Formation of amyloid fibrils by bovine carbonic anhydrase

Anshul Rana, Teemish Praveen Gupta, Saurabh Bansal, Bishwajit Kundu *

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas. New Delhi 110016, India

**ABSTRACT**

Amyloids are typically characterized by extensive aggregation of proteins where the participating polypeptide chains are involved in formation of intermolecular cross β-sheet structures. Alternate structure attainment and amyloid formation has been hypothesized to be a generic property of a polypeptide, the propensities of which vary widely depending on the polypeptide involved and the physicochemical conditions it encounters. Many proteins that exist in the normal form in-vivo have been shown to form amyloid when incubated in partially denaturing conditions. The protein bovine carbonic anhydrase II (BCA II) when incubated in mildly denaturing conditions showed that the partially unfolded conformers assemble together and form ordered amyloid aggregates. The properties of these aggregates were tested using the traditional Congo-Red (CR) and Thioflavin-T (Tht) assays along with fluorescence microscopy, transmission electron microscopy (TEM), and circular dichroism (CD) spectroscopy. The aggregates were found to possess most of the characteristics ascribed to amyloid fibers. Thus, we report here that the single-domain globular protein, BCA II, is capable of forming amyloid fibrils. The primary sequence of BCA II was also analyzed using recurrence quantification analysis in order to suggest the probable residues responsible for amyloid formation.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Amyloid fibrils are highly ordered structures formed by aggregation of polypeptide chains, based upon long range repetitive intermolecular interactions [1]. Initially amyloids were discovered as fibrillar protein aggregates associated with neurodegenerative diseases like Alzheimer’s and Parkinson’s disease in humans and prion diseases in animals [2–5]. Amyloid fibrils have also been found in many systemic diseases like primary systemic amyloidosis [6]. Recently, amyloid formation has been hypothesized to be a property common to all polypeptide chains [2,6,7]. This hypothesis is based on the lack of any apparent structural, functional or sequential similarity between the observed amyloidogenic proteins [8]. It is further strengthened by the observation of amyloid formation, under suitable conditions, by several proteins unrelated to any known diseases [9–11]. Despite significant differences in the parent proteins involved, the fibrils formed display a high degree of orderliness and show remarkable similarities in a number of physicochemical, morphological and structural properties [12–14]. The formation of ordered aggregates by unrelated and dissimilar proteins points to some intrinsic uniqueness. Evidence for similarity in the lag-times and growth rates ratio for unrelated proteins has also been reported [15]. It also suggests that such structured aggregation may proceed by a general mechanism [16–18]. The actual mechanism has thus far eluded researchers, though there have been some advances in understanding fiber elongation and growth processes [19,20] as well as structural changes accompanying amyloid formation [21]. The process of fiber formation becomes more important because it has been shown that oligomers formed in the preaggregation stage are toxic to the cells rather than the fibrils [22–25]. Furthermore, the non-crystalline and insoluble character of amyloid fibrils makes them poor candidates for X-ray crystallography and solution NMR. Recently, solid state NMR methods have helped in understanding the structural characteristics of several amyloid proteins [26,27]. However, researchers have often had to depend on computational and predictive methods [28] resulting in postulates to explain amyloidogenicity of proteins. None of these methods unfortunately have been able to explain all the observations related to amyloid formation satisfactorily.

Carbonic anhydrase (CA) is a protein found in almost all animals and photosynthesizing organisms. In animals, its major function is to catalyze the reversible conversion of carbon dioxide to carbonic acid in red blood cells where it is found in abundance. CA performs other physiological functions such as acid secretion in stomach, pH maintenance of alkaline pancreatic secretions and saliva, osmoregulation in kidneys and eyes [29,30]. As a subject of enzyme research, CA is important for its extremely high turnover rate [31]. BCA II has no disulphide linkages [32], and complete denaturation of the protein happens at a guanidinium hydrochloride (GdnHCl) concentration of 4.0 M.

Apart from its biological and scientific significance as mentioned above, BCA II emerged as a favored molecule for amyloidogenic studies owing to some additional characteristics; i) All known CAs and their isozymes are structurally similar containing a 10-stranded twisted β-sheet with a few small helices [33,34]. ii) It was shown that
proteins displaying amyloidogenic properties, when screened through the recurrence quantification analysis (RQA) method were mostly α-helical in nature [35]. BCA II being a β-sheet-rich protein makes the subject even more interesting. iii) The molecule is nearly spherical and is essentially a single-domain protein. Small, single-domain, globular proteins are likely to form disordered amorphous aggregates upon destabilizing physicochemical conditions as shown earlier [36].

In this paper the technique of RQA was initially applied to determine the amino acid residues that might be involved in initiation of amyloid forming interactions [35,37]. The stretches predicted by RQA lie at the C-terminus of the protein. Interestingly, some residues at the C-terminus are quite hydrophobic in nature and have also been proposed to be involved in the initial folding events of this protein [38].

Here, we show that BCA II when incubated under mildly denaturing condition of GdnHCl concentration of 1.5 M and a pH of 3.5 (slightly below its isoelectric point), forms ordered aggregates that are characteristically distinct from amorphous aggregates. These conditions were selected by repeated experiments to find out the mid point of unfolding transitions under varying GdnHCl and pH conditions. At early stages of incubation, aggregates displayed a linear arrangement of beaded structures which eventually appeared to coalesce to form fibers of slightly thicker dimensions. Increase in the overall β-sheet content of the aggregated form of the protein is also evident from the CD data. The ordered aggregates thus obtained display most of the characteristics assigned to amyloids such as Thioflavin-T and Congo-Red binding which are markedly distinct from amorphous aggregates.

2. Materials and methods

Bovine Carbonic Anhydrase II, Guanidinium hydrochloride, Congo-Red (CR) and Thioflavin-T (ThT) were purchased from Sigma chemical Inc. All other buffers and chemicals were from local suppliers.

2.1. Formation of amyloid fibers

BCA II was incubated at a concentration of 4 mg/ml (133 µM) in 10 mM sodium acetate buffer at pH 3.5 in the presence of 1.5 M GdnHCl and allowed to stand at 37 °C without agitation. Aliquots were periodically drawn for analysis using CR and ThT-binding assays.

2.2. Formation of thermal aggregates

Thermal aggregates were formed by heating a 4 mg/ml solution of BCA II at 75 °C for 5 min in a 20 mM potassium phosphate buffer, pH 6.5. For electron and fluorescence microscopy experiments, pelleted samples of thermal aggregates were taken and directly deposited either on transmission electron microscopy (TEM) grids or microscopic slides.

2.3. Congo-Red assay

A stock solution of 0.5 mM CR was prepared in 20 mM potassium phosphate buffer, pH 7.5. Samples for spectrophotometry were prepared by the addition of 30 µl of CR stock solution to 16 µl of an incubated BCA II sample. The final volume was made up to 600 µl by adding water and phosphate buffer to bring the concentration of the latter to 20 mM. The effective concentrations of protein and CR in the mixture were 3.5 µM and 25 µM respectively. Samples prepared in this way were incubated for 30 min at room temperature followed by an absorption scan from 400–600 nm. All absorption measurements were done in quartz cuvettes of 1 cm path length in a Spectroscan V spectrophotometer operated at a bandwidth of 5 nm. Controls were made with the same composition but without CR in order to measure the absorbance and scattering effects, if any, of the protein.

2.4. Thioflavin-T assay

A stock solution of 2.5 mM ThT was prepared in 10 mM potassium phosphate buffer, pH 6.5. Samples for fluorometry were prepared by the addition of 20 µl of ThT stock solution to 25 µl (0.1 mg/ml, assuming a homogeneous solution) of an incubated BCA II sample. The final volume was made up to 1000 µl by the addition of water and phosphate buffer to bring the concentration of the latter to 10 mM. The effective concentrations of protein and ThT in the mixture were 3.5 µM and 50 µM respectively. Samples prepared in this way were incubated for 30 min at room temperature followed by an emission scan from 470–560 nm with an excitation wavelength of 450 nm. All

Fig. 1. (A) Absorption spectrum of CR binding by BCA II. The dotted line shows the specific shoulder peak representative of CR binding by incubated samples, solid line is the control spectrum of non-incubated BCA II sample. Inset shows a difference spectrum of the peak at 548 nm. (B) Fluorescence spectrum of ThT bound BCA II amyloids after 12 days of incubation (dotted line) as compared to control (solid line). (C) Kinetics of amyloid formation as observed by increase in ThT fluorescence with increasing periods of incubation, experimental results of five sets of repeats are plotted. (D) Far-UV CD spectra of BCA II at 0 h (solid line), and after incubation for 4 h (dash and dotted line), 10 h (dotted line) and 28 h (dashed line) respectively.
fluorescence measurements were done in quartz cuvettes of 1 cm path length in a Perkin-Elmer Lambda-45 fluorimeter operated with excitation and emission band-widths of 10 nm.

2.5. Structural changes in BCA II upon incubation

Circular dichroism spectrometry was done using a Jasco J-810 spectropolarimeter. Incubated protein samples were diluted to a final protein concentration of 0.1 mg/ml in 10 mM sodium acetate buffer pH 3.5 and far-UV CD spectra were taken in 0.1 cm path length cuvette at varying intervals of time.

2.6. Fluorescence microscopy

Incubated protein samples were centrifuged and the pellets were collected and placed on a glass slide. A 20 µM ThT solution was then added to the pellet material and covered with a glass slip. The slide was then observed at 1000x magnification under light and dark fields to detect the presence of clusters of amyloid fibers in an Olympus IX-51 fluorescence microscope using a 450–500 nm filter. A sample of thermal aggregates was used as a control.

2.7. Transmission electron microscopy

Proteins samples incubated with GdnHCl at pH 3.5 were periodically removed and placed on TEM grids. As a control thermal aggregates of BCA II (as obtained earlier) and a native protein solution were also placed on the grids. Each sample (10 µl) was placed on a copper grid and was air dried for 10 min to ensure adsorption of the protein on the grid. For negative staining of the samples, a saturated uranyl acetate solution (2 µl) was added on to the copper grid and allowed to air dry. Samples were then observed under a Phillips C12 microscope operating at 800 kV.

2.8. Recurrence quantification analysis

Windowed RQA was performed on the hydropathy sequence of BCA II using the software developed earlier [38]. The parameters used were delay of 1, epoch size of 36, data shift of 1, maximum radius scaling and line parameter of 2. A plot of % DET versus residues for varying radius values for the entire protein sequence was plotted to search for characteristic singularities [35].

3. Results

Formation of amyloids by incubated samples of BCA II was monitored by CR and ThT-binding assays as given in the Materials and methods section. CR binding is detected as a characteristic red shifted peak at 540 nm from 490 nm with samples incubated for 15 days as shown in Fig. 1A. Thioflavin-T is reported to bind specifically to amyloids and show an increase in the fluorescence intensity at 482 nm when excited at 450 nm [39]. This is observed in the case of BCA II samples after 12 days of incubation as in Fig. 1B. A progressive increase in ThT fluorescence was observed with increasing periods of incubation as in Fig. 1C. A prominent lag period of about 10 h was observed where there were no ThT-binding aggregates.

Fig. 2. Fluorescence microscopy of BCA II aggregates after incubating with ThT and washing. Amyloids were observed in bright field (A) and dark field (B) respectively. For comparison, the thermal aggregates were equally treated with ThT and observed under bright (C) and dark field (D) respectively.

Fig. 3. Electron micrographs of BCA II following different kinds of treatment. (A) Non-incubated, non-aggregated BCA II solution. (B) Sample after 12 days shows organized chain of beads (considered as amyloid precursors). (C) Emergence of fibrillar structure after 20 days, arrow shows merging of beads to form fibers. (D) Fibrillar assemblies interconnected with globular supra-molecular structures (arrow heads) after 30 days of incubation. (E) Intertwined thick fibers (arrow) formed by fusion of individual fibers visible after 45 days of incubation. (F) Disordered thermal aggregates of BCA II.
A change in protein secondary structure during initial hours of incubation was monitored by far-UV CD spectroscopy. Scans from 250 nm to 205 nm for samples incubated for different time periods are shown in Fig. 1D. A progressive increase in signal with increasing time of incubation was observed suggesting an increase in the β-sheet content. Spectra beyond 28 h of incubation were not taken as the data became inconsistent probably because of aggregation.

Visual distinction of the ThT-binding fibrils from amorphous aggregates was done by fluorescence microscopy. The BCA II samples incubated for a month displayed clear visibility due to increased ThT fluorescence in the dark field, while the thermal aggregates were poorly visible under similar conditions as in Fig. 2.

To verify that the aggregates obtained have the morphological characteristics typical of amyloid fibrils, TEM was done, results of which are shown in Fig. 3. Image of amorphous aggregates obtained after thermal denaturation is also shown for comparison [Fig. 3F]. For samples incubated for 12–15 days, large, globular, linearly-arranged, beaded structures were the conspicuous features in most of the grids scanned. The size of the beads was in the range of 100–200 nm in diameter [Fig. 3B]. Emergence of fibrillar structure by possible fusion of beads were observed after incubation for 20 days as shown in Fig. 3C. Although scantly populated, fibrillar structures were visible in samples incubated for 1–2 months, resembling morphologies similar to other amyloid fibrils as in Fig. 3D and E.

A RQA was done on the entire sequence of BCA II. Graphs were plotted for the full sequence and the one showing characteristic singularities extending from residues 186 to 235 are shown in Fig. 4.

4. Discussion

Most proteins including BCA II exist in its stable, globular and soluble form under physiological conditions. However, formation of amyloid fibril requires partially unfolded intermediates [40]. Hence, a variety of partially denaturing conditions like low pH and varying GdnHCl concentrations were tried for incubating the protein. Finally, a pH of 3.5 and 1.5 M GdnHCl concentration were found to be optimal for amyloid formation. Under these conditions, BCA II formed visible aggregates in a time period of about 10–15 days. Interestingly a GdnHCl concentration of 1.5 M has previously been found to lead to the formation of molten globule state of BCA [41]. Formation of molten globules has also been reported around a pH of 3.5 [42]. It can be inferred that under these conditions, BCA II exists in a partially unfolded state with exposed stretches of high hydrophobicity. These stretches, in a concentration-dependent manner, encounter increased collisional interaction to form intermolecular contacts. Hence, under these conditions the partially structured protein molecules get partitioned into an aggregated and a soluble state. The aggregates formed by BCA II were tested for CR binding for preliminary characterization. They were found to produce a notable shift in the absorption spectrum of CR, as shown in Fig. 1A. The appearance of a shoulder peak in the case of BCA II is quite sharp as is reported for other protein amyloids [43]. The shape and sharpness of the shoulder peak may be an attribute of the protein in question. A conspicuous peak around 540 nm in the difference spectrum reinforces the above point (see inset Fig. 1A). Since the specificity of CR binding to amyloids is frequently questioned, fluorescence emission enhancement of ThT upon binding to amyloid was also used in our study [44]. To avoid interference by ThT in amyloid formation, aliquots of BCA II incubated samples were mixed with ThT just prior to taking the emission measurements. An increase in fluorescence emission for the 12 days incubated BCA II sample confirmed the presence of amyloids. Although kinetics of amyloid formation has previously been studied by following ThT fluorescence [45], we could get reliable kinetic data only for the initial phases of incubation as in Fig. 1C. The change in fluorescence was monitored up to 5 days, where it showed a progressive increase with a lag period of 10 h. For the later phases kinetic data could not be measured reliably as relative fluorescence data varied largely because of aggregation. A concomitant increase in the β-sheet content was observed by CD spectroscopy [Fig. 1D] starting at 4 h, while ThT binding was observed only after 10 h incubation (fluorescence data). This suggests that structural alteration and an increase in β-sheet content precipitates intermolecular association to form ThT-binding amyloid.

A direct visual confirmation of ThT-binding to BCA II amyloids was done by fluorescence and transmission electron microscopic experiments. To emphasize the difference between an amorphous aggregate and an amyloid, thermally denatured BCA II aggregates were used as control. Thermal aggregates display a random association of protein molecules in TEM [Fig. 4F], and lacks ThT-binding capacity [Fig. 3B]. On the contrary, incubated BCA samples associate to form ordered fibrillar structures and were capable of binding ThT. At an early stage of incubation (10–100 h), failed to display any ordered structure when observed under TEM. They however exhibit some globular features reminiscent of ordered arrangements as is seen on further incubation (data not shown). Only after incubation of more than 12 days, did ordered beaded ThT-binding structures start appearing. These globules were considered to be the originators of fibrillar structures as they display significant ThT binding, which eventually coalesce to form the fibrils as evidenced from the electron micrograph picture [Fig. 3C]. Such structures have previously been reported in case of HaPrP and Aβ amyloidosis [46] and lend support to the proposal that ultrastructural, supra-molecular; multimeric assemblies precede amyloid formation [47]. Under the non-agitated conditions followed in our studies, the few fibers formed, tend to merge together by intertwining giving rise to thicker fibers sparsely populated on the EM Grids as shown in Fig. 3D.

The results conclusively prove the formation of amyloid fibrils by BCA II under the described conditions. To indicate the residues that might be involved in amyloid formation, the statistical method of RQA was applied to the primary sequence of BCA. This analysis revealed the presence of a characteristic singularity in the hydrophobicity pattern near the C-terminal. Such singularities have previously been shown to predispose proteins towards amyloid formation [35] determined by their ability to form local contacts between hydrophobic stretches. The hypothesis that specific hydrophobic stretches on proteins may significantly alter their amyloidogenic propensities is strongly supported by recent results [47]. It is notable that the singularities for BCA II were observed over a much longer stretch as compared to most other known amyloid forming proteins that have been analyzed [35]. As stated earlier, residues from 186 to 235 at the C-terminus of the protein have been proposed to be involved in the initial hydrophobic collapse. Interestingly, these residues form a typical penultimate β-sheet towards the C-terminus, which forms intimate contacts with the other two β-sheets (from crystal structure) [34]. Two residues, Thr 198 and Thr 197, within the 186–235 stretch are also in the active site of the protein. This may be indicative of their high contact forming propensity, a fact that is indicated by the RQA results. This is in
contrast to amyloidogenic stretches for human prion conversion which typically lie at the N-terminus of the protein [48]. Unlike most proteins known to form amyloids, BCA II is a predominantly β-sheet protein. Only few examples of amyloid formation by β-sheet-rich proteins are known, like the hydrophobin and β2-microglobulin. Hydrophobins are a class of small fungal proteins that naturally self-assembles to form polymeric monolayers [49,50]. β2-microglobulin, a component of the major histocompatibility class complex I have been shown to undergo self-association by edge-to-edge interaction at pH 5.7 [51]. Small, natively unfolded monomeric protein, like α-synuclein also undergoes self-association and fibrillation [52]. BCA II, in comparison, is a monomeric, globular, single-domain protein and, hence, no such association-seeking tendency is expected to be built into it. β-sheet proteins in general impede aggregation by edge strand adaptations such as charged stretches, proline residues and β-turns etc. [53]. Interestingly, the β-strand stretch indicated by the RQA on BCA II is the penultimate one according to available literature [33,34] and is not expected to have any such adaptation. Hence, a natively β-sheet-rich, reversibly folding, monomeric, protein BCA II forms amyloids. Formation of amyloids clearly requires a certain degree of unfolding before aggregation can be initiated. Such a picture of amyloid formation by BCAIIs fit well with the existing understanding of amyloidogenesis [54].

Acknowledgements

We acknowledge the financial support provided by Indian Institute of Technology, Delhi in the form of the ‘summer undergraduate research award’. We are grateful for the help extended by Dr. P. Guptasarma and Shabbir Ahmed for CD and fluorescence microscopy. We are grateful for the help extended by Dr. P. Aditya Mittal, Ms. Guptasarma and Shabbir Ahmed for CD and useful discussions. We also thank Dr. D.A. Dolgikh, A.P. Kolomiets, I.A. Bolotina, O.B. Ptitsyn, for their help extended by D. A. Dolgikh, A. P. Kolomiets, I. A. Bolotina, O. B. Ptitsyn, for the help extended by D. A. Dolgikh, A. P. Kolomiets, I. A. Bolotina, O. B. Ptitsyn, for the help extended by D. A. Dolgikh, A. P. Kolomiets, I. A. Bolotina, O. B. Ptitsyn, for the help extended by D. A. Dolgikh, A. P. Kolomiets, I. A. Bolotina, O. B. Ptitsyn, for the help extended by D. A. Dolgikh, A. P. Kolomiets, I. A. Bolotina, O. B. Ptitsyn.

