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Structural model of amyloid fibrils for amyloidogenic peptide from Bgl2p–glucantransferase of *S. cerevisiae* cell wall and its modifying analog. New morphology of amyloid fibrils

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ABSTRACT

We performed a comparative study of the process of amyloid formation by short homologous peptides with a substitution of aspartate for glutamate in position 2 – V**D**SWNVLVAG (AspNB) and V**E**SWNVLVAG (GluNB) – with unblocked termini. Peptide AspNB (residues 166–175) corresponded to the predicted amyloidogenic region of the protein glucantransferase Bgl2 from the *Saccharomyces cerevisiae* cell wall. The process of amyloid formation was monitored by fluorescence spectroscopy (FS), electron microscopy (EM), tandem mass spectrometry (TMS), and X-ray diffraction (XD) methods. The experimental study at pH 3.0 revealed formation of amyloid fibrils with similar morphology for both peptides. Moreover, we found that the morphology of fibrils made of untreated ammonia peptide is not mentioned in the literature. This morphology resembles snakes lying side by side in the form of a wave without intertwining. Irrespective of the way of the peptide preparation, the rate of fibril formation is higher for AspNB than for GluNB. However, preliminary treatment with ammonia highly affected fibril morphology especially for AspNB. Such treatment allowed us to obtain a lag period during the process of amyloid formation. It showed that the process was nucleation-dependent. With or without treatment, amyloid fibrils consisted of ring-like oligomers with the diameter of about 6 nm packed either directly ring-to-ring or ring-on-ring with a slight shift. We also proposed the molecular structure of amyloid fibrils for two studied peptides.

1. Introduction

The study of the process of amyloid formation is an important field of research on protein behavior. Many proteins and peptides capable of forming amyloids are associated with severe human neurodegenerative diseases such as Parkinson's and Alzheimer's disease and others. Amyloids are fibril formations generating deposits or amyloid plaques in different tissues of organisms [1]. The principal characteristic of amyloids is supposed to be the presence of a cross-β structure [2]. This structure can be revealed with the use of X-ray diffraction and reflects the specific organization of fibrils, where β-layers lying parallel to the fibril axis at a distance of 9–11 Å from each other consist of β-strands that are at a distance of 4.6–4.8 Å from each other and perpendicular to the fibril axis.

However, there are proteins and peptides that can form amyloids nontoxic for a human organism – functional amyloids [3,4]. Some human hormones exist for a long time as amyloid structures in secretory granules and can leave them as monomer molecules without losing their functional activity [5].

It has been shown earlier that one of the proteins from the *Saccharomyces cerevisiae* cell wall (glucantransferase Bgl2) forms amyloid structures after isolation from cell wall [6]. The pH-dependent capacity of this protein to form fibril structures was studied. The capacity of potentially

Abbreviations: "NB", unblocked peptide termini; Bgl2p, glucantransferase Bgl2p; ThT, thioflavin T; PAD, potential amyloidogenic determinant; FS, fluorescence spectroscopy; EM, electron microscopy; XD, X-ray diffraction; TMS, tandem mass spectrometry.

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amyloidogenic regions (PADs) in the Bgl2p sequence to generate amyloid formation was examined using the bioinformatics analysis [7]. Short amyloidogenic regions of the sequence can be predicted in many proteins. The analysis of their capacity to form amyloids and understanding of the dependence of amyloid properties on different conditions (synthesis, pH, temperature, ionic strength, etc.) are important for the understanding of general mechanisms of amyloidogenesis in proteins. The tendency to form amyloids is frequently revealed with bioinformatics methods and is associated with self-recognition elements in the sequence [8]. Such elements of the primary structure constitute the backbone of amyloid fibrils [9,10] and are hot spots for aggregation of native proteins with the subsequent formation of amyloid fibrils [11]. These structures have typical characteristics of amyloids. 1) Morphology can be revealed in electron microscopes. Fibrils are highly elongated and mostly not branched protease-stable polymers up to 20 μm in length and from 2 to 20 nm in diameter in average. They consist of several filaments, which can be intertwined or packed in parallel. Moreover, morphological polymorphism of fibrils is frequently observed within the same sample. 2) Fibrils are capable of binding some stains (for example, ThT) with different intensity, which can be registered using fluorescence spectroscopy. 3) At last, the final answer to the question whether it is the amyloid or amyloid-like structure is obtained using the X-ray diffraction analysis. Most amyloids have the characteristic patterns of X-ray diffraction [2].

The existence of a cross-β structure was demonstrated for protein glucantransferase Bgl2p by the method of fluorescence spectroscopy with the use of thioflavin T and it was shown with the use of electron microscopy that the formed aggregates had a fibril structure [6].

A significant aspect of studying amyloid structures is determining conditions and factors that affect the process of amyloid formation, such as temperature, pH, ionic strength, the presence of ligands and others. A more challenging task is to identify amyloidogenic regions, which are critical to amyloid formation in a biological context, and to predict the effects of protein mutations and modifications on propensity to form amyloids in vivo. It is possible that in spite of a great variety of experimental data on this issue, there are general key mechanisms for all proteins. To exclude the effect of different contexts in proteins, small model peptides are used, the most part of their sequence being a priori the amyloidogenic region. For prediction of such regions different computation algorithms are used [12]. In our previous study we successfully used model peptides from Bgl2p to analyze the dependence of amyloid formation on pH values [7]. We examined the ability of peptides to form amyloid fibrils in the acidic pH range, since it was previously shown that Bgl2p is capable of forming fibrils at pH values below 6.0 [6]. It is known that the acid treatment of yeast cells leads to the occurrence of an apoptotic phenotype and induction of general stress response pathways that may include the mechanisms of cell wall integrity control [13–16]. It can be assumed that the ability to form fibrils facilitates yeast cell protection against oxidative stress.

To fully understand the mechanism of amyloidogenesis, it is necessary to know not only the reasons why proteins undergo these conformational changes, but also how these changes occur. It is believed that formation of amyloids goes through formation of a nucleus, to which other monomer molecules are attached later. We developed a model that makes it possible to estimate the size of an amyloid nucleus on the basis of kinetic data obtained with fluorescence spectroscopy [17]. For insulin and Aβ peptide the possible mechanism of amyloid formation was proposed: monomer → oligomer → amyloid fibril and it was demonstrated that the amyloid fibrils consist of oligomer structures [18–20].

In this study we analyzed the process of amyloid formation by the peptide that was predicted as a potentially amyloidogenic region of protein Bgl2p V**D**SWNVLVAG (residues 166–175) by using three programs: Aggrescan, DHPred, and FoldAmyloid. Other programs: PASTA, Tango and Waltz did not predict this region as an amyloidogenic one [7]. In addition to the mentioned peptide, we also synthesized a peptide with the Asp2Glu substitution. Using the methods of fluorescence spectroscopy, electron microscopy, tandem mass spectrometry and X-ray structural analysis, the characteristics of the aggregates obtained at pH 3.0 were analyzed and the dependence of amyloid formation on the peptide concentration in solution was studied. The comparison of the data, obtained for the two peptides, allowed us to conclude that substitutions of D for E affected the process of amyloid formation. Using the electron microscopy method, we also described new amyloid morphology not mentioned in the literature and presented the molecular structure of amyloid fibrils. We found that amyloid fibrils consisted of ring-like oligomer structures and possible mechanism of amyloid formation was proposed for the studied peptides.

2. Materials and methods

*2.1. Synthesis of peptides: V**D**SWNVLVAG (AspNB) and V**E**SWNVLVAG (GluNB)*

Peptides were obtained by solid-phase peptide synthesis. The strategy of the synthesis was based on the 9-fluorenylmethyloxycarbonyl/*t*-butyl ether (Fmoc/*t*Bu) protocol using an alkoxybenzyl alcohol resin as the solid phase. The following side-chain protecting groups were applied: *t*Bu (*tert*-butyl) for Asp, Glu and Ser, Trt (trityl) for Asn, Boc (*tert*-butyloxycarbonyl) for Trp. The first amino acid (Gly) was coupled with the polymer by dicyclohexylcarbodiimide (DCC) in the presence of 4-dimethylaminopyridine (DMAP). After attachment of the first amino acid, residual hydroxyl groups of the polymer were blocked by benzoyl chloride. All coupling reactions were performed step by step with the 2-(1-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) method, using a solution of 2.5 equivalents of each Fmoc-amino acid derivative in *N*-methylpyrrolidone (NMP). Double coupling of the Fmoc-amino acids was carried out to ensure the reaction completion, which was monitored at each stage using ninhydrin tests. The presence in a sequence of more than four hydrophobic residues requires increasing the time of reaction to 12 h and the number of couplings in succession. The peptides were detached from the polymer with a freshly prepared mixture of trifluoroacetic acid (TFA)-triisopropylsilane (TIS)-water (95:2.5:2.5) during 2 h under Ar. The resin was filtered and washed with TFA and dichloromethane. The peptides were precipitated using cold diethyl ether, dried and purified by reversed phase high performance liquid chromatography (RP-HPLC). Chromatography was carried out on a high pressure chromatograph (Waters with Waters 2487 Dual Absorbance Detector and Waters 1525 Binary HPLC Pump). Semipreparative purification was performed on a Diasorb 130 C16T 6 Å (250 × 8 mm) column in acetonitrile gradient in 0.1% TFA/water at a flow of 2 ml/min, the wave length of detection was 226 nm. The samples were dissolved in dimethylsulfoxide (DMSO) and applied on the column. Analytical HPLC was carried out on Luna 5u C18 (2) 100 Å (250 × 4.6 mm). The purity of the products was 99.8%. The molecular mass and homogeneity of the synthesized peptides were confirmed by mass spectrometry (major peak I + H^+: *m/z* 1054.3 – calc. *m/z* 1053; and major peak II + H^+: *m/z* 1067.5 – calc. *m/z* 1067).

2.2. Sample preparation

Peptide samples were prepared in two ways. (1) The preparations for the studies were preliminarily dissolved in 100% DMSO (with regard to the final concentration of 3–5%) and adjusted to the required concentration (0.2–2.0 mg/ml) with 5% acetic acid (pH 3.0). The dilution of the samples was performed on ice. (2) To obtain a monomer state of the peptides, the samples were preliminarily dissolved in ammonia (NH_4OH), the concentration was measured, and aliquots of the required concentration (0.4–0.7 mg/ml) were obtained and lyophilized. Prior to the experiment, the aliquots were dissolved in DMSO (the final concentration 5%), then adjusted to the final concentration 5% with acetic acid. The dilution of the samples was conducted on ice. For kinetic studies,