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Biophysics

Study of the Aggregation Rate of Amyloid Smitin Using Dynamic Light Scattering and Transmission Electron Microscopy Methods

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Abstract. In this study, we report that chicken stomach smooth muscle titin (smitin) forms amyloid aggregates *in vitro*. This finding is supported by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Dynamic light scattering data indicate that smitin forms amyloid aggregates with a hydrodynamic diameter (d) ranging from approximately 346-4169 nm *in vitro*. The zeta potential of the amyloid form of smitin was -16.1 mV and remained virtually unchanged for 24 h. From the zeta potential measurements, it can be concluded that the amyloid forms of smitin retain the ability to aggregate. Transmission electron microscopy showed that smitin in the native state is presented by a bundle of linear fibrils, while its amyloid forms large amorphous aggregates. *© 2025 Bull. Georg. Natl. Acad. Sci.*

Keywords: amyloid, amyloidosis, smooth muscle titin

Introduction

Amyloidoses are a large group of diseases characterized by protein deposits in the form of insoluble fibrils in various organs and tissues, formed as a result of hereditary or acquired protein folding disorders [1,2]. Besides amyloid fibrils, which mostly form in the brain, other types of protein aggregates can also cause blood clots or floaters in the vitreous of the eye [3]. Their accumulation destroys the structure and functioning of organs and tissues, leading to disease and death. Amyloid deposits are found in Alzheimer's disease, Parkinson's disease, Down syndrome, type 2 diabetes, hereditary amyloid polyneuropathy, systemic amyloidosis, etc. [1,4,5]. There are many proteins known to form amyloid fibrils, such as tau protein, Aβ-peptide, acylphosphatase, myoglobin, amylin, transthyretin and others [6-9]. Despite the differences in the amyloid precursor proteins, amyloid fibrils have common properties: β-sheet structure with individual β-sheets oriented parallel to the main axis of the fibril, insolubility *in vivo,* specific binding to Congo red and thioflavin T dyes [9,10]. It is important to note that amyloid precursor proteins undergo an α-helix–β-sheet transformation, which is necessary for the formation of amyloid fibrils [2].

Clarification of the molecular mechanisms of amyloidosis, establishment of the protein nature of deposits and their properties, development of therapeutic methods of treatment and prevention of these diseases, as well as development of their lifetime diagnostics are urgent tasks. Successful solution of these tasks largely depends on fundamental knowledge of amyloidogenesis: clarification of the properties of amyloids of different proteins, knowledge of the factors regulating their formation and destruction, their effects on the vital activity of different cells, etc. This applies to muscle amyloidosis to the greatest extent. Amyloid deposits have been found in cardiomyopathies, myocarditis and myositis, affecting muscles and blood vessels [11].

In vitro experiments with many proteins have shown that prior to amyloids formation, the molecules must undergo a transformation the "α-helix to β-fold" type, which, as a rule, requires long-term incubation and harsh conditions incompatible with *in vivo* conditions, such as low pH, high temperatures, addition of a number of substances not present in the cell, etc. Amyloid precursor proteins can have a β-structure, an α-helix, or contain both structures. The transition of the soluble form of prion protein to the fibrillar is accompanied by a decrease in the α-helix content and an increase in the β-structure. The Aβ-peptide also undergoes a transformation of the structure from the α-helix to the β-structure during the formation of amyloid fibrils. All these data indicate that proteins whose secondary structure is represented by α-helixes undergo a transformation of the type "α-helix to β-structure" before or during fibril formation [12,2]. Structural investigations of amyloids began in the 1930s using X-ray diffraction. Hence, Astbury and Dickinson [13] were the first to note the distinctive X-ray fiber diffraction pattern, later termed cross-β. Active using of the X-ray diffraction method allows to conclude that amyloids are composed of polypeptide chains extended in the so-called cross-β conformation. In the cross-β structure individual strands of each β-sheet run perpendicular to the fibril axis (4.7 Å spacing), whereas the β-sheet (10 Å spacing) are parallel to the fibril axis. In 2002, smooth muscle titin (SMT; initially called smitin) was found in the smooth muscle extract of chicken gizzard [14].

Further studies showed that titin from smooth and cross-striated muscles is a product of the same gene, alternative splicing of which leads to the formation of isoforms of 700−2000 kDa in smooth muscle [15]. Western blotting demonstrated titin of approximately 500 kDa in the human aorta, which the authors [15] suggested to be either a titin fragment ('truncated' titin) or an isoform. As is well known, titin from cross-striated and smooth muscles consists of immunoglobulin (Ig)-like and fibronectin III (FnIII)-like domains with a *β*-sheet structure. It was demonstrated that approximately 10% of Ig and FnIII domains have more than 40% sequence identity [16]. It was also shown by dynamic light scattering (DLS) that the aggregation rate of TI I32 and TI I27 domains with sequence identities of 42% is higher than that of domains with a lower sequence identity. Bobulev at el. showed the ability of myosin binding protein-C containing Ig like and Fn-like domains to form aggregates with amyloid-like properties *in vitro* [17]. Based on the data obtained, they proposed that SMT can form amyloid aggregates *in vitro*.

In this work, the formation of the amyloid structure of chicken gizzard smooth muscle titin *in vitro* and the ability of smooth muscle titin to form aggregates were studied using structural analysis methods. In the paper, the kinetics of aggregation was studied to understand the features of the rate of aggregation of chicken smitin. For this purpose, dynamic light scattering (DLS), the simplest and most convenient method for studying the kinetics of aggregation of amyloid proteins, was used [18-20].

The morphology of titin aggregates formed *in vitro* was studied using electron microscopy: with an ionic strength below physiological values (0.15 M glycine-KOH, pH 7.0-7.5).

Materials and Methods

Smitin was isolated from the chicken smooth muscle, particularly from the stomach according to Keller with our modification [21] Myofibrils were obtained from chicken stomach according to Wang [22]. Homogenized myofibrils (100 g) were washed three times in A buffer (2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DDT, 2 mM PMSF, 10 mM imidazole, 10 mM trypsin inhibitor, 50 mM KCl, pH 7.0) by centrifugation (5000 g, 10 min, 4°C) and resuspended in extraction buffer B (2 mM $MgCl_2$, 1 mM EDTA, 0.6 M KCl, 4 mM ATP, 0.5 mM DTT, 0.2 mM PMSF, 10 mM trypsin's inhibitor, 10 mM imidazole, pH 7.0). The extracted myofibrils were sedimentated by centrifugation (15 000 g, 30 min, 4°C) and the supernatant was applied to the Toyopearl-65 column (1.5 cm, 90 cm). The Toypearl-65 column was equilibrated with buffer (0.2 M KCl, 10 mM imidazole, 1 mM EGTA, 0.5 mM EDTA, 0.2 mM DTT, pH 7.5). The first fraction contained native smitin only, the second fraction contained smitin + myosin and the third fraction contained only myosin. Purity of the proteins was examined by the electrophoresis method in the polyacrylamide gel gradient (3-15%) in the presence of Na-dodecyl sulfate by Laemmly method [23]. For fixing smitin transition into amyloid form we used Congo Red dye, that stains amyloids into rose or red colors. Congo Red solution was added to amyloid protein suspension at the ratio 1: 2. The difference between absorption spectra of Congo Red and Congo Red + amyloid protein complex indicates to protein transition into amyloid form. For fixing smitin transition into amyloid form we used Congo Red dye, that stains amyloids into rose or red colors. Congo Red solution was added to amyloid protein suspension at the ratio 1: 2. The difference between absorption spectra of Congo Red and Congo Red + amyloid protein complex indicates to protein transition into amyloid form.

Dynamic light scattering experiments. In this work, solutions of native smitin and amyloid aggregates of smitin were studied by DLS. Measurements were carried out on a Zetasizer Nano - ZS device (Malvern Instruments Ltd.) in a cell maintaining a temperature of ±0.5°C at a wavelength $\lambda = 633$ nm, at a scattering angle of 173°) and sample cuvette temperature control (10°C). Autocorrelation functions were converted into particle-size distributions, using the "General purpose" algorithm provided with the Zetasizer. SMT samples for DLS were prepared as follows: Purified SMT at 0.2 mg/ml in column buffer (0.6 M KCl, 30 mM KH_2PO_4, 1 mM DTT, 0.1 M NaN_3, pH 7.5) was dialyzed at 4°C against 0.15 M glycine-KOH, pH 7.0-7.5. Samples for DLS were collected after: 3 h and 24 h.

Dynamic light scattering of smooth muscle titin amyloid aggregates. To study the time dependence of the formation of smitin aggregates, the DLS method was used to detect and characterize the aggregates of different size. Figure 1 shows the change in the sizes upon formation of SMT aggregates at pH 7.0 over 24h.

In the native state, before the formation of smitin aggregates, one well-resolved peak was observed with an average hydrodynamic diameter d = 136.2 nm (91.6% intensity). The second peak d = 3857 nm indicates the presence of a small amount (8.4%) of large smitin molecules. During the first 3-hour incubation, three peaks were observed: an average d = 59.24 nm (minor peak of approximately 5.6%) d = 346 nm (the dominating peak of approximately 49.8%), and d = 4169 nm (44.6%). The diameter of 4169 nm, which is outside the measurement range, and its intensity of 44.6% indicates the formation of large smitin aggregates. A peak with a diameter of 59.24 nm indicates the presence of smitin oligomers, which likely contribute to the acceleration of aggregation and the appearance of a peak with a diameter of 4169 nm, with a high percentage of intensity. When the experiment terminated (after 24 h), three peaks with diameter approximately d = 189.3 nm (30.7%), d = 981.3 nm (46.2%),

and d=4847 nm (23.1%) were observed. The values of the diameters of the obtained peaks indicate the continuation of aggregation and an increase in the size of smitin molecules. It should be noted that the peaks with diameter approximately 4169 nm and 4847 nm were at the limit of the range of this method. Therefore, further the formation of larger smitin aggregates cannot be excluded.

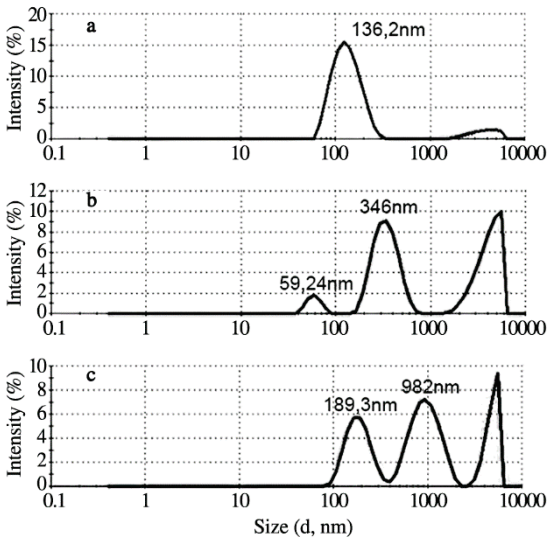

Fig. 1. Size distribution of native smitin (a), smitin amyloid aggregates after 3 h (b) and after 24 h (c).

Zeta potential. Zeta potential is the total charge of particles acquired in a particular environment. The value of the zeta potential allows us to judge the potential stability of the colloidal system. If all particles have a large negative or positive zeta potential, they will repel each other, and this is called dispersion stability. If particles have low zeta potential values, then it is impossible to prevent the particles from connecting, and this is called dispersion instability. It is assumed that particles with zeta potentials above +30 mV and below -30 mV are generally considered stable, respectively particles with zeta potentials in the range from -30 mV to +30 mV are considered unstable.

To determine the tendency of amyloid smitin to aggregation using dynamic laser scattering, we measured the zeta potential of smitin solutions after dialysis for 3 and 24 hours at 4°C against a solution containing 0.15 M glycine-KOH, pH 7.0-7.5. DLS analysis can be used to assess or predict the stability of the protein as a function of time. The value of the zeta potential allows one to judge the potential stability of smitin solutions. The results of measurements of zeta potentials of native smitin and aggregated samples are presented in Fig. 2. The value of the zeta potential of native smitin -6 mV means the protein's tendency to aggregation. During the transition to the amyloid form within 3 hours from the beginning of incubation, the zeta potential value decreased to -16.1 mV and remained virtually unchanged -16.6 mV after the end of the experiment for 24 hours. As a result of zeta potential measurements, it can be concluded that the amyloid forms of smitin retain the ability to aggregate.

Thus, the method of DLS revealed a relatively high rate of smitin aggregation.

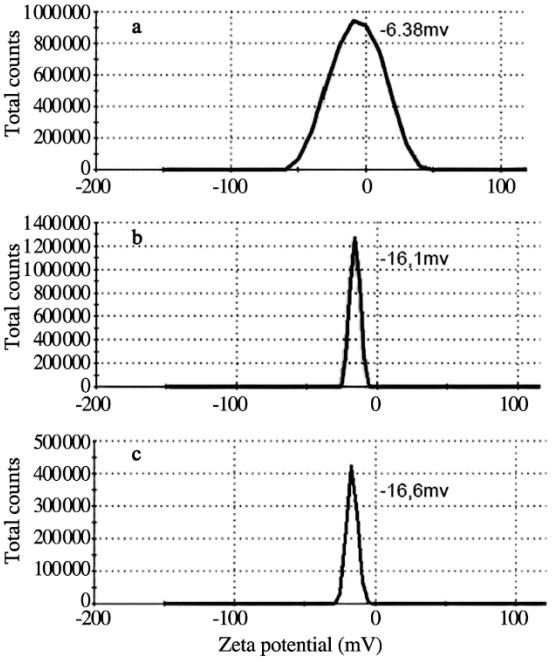

Fig. 2. Zeta potential distribution of native smitin (a), smitin amyloid aggregates after 3h (b) and after 24h (c).

Transmission electron microscope method (TEM). Smitin aggregates were formed by dialysis for 3 and 24 hours at 4°C against a solution containing 0.15 M glycine-KOH, pH 7.0-7.5. A drop of suspension at the 0.2-0.4 mg/ml was applied to a

carbon-coated collodion films on copper grids. Samples were examined using JEM-100SX electron microscope. In this work, electron microscopy showed that already 3 hours after the start of dialysis, smitin forms large amorphous aggregates, which indicates a fairly high rate of protein aggregation (Fig. 3b, 3c). The smitin in its native state is represented by a bundle of linear fibrils (Fig. 3a).

a b c

Fig. 3. Smitin in native state(a), amorphous amyloid smitin aggregates after 3 hours (b) and 24h (c) from the start of dialysis.

Our results show that smitin isolated from chicken gizzards smooth muscles can form amorphous aggregates *in vitro* in a solution containing 0.15 M glycine-KOH, pH 7.0-7.5 (Fig. 3b, 3c).

Conclusion

The ability of smitin to form amorphous amyloid-like aggregates *in vitro* has been discovered. It was shown that smitin aggregates are formed over relatively short time intervals. At the same time, electron microscopy revealed that the aggregates have an amorphous morphology. Studying the dynamics of smitin amyloids formation using the DLS method revealed relatively rapid aggregation of protein molecules.

The results obtained indicate the possibility of intracellular smitin aggregation, which may have functional significance. The amyloid properties of smooth muscle titin (smitin) add a new dimension to our understanding of its biological role and involvement in pathological processes. Ongoing research in this area will help to deepen our understanding of the mechanisms of smitin aggregation and its impact on smooth muscle health, which may lead to the development of new diagnostic and therapeutic approaches.

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ამილოიდური სმიტინის აგრეგაციის სიჩქარის შესწავლა დინამიკური შუქგაბნევის და ელექტრონული მიკროსკოპიის მეთოდების გამოყენებით

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შესწავლილია ქათმის გლუვი კუნთიდან (კუჭი) გამოყოფილი (ცილა) სმიტინის ამილოიდურ მდგომარეობაში გადასვლის თავისებურებანი *in vitro* (ცილის ამილოიდურ მდგომარეობაში გადაყვანა მოხდა 0,15 M გლიცინ / KOH, pH 7,0-7,5, I=0,1-ის ხსნარის გამოყენებით). სმიტინის აგრეგატების წარმოქმნის კინეტიკა შევისწავლეთ დინამიკური შუქგაბნევის (DLS) და ელექტრონული მიკროსკოპიის მეთოდების გამოყენებით. DLS -ის მონაცემებიდან ჩანს, რომ სმიტინი აყალიბებს ამილოიდურ აგრეგატებს ჰიდროდინამიკური დიამეტრით (d) დაახლოებით 346-4169 ნმ *in vitro*. სმიტინის ამილოიდური ფორმის ზეტა პოტენციალი შეესაბამება -16,1 mV და პრაქტიკულად უცვლელი რჩება 24 საათის განმავლობაში, რაც ადასტურებს სმიტინის აგრეგაციისადმი მიდრეკილებას. ელექტრონული მიკროსკოპიის მეთოდით მიღებულ სურათებზე ჩანს, რომ დიალიზის დაწყებიდან 3 საათის შემდეგ ამილოიდური სმიტინის მოლეკულები აყალიბებს მსხვილ ამორფულ აგრეგატებს, ნატიურ მდგომარეობაში კი სმიტინი წარმოდგენილია ხაზოვანი ბოჭკოების სახით.

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