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Amyloidogenic Properties of the Artificial Protein Albebetin and Its Biologically Active Derivatives. The Role of Electrostatic Interactions in Fibril Formation

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Abstract—The artificial protein albebetin (ABB) and its derivatives containing biologically active fragments of natural proteins form fibrils at physiological pH. The amyloid nature of the fibrils was confirmed by far UV circular dichroism spectra indicating for rich β-structure, thioflavin T binding assays, and examination of the obtained polymers by atomic force microscopy. Fusing of short peptides—octapeptide of human α_2-interferon (130-137) or hexapeptide HLDF-6 (41-46) of human leukemia differentiation factor—with the N-terminus of ABB led to increased amyloidogenicity of the protein: the rate of fibril formation increased and the morphology of fibrils became more complex. The presence of free hexapeptide HLDF-6 in the ABB solution had the same effect. Increasing ionic strength also activated the process of amyloid formation, but to less extent than did the peptides fused with ABB or added to the ABB solution. We suggest an important role of electrostatic interactions in formation of ABB fibrils. The foregoing ways (addition of salt or peptides) allow decrease in electrostatic repulsion between ABB molecules carrying large negative charge (–12) at neutral pH, thus promoting fibril formation.

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Key words: fibrilogenesis, amyloid, artificial protein, albebetin, albeferon, hexapeptide HLDF-6, differentiation factor HLDF

Many studies undertaken during the last decade have been devoted to protein folding and its disturbance in amyloid diseases, such as Alzheimer's disease, Parkinsonism, Down's syndrome, prion diseases (Mad Cow and Creutzfeldt–Jakob diseases), hereditary amyloid neuropathy, and systemic amyloidoses. These diseases with frequent lethal outcome are caused by improper folding of protein molecules and their aggregation leading to formation of amyloid deposits [1, 2]. Structures resulting from association of monomeric molecules into stable polymeric chains capable of unlimited elongation were named amyloid fibrils. The overall weight of amyloid deposits in human and animal tissues and organs can achieve several kilograms, in particular, in the case of fibrillar agglomerates of lysozyme in liver [3]. Curing of patients with this disorder is impossible without liver transplantation.

In this study, we investigate fibrilogenesis of the artificial protein albebetin (ABB) and its derivatives. These proteins have no analogs in nature, but can form fibrils, so investigation of these proteins is important for general understanding of molecular mechanisms of fibrilogenesis as a fundamental process depending on properties of the polypeptide chain and for understanding the nature of interactions leading to fibril formation.

Abbreviations: ABB) artificial protein albebetin; ABB-df) albebetin with adjoint fragment ^{41}TGENHR^{46}; ABBI) albebetin with adjoint octapeptide LKEKKYSP of human α_2-interferon; AFM) atomic force microscopy; HLDF) human leukemia differentiation factor (produced by the human promyelocytic leukemia cell line HL-60); HLDF-6) hexapeptide fragment ^{41}TGENHR^{46} of the HLDF molecule.

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Amyloidoses are associated with proteins such as transthyritin, Aβ-peptide (β-Amyloid Peptide), prion proteins, insulin, lysozyme, and so on. These proteins have non-homologous amino acid sequences and differ from each other in their secondary and tertiary structures. However, fibrils formed by all these proteins have similar structure characterized by crossed β-sheets in central core [4, 5].

Fibrilogenesis is a cause of various pathologies, but, at the same time, is a functional process of critical importance for some organisms [6]. For example, in spinning glands of spiders, spidroin is transformed into β-structured fibrils [7, 8], and extracellular fibrils of *Escherichia coli* and *Salmonella* are found to facilitate formation of bacterial colonies [9]. Mammalian melanocytes produce the glycoprotein Pmel17, which is polymerized into amyloid-like fibrils [10]. Many proteins that are not associated with amyloid diseases can nonetheless form fibrils *in vitro* [11-15]. It was found recently that some artificial proteins can form amyloid as well [16]. Thus, it is arguable that fibrilogenesis is a common characteristic of protein molecules, which are not confined to a narrow class of so-called "disease-producing" proteins [11].

In this study, we have investigated amyloid properties of the artificial (or *de novo*) protein ABB and its biologically active derivatives. This protein possessing unique topology was constructed on the basis of theoretical principles of globular protein structure formation [17]. Two α-helices of albebetin are juxtaposed to one of the planes of antiparallel β-sheet formed by four bands (Fig. 1). Compactness of ABB is provided by interactions between polar residues and hydrophobic interactions in the central β-sheet. The number of possible conformations is limited due to the presence of short loops connecting secondary structure elements. Detailed examination of ABB by physicochemical methods has shown that this artificial protein has predetermined secondary structure and nearly predetermined spatial structure, and its stability is little less than those of natural globular proteins of similar molecular mass [17, 18].

ABB has been used as an inert carrier matrix for biologically active fragments of natural proteins [19-25]. Albebetin derivatives were constructed by adjunction of short sequences to its N-terminus (Fig. 2). ABBI contains the octapeptide ^{130}LKEKKYSP^{137} from the consensual sequence of human α_2-interferon [18, 19], and ABB-df [20] contains the hexapeptide HLDF-6 ^{41}TGENHR^{46} [21] from the human leukemia differentiation factor (HLDF) produced by the human promyelocytic leukemia cell line HL-60 [22]. The derivative proteins maintain predetermined secondary structure, seem to maintain flexible tertiary structure of ABB, and possess functions proper to the biologically active fragments. ABBI, called "albeferon", possesses activity of interferon-α_2 causing blast transformation of thymocytes [23], and ABB-df induces differentiation and suppresses proliferation of the human promyelocytic leukemia cell line HL-60 [20]. Moreover, it was shown that both albebetin and albeferon have a relatively low immunogenicity [24, 25].

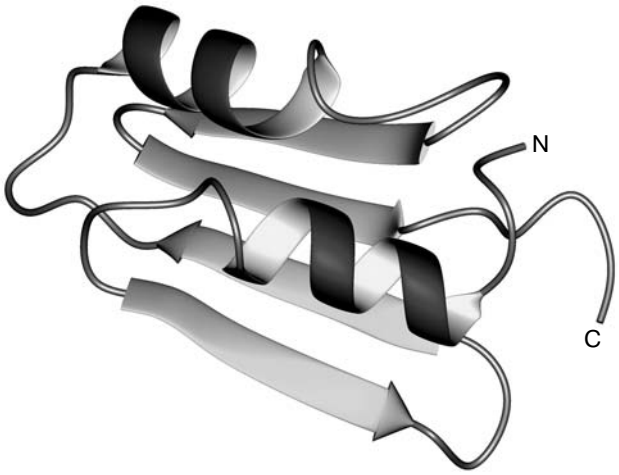

Fig. 1. Proposed spatial structure of ABB.

In the study presented, we show that ABB can form fibrils in spite of good solubility and high negative charge of the protein at neutral pH. We have also examined kinetic and morphological alterations in ABB fibrilogenesis due to adjunction of short sequences to the N-end of the protein molecule or addition of free hexapeptide to the protein solution or changing of ion strength.

MATERIALS AND METHODS

Materials. Genes encoding ABB and its biologically active derivatives were expressed in *E. coli* strain BL21(DE3)pLysS as hybrid constructions with the thioredoxin gene. The artificial proteins were isolated and purified using a combination of ion exchange and metal chelating chromatography [20]. Following the site-specific cleavage of thioredoxin by Xa factor, the artificial proteins were purified using metal chelating chromatography and dialysis. HLDF-6 hexapeptide was synthesized by the solid-phase Boc/Bzl method. The peptide was purified by FPLC on a PepRPC^{TM} column (FPLC-System; Pharmacia, Sweden). The purity of the resulting sample was evaluated by amino acid and mass-spectrometry (MALDI-MS) analyses [21].

Fibril formation of artificial proteins was carried out at the protein concentration of 20 mg/ml in 20 mM Tris-buffer, pH 7.3, containing 0.2% NaN_3 at room temperature or under heating at 57°C. The hexapeptide HLDF-6 was also incubated at 20 and 57°C for 4 weeks at the concentration of 30 mM.

Circular dichroism spectra. CD spectra were recorded on a Jasco J720 CD UV spectropolarimeter (Jasco,

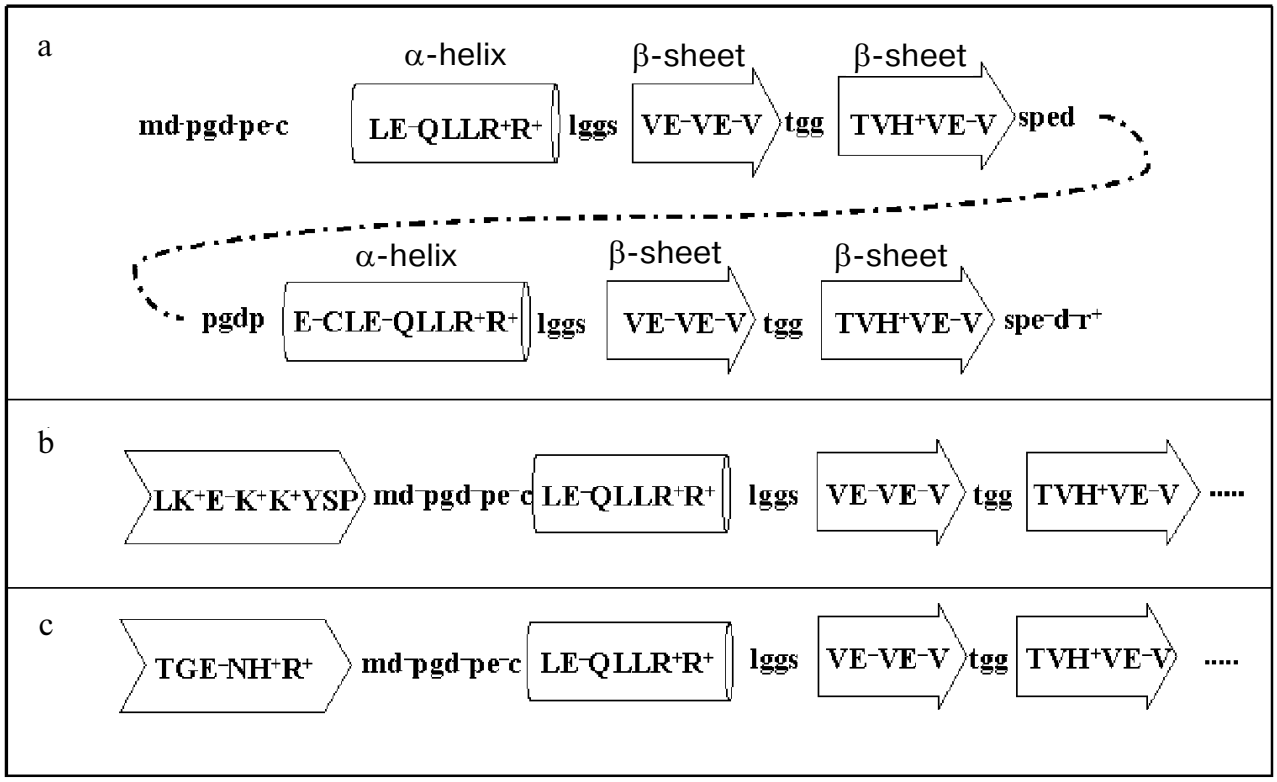

Fig. 2. Amino acid sequences of ABB (a) and its derivatives. Adjoint peptides of α_2-interferon (b) and human leukemia differentiation factor (c) are indicated by arrows. Positively and negatively charged amino acids are marked by signs + and –, respectively.

Japan) with a thermostatted cell. The samples were placed into quartz cells with light path of 0.01 cm (Hellma, Germany). The far UV CD spectra (190-250 nm) were recorded in the temperature range from 20 to 80°C with step of 3-5°C. The samples were incubated for 10 min before the measurement until the thermal equilibrium was established.

Fluorescence assay. The fibril formation kinetics was studied via the binding of the fluorescent dye thioflavin T according to a modification of the method of Levine [26]. The aliquots containing 10 μM protein were added to the dye solution. Fluorescence was measured on a FluoroMax-2 spectrofluorimeter (JOBIN YVON/PSEX Instruments S. A., USA). The excitation wavelength for thioflavin T was 440 nm. The absorption spectrum was registered in the wavelength range from 450 to 550 nm and slit width of 5 nm.

Atomic force microscopy (AFM). Morphological analysis of fibrils was carried out by atomic force microscopy. A PICO PLUS atomic force microscope (Molecular Imaging, USA) was used. For microscopic analysis, the protein solutions were diluted from 200 to 1000 times to the final concentration 20-100 μg/ml. A drop of sample containing fibrils was applied on the surface of freshly cleaved mica (GoodFellow, UK). After incubation for 3 min, the sample was washed three times with deionized water and air-dried overnight at room temperature. Silicon probes were used in the experiment for intermittent contact with the cusp curve radius <10 nm (Atomic Force, Germany). Their resonance frequency varied among probes in 120-160 kHz range. The sample was scanned at frequency from 1 to 3 kHz and resolution 256 × 256 or 512 × 512 points. Height, amplitude, and phase images were obtained simultaneously. To avoid scanning artifacts, the samples were scanned with direct and reverse motion of the probe. Parameters of fibrillar structures obtained in height image were measured using a computer program. The accuracy of measurements of fibril structures was achieved by replication of multiple cross-sections of each object [27].

RESULTS

AFM study of fibril formation. The fibrilogenesis process was visualized by AFM (Fig. 3). Concentrated solutions of ABB and its biologically active derivatives were incubated at room and elevated temperatures to produce fibrils.

Fibril formation at 57°C. During the first hours of incubation at 57°C, the artificial proteins formed globular oligomers 1.2 nm in height. Their density increased with

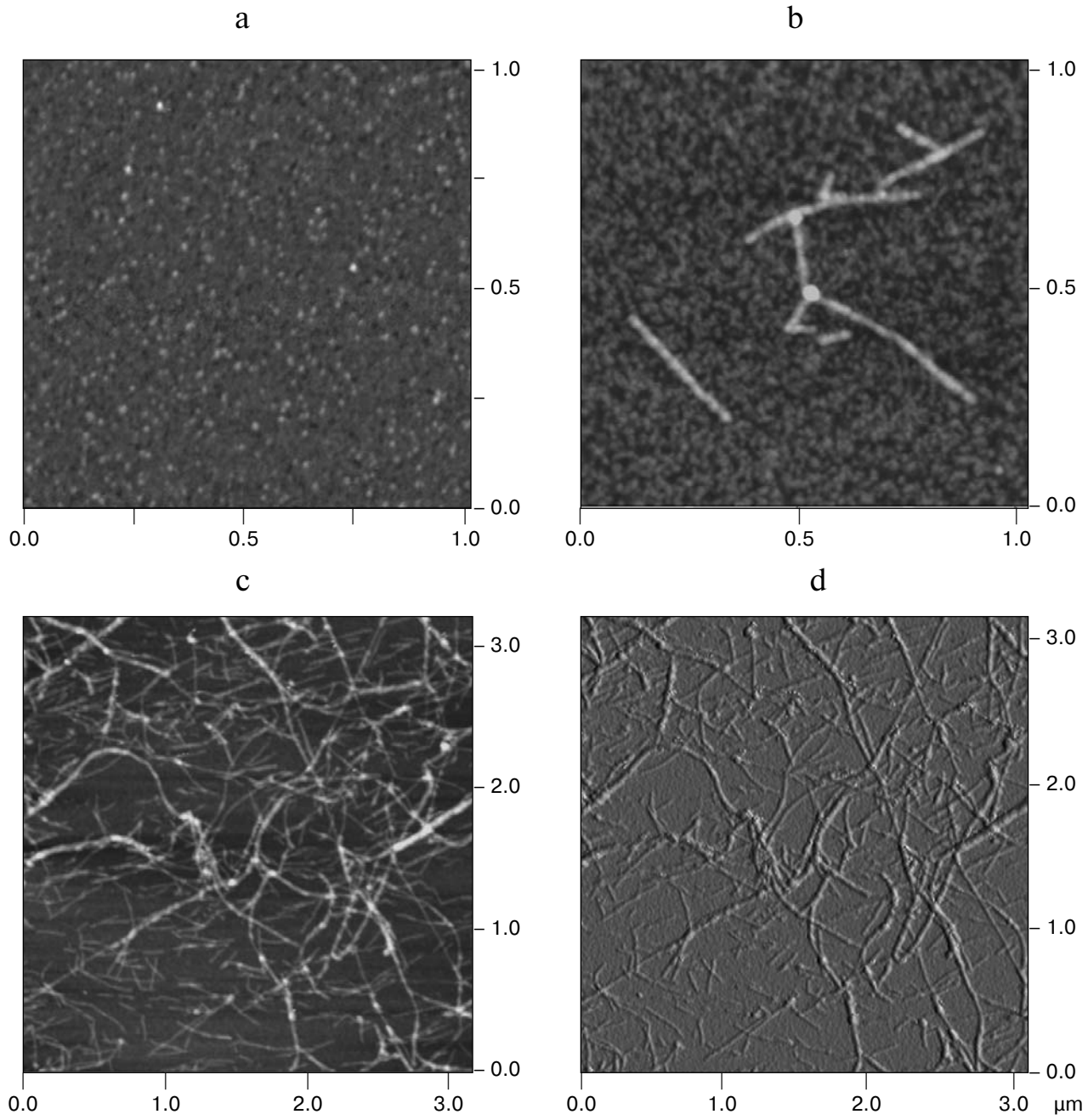

Fig. 3. Amyloid structures of ABB observed by AFM after incubation in 10 mM Tris-buffer, pH 7.3, containing 0.2% NaN_3 at 57°C: a) ABB oligomers (one day of incubation), height presentation; b) ABB protofilaments (7 days of incubation), height presentation; c) net of mature ABB-df fibrils, height presentation; d) phase presentation of ABB-df fibrils.

the lapse of time, and they began to associate with each other. It took a day to form large globular aggregates of albebetin (Fig. 3a), whereas this process took only a few hours in solutions of ABBI and ABB-df.

A subtle fibrillar net of ABB appeared in 4-5 days and consisted of amyloid structures 2 nm in height and 50-100 nm in length (Fig. 3b) corresponding to minimal parameters of single-stranded fibrils designated as protofilaments [28]. These parameters of ABB fibrils remained constant with time (Fig. 3, c and d), whereas fibrils of ABBI and ABB-df continued to grow.

ABBI and ABB-df protofilaments can be observed only during the first 2 h of incubation; their height was 2 nm and length 50-200 nm. Development of the fibrillar net was very quick: the ABB derivatives formed distinct filamentous structures during one day of incubation.

Their length achieved several micrometers and height 2-12 nm. Mature fibrils were formed via protofilament prolongation and their mutual convolution (Fig. 3, c and d). Periodicity of the convolution varied from 20 to 70 nm.

Fibril formation at room temperature. There was no fibril formation in ABB solution at room temperature. The protein formed only large granular aggregates 2-4 nm in height and higher even after 24 days of incubation.

ABBI and ABB-df at room temperature formed structures identical to those formed at 57°C, but the rate of mature fibril formation was markedly lower. Sphere-shaped oligomers, both free and associated with each other, dominated in the samples even after four days of incubation, and only later a rare fibrillar net began to appear.

Influence of HLDF-6 hexapeptide on fibril formation. Solutions of proteins *de novo* were incubated in the presence of HLDF-6 hexapeptide to analyze its effect on the process of fibrilogenesis. The AFM method did not show any morphological alterations in ABBI and ABB-df fibrils. However, in the presence of HLDF-6 peptide, albebetin acquired the ability to form structures that were as complex as ABBI and ABB-df fibrils (Fig. 3, c and d).

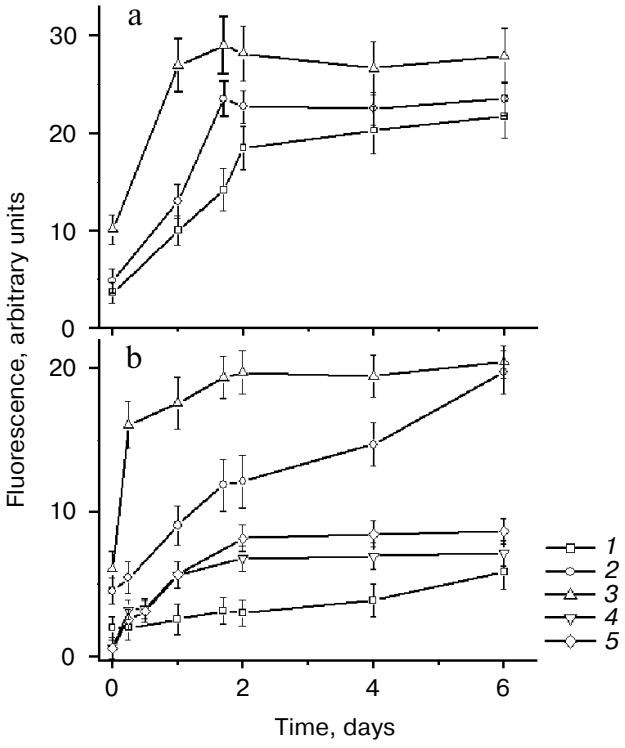

Fig. 4. Kinetics of ABB fibril formation (*1*) in the presence of 100 mM (*4*) and 200 mM (*5*) NaCl and of ABB derivatives ABBI (*2*) and ABB-df (*3*). The data are given as time dependence of fluorescence of amyloid dye thioflavin T in absence (a) and in the presence (b) of HLDF-6 hexapeptide. Fluorescence intensities are given as M ± SD. Each measurement was done at least in triplicate.

Interestingly, HLDF-6 hexapeptide itself did not display ability to form fibrils: it did not form fibrils either at room temperature or at 57°C during four weeks of incubation.

Effect of ionic strength of the solution on protein fibril formation. Increasing ionic strength up to 100-200 mM by the addition of sodium chloride to the protein solution did not result in structural alterations in either ABBI or ABB-df fibrils. However, ABB under high ionic strength conditions formed a fibrillar net resembling that observed in the case of its derivatives (Fig. 3, c and d) or ABB in the presence of HLDF-6 hexapeptide.

Kinetics of fibril formation. The kinetics of fibril formation was studied using the typical amyloid dye thioflavin T. Protein solutions were incubated at 57°C, and aliquots were sampled in regular time intervals. The increase in fluorescence of thioflavin T (Fig. 4) is evidence for gradual accumulation of amyloid structures developing in the samples. It turns out that, despite structural similarity, the artificial proteins differ in kinetic parameters of fibril formation.

Start of fibril formation. The process of amyloid formation in ABB solution began after a prolonged lag-period (1 day) (Fig. 4a). Amyloid structures accumulated thereafter, which bound to thioflavin T, inducing its fluorescence. In the time range chosen in our experiments (6 days), ABB derivatives began to form fibrils without any lag-phase; this was reflected by rapid growth of the thioflavin T fluorescence signal (Fig. 4a).

Rate of fibril formation. The highest rate of fibril formation was observed in ABB-df solution, in which the thioflavin T signal achieved a plateau of the fluorescence curve at the second day (Fig. 4a). In ABBI samples, the fluorescent signal achieved a plateau only on the sixth day of incubation, and the minimum rate of fibril formation was in ABB, as evidenced by very slow and monotonous increase in fluorescence intensity during 7-8 days of incubation.

Stationary phase of fibril formation. The studied proteins also differed in final amounts of amyloid structures capable of binding thioflavin T, as determined by various levels of the plateau on their fluorescence curves. So, the levels of the plateau on ABBI and ABB-df fluorescence curves were higher than the level for ABB (Fig. 4a), this indicating higher density of fibril population in the samples of derivative proteins.

Fibril formation in the presence of HLDF-6 hexapeptide. A stimulatory effect on the process of fibril formation was observed not only when a short fragment was attached to the N-terminus of ABB, but also in the presence of the free peptide in the solution. So, in ABB and ABBI solutions HLDF-6 hexapeptide added in equimolar ratio accelerated significantly the process of fibril formation: the kinetic curve of amyloid growth achieved a plateau on the second day of incubation (Fig. 4b), instead of the sixth and seventhes days in absence of the peptide (Fig. 4a). The amount of amyloid formed by ABBI in the pres-

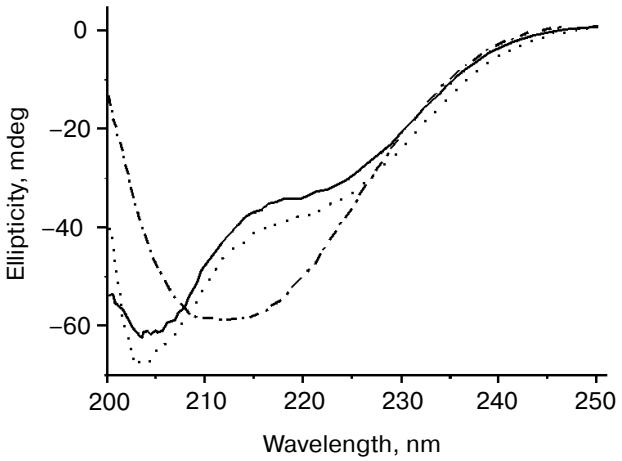

Fig. 5. CD spectrum for ABB-df in far UV region measured at 20°C (solid curve) and at 57°C (dashed-dot curve) in 10 mM Tris-buffer, pH 7.5; the spectrum of amyloid fibrils (dotted curve) was recorded after incubation of the same sample for 4 days at 57°C.

ence of HLDF-6 was greatly increased because the plateau level of the fluorescence curve was elevated (Fig. 4b).

Effect of ionic strength on fibril formation. The protein samples were incubated in the presence of 100 and 200 mM NaCl (Fig. 4a). The increase in ionic strength did not influence the kinetics of fibril growth in ABBI and ABB-df, but accelerated this process in ABB solution. Already on the second day of incubation, the kinetic curve achieved a plateau, which was two times higher than in salt-free ABB solution.

CD spectra. ABB and its derivatives, as shown earlier, are characterized by similar CD spectra [18, 20]. Since heating favors the fibril formation, the effect of elevated temperature was studied on the state of secondary protein structure. As evident from Fig. 5, heating does not induce any changes in the CD spectrum of ABB-df. In the course of fibril formation, substantial changes in secondary structure of proteins occurred leading to the increase in β-sheet content as shown for ABB-df in Fig. 5 (dotted curve).

DISCUSSION

Amyloid properties of *de novo* proteins. The role of molten-globule in fibril formation. The data of the present study indicate that highly charged artificial protein ABB having no natural analogs is capable of forming amyloid fibrils at physiological pH at 57°C, and its derivatives ABBI and ABB-df form fibrils already at room temperature. The fact that even artificial proteins possessing unique structure are capable of forming amyloid structures confirms again that the ability to form fibrils is independent on origin and function of the protein, but is a fundamental property of polypeptide chain [1, 13].

Mis-association of native structure and an inclination to form β-sheets in amino acids composing the protein are supposed to be probable causes of amyloid aggregation [29]. Thus, fibril formation is possible during the protein folding before the point at which it acquires compact rigid structure. In connection with this, a question arises on particular features of intermediate state of the protein preceding formation of amyloid structures. Both ABB and its derivatives in their native state are characterized by substantial flexibility of side residues and possess the main features of conformationally flexible state close to the molten-globule state (distinct secondary and labile tertiary structure) [17, 18, 23, 30].

It is known that the molten-globule state plays an important role in natural processes. It is formed as a necessary stage in the process of protein folding, before the protein assumes its natural conformation [31]. For instance, it is a well-founded supposition that protein molecules being incorporated into membrane or transferred across the membrane pass into the molten-globule state [32] due to negatively charged phospholipid groups on the membrane surface. The proteins receive pH-dependent transition into the molten-globule state via the interaction with a membrane.

It is supposed that the molten globule can be involved in the mechanism of genetic diseases caused by point protein mutations. The mutations result in retardation of the molecule at an intermediate stage of protein folding into the molten-globule state and subsequent mislocalization (mis-targeting) in the cell [31].

The formation of partially unfolded conformation is probably an important stage in the process of fibril formation as well. The state of the molecule close to the molten-globule conformation fulfills the function of a cross-point from which the protein processing can be directed to the "healthy" way of development to its native conformation or to the "pathological" one, i.e., to the formation of amyloid formations.

The exposition to moderately decreased pH values is the most widely distributed method for protein transferring to the molten-globule state. The same conditions stimulate fibril formation in various proteins [33]. An intermediate state preceding fibrilogenesis was described, in particular, for α-lactalbumin, a Ca^{2+}-binding protein from cow milk [34]. It forms amyloid fibrils exclusively under low pH values at which it receives typical molten-globule state. Highly helical α-lactalbumin is transformed herein into the conformation state with dominating β-structure. Fibroblast growth factor also acquires partially unfolded state under denaturing conditions owing to which it can form fibrils [35]. The key role of molecular intermediate state in the fibril formation was shown with lysozyme as an example, which possesses molten-globule properties, i.e., secondary and destabilized tertiary structure [13, 28].

The ability of ABB and its derivatives to form fibrils at physiological pH values is apparently due to the fact

that the artificial proteins in their native state are already in a conformation of molten-globule type [17, 18]. Thus, ABB and its derivatives serve as an adequate model for studies of molten globule as a stage preceding fibril formation. Moreover, the study of amyloid properties in artificial proteins is necessary from a practical point of view, due to the possible application of ABB biologically active derivatives as medicines for therapy.

Role of electrostatic interactions in fibril formation of artificial proteins. The influence of peptides and ion strength on the process of fibril formation is schematically shown in Fig. 6. It is necessary to note that mature ABB fibrils are formed only in the presence of peptides covalently attached to the N-terminus of the molecule or added to the solution. Since the attachment of peptides to the N-terminus induces no significant change in secondary structure of ABB, but increases intensity fibril formation, a question arises on the role of hydrophobic and electrostatic interactions in the process of fibrilogenesis. The total charge of the ABB molecule (−12) is due to the presence of 22 charged amino acid residues (17 Glu and Asp; 5 Arg and Lys). Electrostatic repulsion results in structural instability and provides high solubility of the protein. These particular features block polymerization, and, hence, fibril formation as well. However, being generally hydrophilic, the ABB molecule has hydrophobic sites, such as β-sheets. Most probably they are the first involved in the fibrilogenesis process via formation of amyloid β-bars. Since external and inner β-sheets of albebetin carry like charges −2 and −1, respectively, they have to overcome electrostatic repulsion. It is shown that elevation of temperature favors structural changes resulting in fibril formation. Most probably, the increase in diffusion activity in the molecule and enhancement of interaction between hydrophobic sites alter spatial disposition of like charged residues, decreasing electrostatic repulsion and forming stable β-structure. Thus, heating can change the relationship between hydrophobic forces facilitating fibrilogenesis and electrostatic repulsion forces hindering fibrilogenesis.

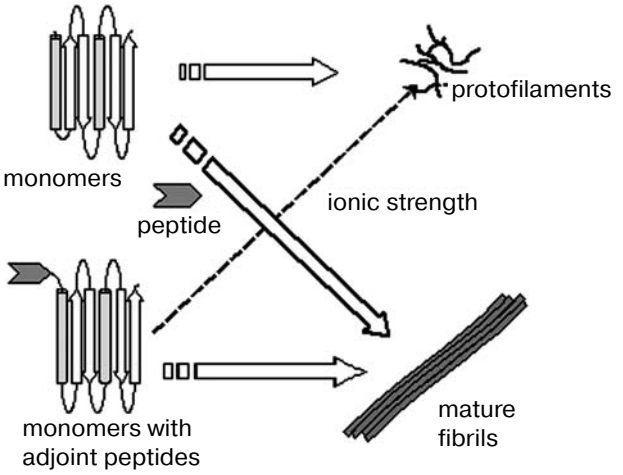

Fig. 6. Scheme of amyloid structure formation in artificial proteins: effect of peptides and ion strength on the process. The main pathways of fibril formation are shown as thick light arrows, and additional pathways as dashed arrows. Peptides are shown as short gray cutoffs.

Effect of exogenous and endogenous peptides on ABB fibril formation. It is important to note that ABB derivatives form amyloid already at room temperature and substantially more intensively at 57°C. Peptide fragments attached to the N-terminus of the artificial protein increase the fibrilogenesis rate, thus enhancing the processes of growth and protofilament convolution.

Although interferon octapeptide stabilizes the ABB molecule by increasing its compactness and cooperative effect of interactions [18, 23] that would counterpart the fibril formation, it nevertheless favors protein polymerization into fibrils. Also, HLDF-6 hexapeptide unable to form amyloid itself exhibits properties similar to the interferon octapeptide as enhancer of fibril formation in ABB (Fig. 4b). We propose the following explanation for fibrilogenesis activation as a result of peptide attachment to ABB. Four antiparallel β-sheets of albebetin carry six negatively charged carboxyl groups oriented outside and forming a negatively charged surface. Electrostatic intra- and intermolecular repulsion of β-chains hinders polymerization (Figs. 1 and 2). Both these peptides are capable of compensating surface charges, particularly ABBI, which carries a positive charge of 2. Aliphatic peptide groups providing additional hydrophobic interactions can also stabilize fibril structure. Compensation of electrostatic repulsion between ABB molecules was achieved by increasing ionic strength in the solution resulting in an increase in fibril formation as well. Thus, ABB fibrilogenesis depends on the balance between the forces of hydrophobic attraction and electrostatic repulsion.

The effect of charges on the ability to form fibrils was also studied on α-synuclein [36], acetyl phosphatase [11], and insulin [37]. So, α-synuclein is negatively charged (−9) at physiological pH and is in native unfolded state due to the repulsion of negatively charged side residues concentrated mainly at the C-terminus of the molecule. It has been shown that cations [38], polycations [39], and anions [36] stimulate fibril formation in this protein. The authors suppose that this results from a decrease in number of uncompensated charges that is a cause of partially unfolded soluble protein conformation and enhancement of hydrophobic bounds as well. These data are in agreement with our results obtained for the highly charged protein *de novo* ABB having partially unfolded native state. The lack of homology between amino acid sequences of α-synuclein and ABB confirms the fundamental process of fibril formation.

Some other proteins are known with αββ-structure that can form fibrils. In particular, acetyl phosphatase

[11] and ribosomal protein S6 [40] belong to this group. It is important to note that ABB has maximum charge among the members of this group.

Thus using a wide set of spectral methods and primarily the method of atomic force microscopy, we have shown the ability to form polymorphous amyloid fibrils in artificial protein ABB and its derivatives under conditions close to physiological. A polymorphism was observed in lengths (from <300 nm to >1 μm) and diameters (from 3 to 10 nm) of amyloid, i.e. in numbers of protofilaments composing one fibril.

The data of the study have shown that peptide attachment to the N-terminus, the addition of peptides to the solution, as well as change in ionic strength of the solution substantially influence amyloid properties of the charged ABB molecule. What this means is the possibility to manage electrostatic interactions in hydrophobic sequences by varying the composition of the solution: by adding charged molecules, by changing ionic strength or pH. We anticipate that complex relationships between the forces of electrostatic repulsion and hydrophobic interactions play a substantial role in the process of amyloid structure formation.

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