed to improve its helical properties, we observed that nonhelical Thr resulting from applied randomization scheme was often found in the selected peptides. Together with the selection of β -structure forming residues at β -strand region and potential core positions, it resulted in generally increased β -structure propensity of the selected peptides, when compared with initial library clones and FSD-1 (Table 1). We suppose that selection proceeded in a concerted way enhancing both hydrophobicity and β -propensity of the peptides, rather than sequence properties observed in FSD-1. Zif268.2 sequence, due to a strong influence of zinc ion on peptide conformation should not be used as reference sequence in such analysis.

Amino acid sequences of eight characterized peptides differ among themselves at five to 10 positions. To find possible sequence-specific reasons for the observed differences in propensities to form amyloids, fibrils, or amorphous aggregates, we analyzed hydrophobicity parameter together

with β - and α -propensities along the peptide sequences. We did not find any sequential patterns, including binary pattern (West et al. 1999), that might indicate why only some of the peptides were able to form amyloids in the simple conditions applied. However, the peptides growing into organized amyloid fibrils showed a higher content of β -structure also in their monomeric state (particularly IIIc.4 and IIIc.9) when compared with those aggregating in a more random way.

Aggregated state of the eight peptides studied in a free form could be divided into three groups: amyloid (3), fibrillar (3) and amorphous (2). Although six of these peptides formed fibrils only for three of them, all hallmarks of amyloid formation could be demonstrated. Remaining three fibrillar peptides did not exhibit β -sheet-like CD spectrum, what we considered as a major feature of amyloid structure. However, two of these peptides were able to weakly bind CR and ThT. We cannot exclude possibility that the three fibril-forming peptides could form amyloids under carefully screened conditions.

Figure 7. Far-UV CD spectra of the aggregated state of the peptides. Spectra were measured in 1-mm cuvette in 10 mM sodium acetate (pH 5.5) at 50 μ M peptide concentration, after 20-d incubation at 1 mM in water or 10 mM sodium acetate (pH 5.5; in case of IVe.2). (A) CD spectra of amyloid fibrils: IIIc.4 (squares), IIIc.9 (circles), and IVe.2 (diamonds). (B) CD spectra of other aggregated peptides: IIIa.1 (asterisks), IIIb.1 (crosses), IIIb.3 (squares), IIIc.7 (circles), and IVe.4 (diamonds).

Figure 8. Thermally induced disassembly of 20-d-old amyloid fibrils monitored by CD. Changes in ellipticity for IIIc.4 (light gray circles), IIIc.9 (dark gray circles), and IVe.2 (solid circles) fibrils were followed in 10-mm cuvette at 215 nm immediately after 200-fold dilution of fibrils in 10 mM sodium acetate (pH 5.5).

Properties of the peptides were investigated also in their monomeric state. Although we applied neat 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), which is known to be an efficient disaggregating agent (Barrow et al. 1992), we had no direct evidence that the peptides retained monomeric properties when they were 100-fold diluted for spectral measurements. Therefore, differences in CD spectra shown on Figure 3, as well as resulting conformational preferences of the monomeric peptides, must be treated with caution. Nevertheless, these spectra reveal serious conformational differences among studied peptides. In case of IIIc.4 and IIIc.9, the CD spectra indicated high presence of β -structure. The IIIc.9 peptide in aggregated state showed the highest T_{dis} value accompanied with the most cooperative disaggregation transition, and could easily reassemble into β -structure-rich aggregate even at low concentration. Based on these features, we think that this is the best amyloidal peptide of studied here and that it can serve as an excellent model peptide in future studies. In contrast, in case of IIIb.3 and IIIc.7 peptides, we observed an α -helix signature in their CD spectra, pointing the possibility that they form some $\beta\beta\alpha$ fold but their high hydrophobicity impairs monomeric structure analysis. Interestingly, these two peptides show the highest α - and the lowest β -propensity in the helical (19–25) segment of the target $\beta\beta\alpha$ fold.

Application of proteolytic selection has been proven to be an efficient method to find folded and stable proteins (Sieber et al. 1998; Finucane and Woolfson 1999; Riechmann and Winter 2000; Martin et al. 2001). In fact, we cannot exclude possibility that our library included natively folded variants. In our case, perhaps a relatively small size of protein drove the selection toward hydrophobic and β -structure-rich peptides that were able to escape proteolysis by interaction with neighboring phage domains rather

than by formation of an independent fold. Because proteolytic resistance of selected clones significantly exceeded those of Zif268.2 and FSD-1, natively folded and less hydrophobic $\beta\beta\alpha$ peptides had little chance to be enriched. The phage display rule “you get what you select for” (Zhao and Arnold 1997) worked superbly in our study.

Figure 9. Negatively stained transmission electron micrographs of 1-month-old aggregates of the selected peptides. A magnification of 40,000 was used for all images. Bars, 400-nm. (A) Amyloid fibrils (IIIc.4, IIIc.9, and IVe.2 peptides). (B) Fibrillar aggregates (IIIa.1, IIIc.7, and IVe.4 peptides). (C) Amorphous aggregates (IIIb.1 and IIIb.3 peptides).

Table 1. Proteolytically selected peptides that were chosen for further characterization in a phage-free form

Library	Peptide	Peptide sequence					P _β ^a	P _β ^b 19–25	P _α ^b 19–25	Hydroph. ^c	pI	Protein res. ^d	Aggregate
		3 4	7 8	11	19–21	23–25							
BBA ^{prot}							0.93	0.97	1.13	0.40 (0.45)	9.76	28	—
BBA ^{IIIa}							0.95	1.08	1.13	0.44 (0.55)	8.74	1300	—
BBA ^{IIIb}	IIIa.1	K P Y I A R F E G R L F S R S D E L R A V I E A H T G E	0.94	1.02	1.21	0.43 (0.53)	7.13	2600	fibrillar				
	IIIb.1	K P F T A R F H G R L F S R S D E L R G I I E K L T G E	0.95	1.04	1.08	0.43 (0.52)	10.37	3500	amorphous				
	IIIb.3	K P Y T A R F K G R L F S R S D E L K R I I E A L T G E	0.95	1.05	1.21	0.42 (0.49)	10.23	3700	amorphous				
BBA ^{IIIc}							0.96	1.08	1.14	0.45 (0.57)	9.59	740	—
	IIIc.4	K P F T A R F E G R I F S R S D E L R A L I T E I T G E	0.96	1.12	1.16	0.45 (0.57)	6.52	2100	amyloid				
	IIIc.7	K P F I A R F E G R L F S R S D E L K A I I K E L T G E	0.94	1.03	1.23	0.46 (0.59)	8.96	1400	fibrillar				
	IIIc.9	K P F L A R V E G R I F S R S D E L R A Y I T A Y T G E	0.99	1.19	1.02	0.45 (0.58)	8.72	1300	amyloid				
BBA ^{IVc}							0.97	1.11	1.14	0.45 (0.57)	10.72	1500	—
BBA ^{IVe}	IVc.5	K P F I A R L S G R I F S R S D E L R T F I A K L T G E	0.99	1.14	1.12	0.48 (0.64)	11.07	5700	not expr.				
							0.99	1.13	1.10	0.45 (0.56)	10.95	2000	—
	IVe.2	K P F T A R I S G R L F S R S D E L K T I I A T I T G E	1.00	1.25	1.07	0.48 (0.65)	10.35	4900	amyloid				
	IVe.4	K P F L A R F R G R I F S R S D E L R T L I A A F T G E	0.99	1.15	1.15	0.48 (0.65)	11.46	2300	fibrillar				
Zif268.2		K P F Q C R I C M R N F S R S D H L T T H I R T H T G E	1.01	1.12	0.94	0.39 (0.40)	9.94	250	no				
FSD-1		Q Q Y T A K I K G R T F R N E K E L R D F I E K F K G E	0.96	0.99	1.21	0.33 (0.38)	10.50	5.5	no				

Table includes only phage clones that were further characterized in a phage-free form and the respective library pools. Randomized positions are shown in bold. Parameters given for library pools are mean values for all single clones analyzed for a given pool.

^a β-structure propensity according to Chou and Fasman (1978) calculated for the whole sequence.

^b β- or α-structure propensity according to Chou and Fasman (1978) calculated for the 19–25 residue segment.

^c Zero to one normalized hydrophobicity calculated according to Kyte and Doolittle (1982) for the whole peptides and for the randomized positions (shown in brackets); the higher value indicates the more hydrophobic sequence.

^d Proteolytic resistance of phages presenting the peptides shown relative to resistance of the control (pProtSel) phages.

Materials and methods

Materials

Bacterial strains, *E. coli* XLI-blue, TG1, and BL21(DE3)pLysS were purchased from Stratagene. KM13 helper phage and pK1 vector (Kristensen and Winter 1998) were a gift of Dr. G. Winter (University of Cambridge, UK); pComb3H_{SS} (Barbas III and Wagner 1995), from Dr. C.F. Barbas (The Scripps Research Institute, California); and pAED4 expression vector, from Dr. P.S. Kim (MIT, Cambridge, MA).

Phage display procedures

ββα Library construction

The ββα library was introduced into pProtSel vector, which was a modified pK1 vector. Two *Xho*I restriction sites present in pK1 were removed, and *Xho*I and *Spe*I restriction sites were introduced in N₂-C linker region of M13 coat protein III gene by using Pfu Turbo DNA polymerase-based mutagenesis to obtain the pProtSel vector. The library was subcloned into introduced *Xho*I and *Spe*I sites. For control experiments, Zif268.2 and FSD-1 genes were annealed from synthetic oligonucleotides and subcloned into *Xho*I and *Spe*I sites of pProtSel.

The library was constructed through cassette mutagenesis. The ββα library insert was annealed from two oligonucleotides (with complementary region underlined): prLib^{Xho}I[fwd], GAC GCT CGA GCG GCA AAC CGT [AT]C[AT] [CT]AG CGC GT[ACGT] TC[ACG] [AG][AT]G GCC GT[AT] [CT]A TTC AGC CGC AGC GAT GAG CTG, and prLib^{Spe}I[rev], GTC GTC ACT AGT GGA TCC ACC CTC GCC GGT A[AT][ACGT] C[GT][CT] C[GT][CT]

GAT A[AT][ACGT] T[CGT][CT] T[CT]T CAG CTC ATC GCT GCG GCT G. The annealed oligonucleotide pair was converted to fully double-stranded by using Sequenase (version 2.0), digested with *Xho*I and *Spe*I, and ligated to pProtSel vector. The ligation product was electroporated to *E. coli* TG1 and spread on 25 × 25-cm agar plates containing 2% glucose enriched 2× TY^{amp} (1.6% peptone, 1% yeast extract, 0.5% NaCl, 100 μg/mL ampicillin) medium. After 8 h at 37°C, the cells were scraped from the plates, and the library was stored as 30% glycerol stock. To obtain the library presenting phages, bacterial stocks (10¹⁰ cells) were transferred to liquid medium and infected with KM13 helper phage. The phages were propagated 4 h at 37°C and precipitated by using standard procedures (Kiczak et al. 2001).

Library selection

The selection of ββα library was based on proteolytic digestion of phage pools with one of the following proteinases: BTP, BCh, or proteinase K. Digestions of 10¹¹ to 10¹² cfu/mL library phages were performed in 50 mM sodium phosphate (pH 7.4) or 50 mM sodium acetate (pH 5.5) by using various enzyme concentrations and incubation temperatures. After 3 h of incubation, the log phase *E. coli* TG1 were infected with the digestion mixture. In the second selection series, the 1 : 1000 phage to bacterial cell ratio was maintained, otherwise standard 1 : 100 ratio was used. The cells were then grown for 8 h on 25 × 25-cm agar plates containing 2% glucose-enriched 2× TY^{amp} medium. Further procedure was similar as in the case of the initial library phage propagation. After each selection round, the titers of the initial and digested phage pools were verified to follow the selection process. After a few rounds of enrichment, single clones were sequenced and analyzed for their resistance against proteolysis. Determination of proteolytic resistance of single clone phages (10¹¹ cfu/mL) was performed at 4°C

in 50 mM sodium phosphate (pH 7.5) or 50 mM sodium acetate (pH 5.5) containing 10^{-7} M bovine trypsin.

Peptide characterization

Peptide expression and purification

Zif268.2 was subcloned into pGex-2T vector, expressed in *E. coli* BL21(DE3) pLysS, and initially purified using standard procedure for glutathione-s-transferase fused proteins. After factor Xa cleavage of a fusion protein, the peptide was purified with high-performance liquid chromatography (HPLC) on a C₁₈ column (Vydac) with acetonitrile gradient in 0.1% TFA.

FSD-1 and library clones selected for further characterization in a phage-free form were expressed in *E. coli* BL21(DE3)pLysS in T7 promoter-based system of pAED4 vector as fusion with hydrophobic leader sequence. Fusion proteins were purified from inclusion bodies and cleaved with cyanogen bromide, and free peptides were double purified with HPLC on C₁₈ column with acetonitrile gradient in 0.1% TFA. The peptide identity was verified by using MALDI mass spectrometry. All measurements were performed with freshly HPLC purified and lyophilized peptide samples to avoid uncontrolled aggregation resulting from drying or storage conditions. Peptide concentration was determined through peak area integration of chromatographs obtained from linear acetonitrile gradient HPLC runs and was corrected for differences in number of aromatic acid residues. For measurements in a disaggregated state, the peptides were additionally incubated 24 h at room temperature in HFIP at a concentration of 5 mg/mL. The solution was partially evaporated and diluted at least 100 times directly before measurements. When the aggregated state of the peptides was to be characterized, 1 mM peptides were incubated for up to a month at 4°C in deionized water or 10 mM sodium acetate (pH 5.5), and were diluted to concentrations needed for given analysis methods directly before measurements.

Resistance against proteolysis

Samples of 50 μ M peptides were incubated 1 h at 4°C in 50 mM sodium acetate (pH 5.5) or 50 mM sodium phosphate (pH 7.5) with various concentrations of bovine trypsin (up to 8×10^{-6} M at pH 5.5 and up to 8×10^{-7} M at pH 7.5), and analyzed on SDS gel electrophoresis according to (Schägger and von Jagow 1987).

CD measurements

CD spectra of the peptides were recorded in 190- to 250-nm wavelength range in 10 mM sodium acetate (pH 5.5) at 4°C on a Jasco J-715 spectropolarimeter. Measurements were performed at a protein concentration of 50 μ M using a 1-mm cuvette. Spectra were averaged from 10 separate scans. Thermal disassembly was monitored after the ellipticity at 215 nm by using band slit of 2 nm and 16-s response time. Automatic Peltier accessory, PFD 350S, allowed continuous monitoring of the thermal transition at a constant rate of 45 deg/h. Temperature of the sample was monitored directly by using a probe immersed in the cuvette and controlled with PFD-350S/350L Peltier type FDCD attachment.

CR and ThT binding

Absorption spectra of free CR (10 μ M, $\epsilon_{505} = 5.93 \times 10^4$ AU/cm/M; Klunk et al. 1999) and in the presence of peptide aggregates (30 μ M) were measured in 10 mM sodium phosphate (pH 7.5) and 150 mM NaCl. For CR birefringence experiments, 1 mM peptide

solution was incubated as a hanging drop (2 μ L) on a microscopic slide in 4°C, stained with 2 μ L of 250 μ M CR in 10 mM sodium acetate (pH 5.5), and visualized in bright and polarized light at 65-fold magnification.

ThT binding experiments were performed in 20 mM sodium phosphate (pH 7.5) containing 10 μ M ThT alone or with up to 5 μ M peptide; 450- to 600-nm fluorescence emission spectra were measured after 440-nm excitation (5-nm band width) by using a Jasco FP-750 fluorescence spectrophotometer.

Transmission electron microscopy

Samples of 1 mM peptide solution were incubated for 1 month at 4°C. Prior measurements were fivefold diluted, and 2 μ L of each were mixed with equal volume of 0.5% phosphotungstic acid in 70% ethanol. The mixtures were applied to a Formvar and carbon-coated nickel grids (400 mesh), air dried, and examined in a Zeiss EM 900 transmission electron microscope at an accelerating voltage of 80 kV.

Acknowledgments

This work was supported by grant 6 PO4B 014 17 from the Polish Committee for Scientific Research. We thank the Foundation for Polish Science for support. J.O. is an International Scholar of the Howard Hughes Medical Institute. We thank Dr. Greg Winter for pK1 vector and KM13 helper phage, Dr. Janusz Kubrakiewicz for help with transmission electron microscopy, and Dr. Michal Dadlez for mass spectrometry analysis.

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