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Mechanisms of Ataxin-3 Misfolding and Fibril Formation: Kinetic Analysis of a Disease-associated Polyglutamine Protein

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The polyglutamine diseases are a family of nine proteins where intracellular protein misfolding and amyloid-like fibril formation are intrinsically coupled to disease. Previously, we identified a complex two-step mechanism of fibril formation of pathologically expanded ataxin-3, the causative protein of spinocerebellar ataxia type-3 (Machado-Joseph disease). Strikingly, ataxin-3 lacking a polyglutamine tract also formed fibrils, although this occurred only *via* a single-step that was homologous to the first step of expanded ataxin-3 fibril formation. Here, we present the first kinetic analysis of a disease-associated polyglutamine repeat protein. We show that ataxin-3 forms amyloid-like fibrils by a nucleation-dependent polymerization mechanism. We kinetically model the nucleating event in ataxin-3 fibrillogenesis to the formation of a monomeric thermodynamic nucleus. Fibril elongation then proceeds by a mechanism of monomer addition. The presence of an expanded polyglutamine tract leads subsequently to rapid inter-fibril association and formation of large, highly stable amyloid-like fibrils. These results enhance our general understanding of polyglutamine fibrillogenesis and highlights the role of non-poly(Q) domains in modulating the kinetics of misfolding in this family.

Keywords: ataxin-3; polyglutamine; amyloid fibril; protein aggregation; protein misfolding

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Introduction

Ataxin-3 is a 42 kDa intracellular protein that is expressed throughout the tissues of the body.[1] It consists of multiple domains, including the globular N-terminal Josephin domain, two ubiquitin interacting motifs, and a polyglutamine (poly(Q)) tract towards the C-terminus.[2,3] Expansion of the C-terminal poly(Q) tract beyond 45 consecutive residues causes the neurodegenerative disorder spinocerebellar ataxia type-3 (SCA3), also known as Machado-Joseph disease.[4,5] SCA3 is a member of the poly(Q) disease family, a group of nine progressive neurodegenerative diseases caused by the expansion of poly(Q) tracts within proteins.[6] Aberrant protein misfolding and aggregation was identified as a possible key causative factor in poly(Q) diseases, with the observation that different types of intraneuronal aggregates occurred within patients affected by poly(Q) diseases.[7–9] Nuclear inclusions are the most prominent aggregate type, and have been shown to contain the expanded poly(Q) protein, ubiquitin, and subunits of the proteasome.[7–14] This co-localization of nuclear inclusions with functional units of the quality control systems of the cell emphasizes a role for protein misfolding and aggregation in the disease process. In a process reflecting this intracellular aggregation, expanded poly(Q) tracts have an inherent ability to induce the formation of amyloid-like fibrils across a wide-range of protein contexts.[15–21] Here, we use the term non-expanded to represent poly(Q) proteins with a poly(Q) tract less than 45 repeats and expanded to represent poly(Q) proteins with a poly(Q) tract in excess of 45 repeats, which represents the pathological setting. The *in vitro* and *in vivo* aggregation rates of poly(Q) proteins increase substantially with poly(Q) tract length. Interestingly, kinetic investigations of this relationship have identified it as the probable cause of the inverse relationship between the length of the

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poly(Q) expansion, and the manifestation of disease symptoms.[17,22] Taken together, this and other evidence suggest that poly(Q) diseases are conformational diseases, where toxicity is linked to protein misfolding and self-aggregation.[23]

Despite being a major hallmark of poly(Q) disease, research suggests that nuclear inclusions are not the primary toxic species, and may play a protective cellular role.[24–27] Therefore, as with conformational diseases in general, attention is now focused on the identification of other putative toxic structures formed during protein misfolding and aggregation. A greater understanding of the kinetic mechanisms involved in poly(Q)-mediated misfolding and aggregation is essential in our understanding of poly(Q) diseases in general, and in the development of rational treatments. Currently, in terms of the poly(Q) aggregation pathway, poly(Q) peptide fibril formation has been modeled as a nucleation-dependent polymerization mechanism.[17] Furthermore, it was identified that nucleation was analogous to a thermodynamically unfavorable misfolding event within the poly(Q) tract.[17,28] After nucleation, elongation proceeded by a lock and dock mechanism of monomer addition, which is a mechanism similar to that proposed for amyloid-β and sup35 fibril growth.[17,29–31] However, the aggregation of full-length proteins, such as ataxin-3, is more complicated as the other domains within the protein can themselves aggregate.[19,32,33]

Several groups have studied full-length ataxin-3 aggregation *in vitro*, with both expanded, and non-expanded ataxin-3 forming amyloid-like fibrils under native and destabilizing conditions.[18,19,32–37] Intriguingly, the Josephin domain of ataxin-3 was also shown to form amyloid-like fibrils.[32] This adds an extra dimension of complexity to ataxin-3 fibrillogenesis, with two protein regions capable of instigating the formation of amyloid-like fibrils.[19,32,38,39] We recently showed that full-length ataxin-3 has an inherent propensity to form fibril-like aggregates independently of the presence of the poly(Q) tract.[19] In line with the poly(Q) length-dependence of SCA3, expansion of the poly(Q) tract beyond the disease threshold increased the rate of aggregation.[19] Interestingly, the presence of an expanded poly(Q) tract led to the formation of a second stage of aggregation marked by a large increase in end-stage fibril stability.[19]

In this study, we have performed the first kinetic analysis of a range of full-length ataxin-3 variants in order to gain a greater understanding of the mechanisms of ataxin-3 misfolding and fibril formation. These variants include ataxin-3 without a poly(Q) tract (at3(QHQ)), ataxin-3 in the non-pathological range (at3(Q15)), and an expanded pathological length variant (at3(Q64)). Here, we firstly model the first stage of aggregation to a nucleation-dependent polymerization mechanism. We find that fibril formation is instigated by the formation of a monomeric thermodynamic nucleus. Once nucleated, this stage of ataxin-3 fibril formation proceeds by a monomer addition mechanism. However, the aggregation of at3(QHQ) highlights a role for non-poly(Q) regions in the instigation and modulation of the fibrillogenesis pathway. We lastly show that the formation of large detergent-resistant aggregates by expanded ataxin-3 is kinetically linked to the intrinsic propensity of the protein to misfold. This study therefore provides the first kinetic description of the aggregation pathway of a full-length poly(Q) protein, and highlights that the poly(Q) tract and the presence of other domains within a full-length protein can add significant complexity to the fibrillogenesis pathway.

Results

Ataxin-3 aggregation is modeled by a nucleated growth polymerization mechanism

We have previously shown that changes in ThT fluorescence report the first-stage of ataxin-3 aggregation, correlating with the loss of monomer from solution and the formation of fibril-like aggregates.[19] The aggregation of all ataxin-3 variants displayed a lag phase that could be abolished by seeding,[19] a characteristic shown also by Gales *et al.* for non-pathological length ataxin-3.[33] This has led to the suggestion that ataxin-3 aggregation occurs by a nucleated growth polymerization mechanism.[19,33] In order to test a nucleated growth polymerization model for ataxin-3 more rigorously, we firstly analyzed the concentration-dependence of ataxin-3 aggregation (Figure 1). The aggregation of all ataxin-3 variants, followed by a continuous ThT assay, was concentration-dependent, with a lag phase, the duration of which was decreased with increasing concentration of protein (Figure 1). The rates of aggregation increased with the expansion of the poly(Q) tract into the pathological range.

Recent mathematical analysis by Ferrone, specify that the kinetics of a nucleated growth mechanism will conform to certain parameters. These parameters separate this model from a simpler non-nucleated growth model or more complex models, including aggregation reactions, with secondary pathways which can be caused by branching or fragmentation.[40] The first of these parameters is that the first 10% of the aggregation reaction should display a time-squared (t^2)-dependence, or cosine-dependence. All ataxin-3 variants display a t^2-dependence of aggregation across a broad concentration range (Figure 2). The rates of aggregation derived from the t^2 data also, as expected, decrease significantly with a lowering of the initial protein concentration. For a nucleated growth polymerization model of aggregation, this initial aggregation rate should show a higher order rate-dependence upon protein concentration.[40] To determine this rate dependence, we followed the previous analysis of Chen and colleagues by plotting the log/log relationship between the initial rate of aggregation derived from the t^2 plots and concen-