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Conformational Conversion May Precede or Follow Aggregate Elongation on Alternative Pathways of Amyloid Protofibril Formation

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A major goal in the study of protein aggregation is to understand how the conformational heterogeneity characteristic of the process leads to structurally distinct amyloid fibrils. The small protein barstar is known to form amyloid protofibrils in multiple steps at low pH: a small oligomer, the A-form, first transforms into a larger spherical higher oligomeric intermediate (HOI), which then self-associates to form the elongated protofibril. To determine how the conformational conversion reaction during aggregation is coupled to the process of protofibril formation, cysteine-scanning mutagenesis was first used to identify specific residue positions in the protein sequence, which are important in defining the nature of the aggregation process. Two classes of mutant proteins, which are distinguished by their kinetics of aggregation at high protein concentration, have been identified: Class I mutant proteins undergo conformational conversion, as measured by an increase in thioflavin T binding ability and an increase in circular dichroism at 216 nm, significantly faster than Class II mutant proteins. At low protein concentration, the rates of conformational conversion are, however, identical for both classes of mutant proteins. At high protein concentration, the two classes of mutant proteins can be further distinguished on the basis of their rates of protofibril growth, as determined from dynamic light-scattering measurements. For Class I mutant proteins, protofibril elongation occurs at the same, or slightly faster, rate than conformational conversion. For Class II mutant proteins, protofibril elongation is significantly slower than conformational conversion. Dynamic light scattering measurements and atomic force microscopy imaging indicate that for the Class I mutant proteins, conformational conversion occurs concurrently with the self-association of prefibrillar HOIs into protofibrils. On the other hand, for the Class II mutant proteins, the prefibrillar HOI first undergoes conformational conversion, and the conformationally converted HOIs then self-associate to form protofibrils. The two classes of mutant proteins appear, therefore, to use structurally distinct pathways to form amyloid protofibrils. On one pathway, conformational conversion occurs along with, or after, elongation of the oligomers; on the other pathway, conformational conversion precedes elongation of the oligomers. Single mutations in the protein can cause aggregation to switch from one pathway to the other. Importantly, the protofibrils formed by the two classes of mutant proteins have significantly different diameters and different internal structures.

Keywords: barstar; amyloid protofibrils; soluble oligomers; conformational conversion; alternative pathways

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Abbreviations used: HOI, higher oligomeric intermediate; AFM, atomic force microscopy; ThT, thioflavin T; wt, wild type; DLS, dynamic light scattering.

Introduction

Virtually any protein can self-assemble into amyloid fibrils,[1] and structural heterogeneity is the hallmark of virtually all protein aggregation reactions leading to the formation of amyloid fibrils. Heterogeneity is evident in the different sizes of oligomers that are populated transiently during fibril formation and in the variety of structures (spherical, annular, and worm-like) that these oligomers adopt.[2–12] Heterogeneity is evident in the structures of amyloid fibrils themselves.[2,13–17] While the cross-β motif is a feature common to all amyloid fibrils,[18,19] its exact molecular structure can show variations. A protein can form amyloid fibrils of multiple distinct conformations, not only in response to a change in either fibril growth conditions[13,20,21] or the amino acid sequence of the protein[14] but also under a single growth condition.[2,15] Understanding the origin of the conformational polymorphism seen in amyloid fibril structures, in terms of the underlying mechanism of self-assembly, is a major goal of protein aggregation studies.

The roles of prefibrillar spherical oligomers in the assembly of elongated worm-like protofibrils (whose ends can sometimes close to form annular rings[22]), as well as of long, straight fibrils, are still poorly understood. An important question is whether the conformational conversion reaction leading to the characteristic increase in β-sheet structure occurs in prefibrillar spherical oligomers or in elongated protofibrillar structures, both of which have been implicated as toxic entities in diseases related to amyloid fibril formation.[1,23–25] Determining whether fibrillation drives conformational conversion, or whether conformational conversion drives fibrillation, will help in delineating the pathways of fibrillation[5,7,21,26–28] and the possible roles of alternative pathways in giving rise to alternative conformational variants of amyloid protofibrils and fibrils.[2,14,21,29] Understanding how alternative pathways of aggregation originate, how they differ in intermediate aggregate structures and in the sequence of steps involved, and how aggregation may switch from one available pathway to another is necessary, for example, for understanding strain diversity and amyloidosis in the case of prion protein.[30–32]

Like many other proteins, the small protein barstar forms soluble oligomers (the A form), as well as amyloid protofibrils and fibrils, at low pH. The A form of barstar is a symmetrical aggregate formed by the self-assembly of about 16 protein molecules.[33,34] NMR characterization of the A form has shown that the core of the aggregate is formed by the C-terminal segments of the 16 self-assembled protein molecules.[35] The A form transforms into amyloid protofibrils in a slow stepwise process that is accelerated at higher temperatures.[12,36] The amyloid protofibrils of barstar are elongated worm-like fibrils, and atomic force microscopy (AFM) studies suggest that they are assemblies of the A form oligomers.[11,12] Time-resolved fluorescence studies have suggested that the cores of the aggregates in the A form oligomers and in the protofibrils are similar.[11] This report presents the results of a cysteine-scanning mutagenesis study that investigates the effects of mutations on the kinetics of protofibril formation from the A form. The advantage of using cysteine, instead of proline or alanine, in scanning mutagenesis is that the thiol group of a cysteine can be easily modified. If a site-specific effect on aggregation kinetics is observed upon replacement of an amino acid residue by cysteine, it then becomes easy to obtain further structural information on the importance of the residue position by suitable chemical modification of the cysteine thiol.

The cysteine-scanning mutagenesis study shows that the kinetics of formation of protofibrils from the A form are highly dependent on the position of the mutation. It is seen that single point mutations can lead to the formation of amyloid protofibrils of distinct conformations. The use of multiple structural probes to monitor the kinetics of protofibril formation by a few representative mutant proteins suggests that the formation of distinct protofibril conformations occurs *via* alternative pathways. The sequence of events occurring and differentiating the alternative pathways is described. It appears that single mutations can cause the process of aggregation to switch between alternative pathways and can thereby change the conformation of the amyloid protofibrils formed.

Results

Effect of mutations on the kinetics of amyloid protofibril formation

Figure 1a shows representative data for three different mutant variants of barstar showing that the kinetics of protofibril formation determined by the thioflavin T (ThT) binding assay are monophasic, with no lag phase, as seen previously for wild-type (wt) barstar.[12] Although similar amplitudes of fluorescence change are observed, the observed rate depends on the position of mutation when the concentration of the aggregating protein is 20 μM, but not when it is lower at 5 μM (Supporting Information, Fig. S1; Fig. 1b). The high reproducibility of the data is indicated by the small error in measurement at each time point. Importantly, overlapping kinetic curves are obtained when samples are spun at 20,000*g* for 2 min prior to the ThT assay, and when they are not, indicating that there are no large insoluble aggregates interfering with any of the spectroscopic measurements (data not shown).

The kinetics of protofibril formation by 15 single cysteine-containing mutant variants of barstar, when determined at the higher protein concentration (20 μM), are found to be monophasic, with no lag phase (Fig. S2). The aggregation kinetics fit reasonably well to a single-exponential equation, even though the aggregation reaction is complex and not