The Two-stage Pathway of Ataxin-3 Fibrillogenesis Involves a Polyglutamine-independent Step*

Andrew M. Ellisdon, Bronwen Thomas, and Stephen P. Bottomley

From the Department of Biochemistry and Molecular Biology, Monash University, P. O. Box 13D, Wellington Road, Clayton, Victoria 3800, Australia

The aggregation of ataxin-3 is associated with spinocerebellar ataxia type 3, which is characterized by the formation of intraneuronal aggregates. However, the mechanism of aggregation is currently not well understood. Ataxin-3 consists of a folded Josephin domain followed by two ubiquitin-interacting motifs and a C-terminal polyglutamine tract, which in the non-pathological form is less than 45 residues in length. We demonstrate that ataxin-3 with 64 glutamines (at(Q64)) undergoes a two-stage aggregation. The first stage involves formation of SDS-soluble aggregates, and the second stage results in formation of SDS-insoluble aggregates via the poly(Q) region. Both these first and second stage aggregates display typical amyloid-like characteristics. Under the same conditions at(Q15) and at(QHQ) undergo a single step aggregation event resulting in SDS-soluble aggregates, which does not involve the polyglutamine tract. These aggregates do not convert to the SDS-insoluble form. These observations demonstrate that ataxin-3 has an inherent capacity to aggregate through its non-polyglutamine domains. However, the presence of a pathological length polyglutamine tract introduces an additional step resulting in formation of a highly stable amyloid-like aggregate.

Ataxin-3 is a 42-kDa multi-domain protein consisting of an N-terminal Josephin domain, two ubiquitin-interacting motifs, which are situated next to a polymorphous C-terminal polyglutamine (poly(Q))3 tract (1, 2). Ataxin-3 functions as a de-ubiquitinating enzyme (3, 4) and binds polyubiquitin chains through the ubiquitin-interacting motifs (5–8). Expansion of the poly(Q) tract beyond 45 residues causes spinocerebellar ataxia type 3 (SCA3) also known as Machado-Joseph disease (9, 10). Similar dynamic expansion of poly(Q) tracts within various other proteins causes a further eight autosomal dominant neurodegenerative diseases, collectively termed poly(Q) diseases (2, 11).

Several key observations have led to the conclusion that poly(Q) diseases originate via a toxic gain of function, mediated by poly(Q) tract expansion. Firstly, the manifestation of each poly(Q) disease is directly reliant on a threshold length of consecutive glutamine residues. In all of the diseases, except for SCA6, this threshold is remarkably similar with a tract length in excess of 40 residues associated with disease onset (2).

Secondly, there is a non-linear correlation between an increasing glutamine tract length and an earlier age of disease onset (12). Thirdly, different proteins involved in each of the various poly(Q) diseases share no sequence homology except the presence of the glutamine tract (13). Various studies have suggested that this toxic gain of function is causally linked to aberrant protein aggregation mediated by the extended poly(Q) tract (14). In vitro studies have shown that poly(Q) peptides, fragments, and proteins can form amyloid-like fibrillar aggregates and that the aggregation rate increases with increasing glutamine tract length (15–19). Various types of intraneuronal aggregates, including nuclear inclusions, of the poly(Q) protein in question are also evident upon post-mortem analysis (20–22). These observations, among others, suggest that poly(Q) diseases are conformational diseases, in which toxicity is linked to the ability of the protein to self-associate and form aggregates (23–27).

We and others have demonstrated that under physiologically relevant conditions expanded ataxin-3 can form amyloid-like fibrils (19, 28). Furthermore, under certain conditions non-expanded ataxin-3 can also form amyloid-like fibrils and their precursors (29–33). The conclusion from these studies is that the poly(Q) tract is the main mediator of the aggregation process, with other domains and surrounding residues playing secondary roles in modulating aggregation (34).

Recently however, it was shown that under destabilizing conditions the Josephin domain alone is capable of forming amyloid-like fibrils, with a similar rate to full-length ataxin-3 containing a 15-residue-long poly(Q) tract (29). The authors suggested that the Josephin domain is a major determinant in ataxin-3 aggregation, with the poly(Q) tract playing a role in increasing the rate of fibril formation (29). Although it is clear that the poly(Q) tract is necessary for kinetic instigation of aggregation and, therefore, the disease process, these data and other work have led to the hypothesis that non-poly(Q)-containing domains may play a large role in determining the aggregation characteristics and toxicity of poly(Q) proteins (35–37). We have therefore studied a range of ataxin-3 variants in an attempt to understand how both the poly(Q) tract and protein context kinetically and structurally characterize the aggregation pathway. These variants include ataxin-3 without a poly(Q) tract (at3(QHQ)), a non-pathological length variant with 15 glutamines (at3(Q15)), and pathological length ataxin-3 with 64 glutamines (at3(Q64)). We firstly demonstrate that full-length ataxin-3 has an intrinsic ability to form amyloid-like fibrils independent of the poly(Q) tract. However, we further show that a pathological length poly(Q) tract introduces a second step into the aggregation pathway, which dramatically alters the stability and morphology of the end-stage fibrils.

EXPERIMENTAL PROCEDURES

Materials—Phenylmethylsulfonyl fluoride, thioflavin T (ThT), and β-mercaptoethanol were all obtained from Sigma.
Expression and Purification of Ataxin-3 Variants—All ataxin-3 variants were expressed and purified as described previously (19). Protein was stored at -80 °C and run on a Superose 12 column prior to each experiment to confirm it was monomeric.

Fibrillogenesis Time-course Assays—Ataxin-3 variants were incubated at a concentration of 30 μM at pH 7.4 in Tris-buffered saline (TBS) (100 mM Tris, 80 mM NaCl) containing 10% (v/v) glycerol, phenylmethylsulfonyl fluoride (2 mM), EDTA (5 mM), and β-mercaptoethanol (15 mM). Reactions were performed at 37 °C without shaking and in air-tight containers to eliminate evaporation.

Membrane Filter Trap Assay—The membrane filter-trap assay was carried out as previously described with only minor modifications (16, 17). An aliquot of protein (30 μg) was removed from the fibrillogenesis time-course incubations and diluted into 4% SDS (w/v) and 100 mM dithiothreitol at a 1:1 ratio. The samples were heated for 5 min at 100 °C before being diluted to 200 μl with 0.1% (w/v) SDS. The samples were filtered through a cellulose acetate membrane (Schleicher and Schuell, 0.2 μm) using a Bio-Rad Bio-Dot SF microfiltration unit. After application, the membrane was washed twice by filtering through 200 μl of 0.1% SDS (w/v). The membranes were then blotted with a hexahistidine (His6) antibody (Serotec).

ThT Measurements—ThT fluorescence was measured using a BMG Laboratories FLUOstar Optima fluorescence plate reader. Excitation and emission filters used were for 450 and 490 nm, respectively. All reactions were carried out in black 384-well clear bottom plates with both excitation and emission read from the bottom. Reactions were carried out at 37 °C, without shaking, and the top of the plate was sealed to prevent any evaporation. Plate reader assays contained 20 μM ThT, with all other buffering solutions and additives remaining the same as previously described herein. Discontinuous assays, carried out without the continual presence of ThT, displayed comparable kinetics to the continuous plate reader assays (data not shown).

SDS Stability Assays—An aliquot of 15 μg of end-stage fibrils was removed from the fibril assay and immediately diluted into 200 μl of SDS at the indicated concentration. Samples were incubated for 20 min at room temperature before being filtered through a 0.2-μm cellulose acetate membrane (Schleicher and Schuell). The membrane was pre-equilibrated in TBS, and after filtration 200 μl of TBS was filtered through the membrane as a washing step. The membrane was then probed as previously described for the membrane filter-trap assay.

QBP1 Aggregation Assays—The QBP1 peptide was purchased from AusPep with the same sequence as described previously (3). The peptide was dissolved in Me2SO to a final concentration of 13.5 mM. The peptide was incubated with ataxin-3 aggregation assays at a concentration of 120 μM. Upon final dilution the concentration of Me2SO was <1%. Control assays with identical final Me2SO concentrations were used for comparison with QBP1 assays.

Fluorescence Spectroscopy—All fluorescence measurements were performed by diluting aliquots of sample into TBS (pH 7.4) to a final concentration of 1 μM. Fluorescence emission spectra were immediately recorded on a PerkinElmer Life Sciences LS50B spectrofluorometer with a thermostatted cuvette holder at 25 °C, using a 1-cm path length quartz cuvette. For intrinsic fluorescence measurements an excitation wavelength of 280 nm was used, with measurement of the emission spectra from 300 to 450 nm. Emission and excitation slit widths were set at 2.5 and 3.0 nm, respectively, and a scan speed of 25 nm/min was used.

Size Exclusion Analysis of Fibril Formation—Size exclusion chromatography (SEC) was carried out on an Amersham Biosciences fast protein liquid chromatography system. Fibril formation was monitored by the loss of area of the monomer peak on a Superose 12 column. At indicated time points 20 μg of protein was injected onto the column, and its elution was monitored by absorbance at 214 nm. The area of the peak was calculated using the manufacturers supplied software.

Circular Dichroism—CD analysis was carried out by removing aliquots of protein from the incubating sample and diluting into TBS to 21.4 μM immediately prior to analysis. All far-UV CD spectra were measured on a Jasco-810 spectropolarimeter with a path length of 0.1 mm. Spectra were recorded from 190 to 260 nm, using a 5-s-point signal averaging, and a scan speed of 20 nm/min.

Transmission Electron Microscopy—TEM images were obtained using a Jeol JEM-200CX transmission electron microscope. The acceleration voltage was 100 kV. Samples were adsorbed onto carbon-coated grids and stained with 1% (w/v) uranyl acetate.

Analysis of Seeded Aggregation—End-stage aggregates were added to the fibril reaction immediately prior to incubation at 10% of the total molar concentration. A reaction containing seed only was analyzed, and the result was used to correct kinetics of seeded samples. ThT and membrane filter-trap assays were carried out as previously described herein.

RESULTS

Characterization of Ataxin-3 Variants—We expressed and purified three ataxin-3 variants from Escherichia coli as previously described (19) (Fig. 1A). Using SEC (Fig. 1B) and mass spectrometry (data not shown) we confirmed that the final purified proteins were entirely monomeric and intact. Furthermore, all the variants possessed ubiquitin-protease activity as previously described (data not shown) (3).

Only Expanded Ataxin-3 Forms SDS-resistant Aggregates—One common feature of poly(Q) aggregates is their ability to retain structure upon incubation with SDS (16, 17, 39). We initially analyzed the aggregation of all ataxin-3 variants using the membrane filter-trap assay routinely used to detect SDS-resistant huntingtin exon 1 fibrils (16, 17, 39). Upon incubation of all variants, at a concentration of 30 μM, we observe that only pathological length at3(Q64) forms SDS-resistant aggregates (Fig. 2A). Further analysis of the aggregation with densitometry reveals that at3(Q64) aggregation follows a classic nucleation-dependent time.
Aggregates under both near-physiological conditions (33) and denaturational length poly(Q) tracts have been shown to form amyloid-like stress (29). Furthermore, numerous ataxin-3 variants with non-pathological properties of the three ataxin-3 variants, under identical conditions (Fig. 2). Therefore, it is possible that the protein region responsible for the formation of ThT-positive aggregates involves the N-terminal Josephin domain, which is common to all of the proteins, although further work is needed to clarify this (29, 33).

Inspection of the kinetics derived from the SDS-insolubility and ThT assays (Fig. 2) suggests that pathological length at3(Q64) aggregates in two stages. The first is a ThT-detectable stage common to all variants, and this is followed by a second poly(Q)-length dependent stage in which classic SDS-insoluble poly(Q) aggregates are formed. A small degree of overlap is seen between the two phases, however the bulk of SDS-resistant aggregates do not appear to form until after a significant ThT increase.

Therefore, our data indicate that the three variants form different aggregates based upon the length of their poly-Q tract. If this is the case then the aggregates formed are likely to possess different stabilities. To examine these differences we analyzed the resistance of these end-stage aggregates to SDS. End-stage aggregates of the three proteins were incubated with increasing concentrations of SDS to determine any stability differences (Fig. 3). As can be seen, only at3(Q64) aggregates are stable upon incubation in up to 1% (w/v) SDS. The two shorter variants both lose the ability to be retained on the 0.2-μm filter after incubation in a very low 0.01% (w/v) SDS. This strongly suggests that the aggregates formed from expanded ataxin-3 are structurally different to those of the non-expanded variants. Furthermore, this increased stability of expanded ataxin-3 aggregates may have ramifications in the pathogenesis of the disease, as more stable aggregates are likely to be harder to break down and longer lived.

**QBP1 Inhibits the Second Phase of at3(Q64) Aggregation**—The data presented above suggest a two-step mechanism of aggregation with only the pathological length poly(Q) tract playing a role in formation of SDS-stable aggregates. To confirm this, we have used the QBP1 peptide as a tool to explore the role of the poly(Q) tract in defining the at3(Q64) aggregation pathway. The QBP1 peptide is 11 amino acids in length and has been shown to inhibit poly(Q) aggregation both *in vitro* and *in vivo* (38, 42). In Fig. 4 we followed the aggregation of 30 μM at3(Q64) by the combination of the filter-trap assay and the ThT assay. Upon incubation of the QBP1 peptide, at a molar ratio of 4:1 (peptide:protein), we still observe the initial ThT increase (Fig. 4C). However, the second stage of aggregation is completely eliminated, with no detectable SDS-insoluble aggregates formed in the presence of QBP1 (Fig. 4, A and B). Taken together these data indicate that expanded ataxin-3 aggregates through course, with a defined lag period followed by a rapid growth phase (Fig. 2B). Even upon incubation times of up to 200 h, at3(QHQ) and at3(Q15) did not form SDS-resistant aggregates, suggesting that under physiologically relevant conditions ataxin-3 forms SDS-resistant aggregates in a poly(Q)-length dependent manner similar to that seen in other poly(Q) proteins (15–19).

In contrast to the filter-trap assay we observed that all the variants are able to form ThT-positive aggregates. The rate of aggregation was poly(Q)-length dependent, with at3(Q64) aggregating at a faster rate than either at3(QHQ) or at3(Q15). However intriguingly, both at3(QHQ) and at3(Q15) aggregate with essentially identical kinetics (Table 1). The kinetics display time-squared dependence (data not shown) suggestive of a nucleation-dependent time course (40, 41). As at3(QHQ) contains no poly(Q) tract, it appears that the ataxin-3 protein contains an inherent propensity to form ThT-positive aggregates independently of the poly(Q) tract. In a recent study, the Josephin domain of ataxin-3 has been shown to form amyloid-like aggregates upon thermal stress (29). Furthermore, numerous ataxin-3 variants with non-pathological length poly(Q) tracts have been shown to form amyloid-like aggregates under both near-physiological conditions (33) and denaturational conditions (19, 29–31). Therefore, it is possible that the protein region responsible for the formation of ThT-positive aggregates involves the N-terminal Josephin domain, which is common to all of the proteins, although further work is needed to clarify this (29, 33).

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**TABLE 1**

| Kinetic analysis of the first aggregation stage of ataxin-3 |
|----------------------------------------------------------|
| **ThT** | **SEC** | **Fluorescence** |
|----------|---------|-----------------|
| at3(QHQ) | 23.1 ± 2.5 | 25.7 ± 4.1 | 26.5 ± 4.4 |
| at3(Q15) | 25.4 ± 3.9 | 22.6 ± 3.5 | 25.0 ± 2.4 |
| at3(Q64) | 10.4 ± 1.6 | 11.1 ± 2.5 | 11.8 ± 0.6 |

**FIGURE 3. Expanded ataxin-3 aggregates are highly stable.** The stability of the aggregates was analyzed under increasing SDS concentrations. Equal amounts (15 μg) of the ataxin-3 variants were treated with increasing amounts of SDS for 20 min before being filtered through a 0.2-μm cellulose acetate membrane and detected by Western blotting.

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**FIGURE 2. Aggregation monitored by SDS-insolubility and ThT.** A, aggregation of ataxin-3 variants monitored by the formation of SDS-insoluble aggregates. Shown is a membrane filter-trap assay representative of a typical result following incubation of the ataxin-3 variants at 30 μM (37 °C and pH 7.4). The protein samples were treated as previously described in the experimental procedures. B, analysis of filter trap blots by densitometry. Time courses are shown for at3(QHQ) (dashed line, ■), at3(Q15) (dotted line, ▲), and at3(Q64) (solid line, ★). Values are derived from at least three independent experiments and at3(Q64) was fit to an exponential curve. C, aggregation of 30 μM ataxin-3 monitored by ThT fluorescence. ThT fluorescence was monitored using a fluorescence plate reader with values read every 20 min. Exponential fits are shown for at3(QHQ) (dashed line, ■), at3(Q15) (dotted line, ▲), and at3(Q64) (solid line). All exponential fits have r² values of >0.95 and are derived from at least three independent data sets.
FIGURE 4. QBP1 inhibits the second phase of at3(Q64) aggregation. The effect of the QBP1 peptide on the aggregation of at3(Q64) (solid line without QBP1; dashed line with QBP1), as monitored by the formation of SDS-insoluble aggregates (A), densitometry of blots (B), and ThT fluorescence (C). The QBP1 protein was dissolved in Me2SO to a concentration of 13.5 mM and added to the reaction to give a final concentration of 120 μM. Results shown are representative of at least three independent experiments.

FIGURE 5. Aggregation monitored by intrinsic tryptophan fluorescence and size exclusion chromatography. Fluorescence emission spectra of ataxin-3 (at3(QHQ) (A), at3(Q15) (B), and at3(Q64) (C)) at 0 h (solid line) and 96 h (dotted line). Kinetics of aggregation of at3(QHQ) (D), at3(Q15) (E), and at3(Q64) (F) monitored by intrinsic fluorescence intensity (λ_{ex} = 360 nm) (right axis) and SEC chromatography (left axis). For all intrinsic fluorescence measurements the excitation wavelength was set to 280 nm, and the protein concentration was 1 μM. All spectra were recorded at 25 °C. For SEC, aliquots of protein were removed and analyzed using a Superose 12 column (Amersham Biosciences). The loss of monomer peak area was calculated for each variant using the supplied software.
Ataxin-3 Fibrillogenesis

a two-stage pathway, with only the second stage reliant on the presence of an expanded glutamine tract. We then went on to further structurally and kinetically characterize this pathway using a range of techniques.

Ataxin-3 Aggregation Monitored by Intrinsic Fluorescence and Size Exclusion Chromatography—The two-step mechanism of aggregation proposed above suggests that the N-terminal domain of ataxin-3, which is common to all the proteins, is involved in the first step. To determine if this is the case we exploited the fact that ataxin-3 contains three tryptophan residues, all of which are located within the Josephin domain, as such they act as a highly sensitive reporter groups for the tertiary structure of this domain. We therefore followed the changes in intrinsic tryptophan fluorescence during aggregation for all three proteins. We observe that, upon formation of the final aggregate conformation, all three variants adopt a hyper-fluorescent state in which the emission maxima has shifted from 333 nm in the native state to 340 nm in the aggregate (Fig. 5, A–C). These data suggest that Josephin has undergone a conformational change during aggregation and is in a similar non-native conformation in the final aggregation states for all three variants. For at3(Q64) there was no difference in emission spectra between the SDS-soluble and SDS-stable aggregates, suggesting that the Josephin domain undergoes no detectable structural rearrangement upon the formation of the SDS-stable aggregates. We also used this change in fluorescence to follow the kinetics of aggregation in reference to the conformational changes in the Josephin domain (Fig. 5, D–F). Interestingly, the fluorescence-derived kinetics correlates well with the gain of ThT signal (Table 1). These data further suggest that the Josephin domain plays a crucial structural role only in the first stage of ataxin-3 aggregation.

To further analyze the aggregation pathway of ataxin-3 we used SEC to follow the loss of monomer from solution during aggregation (Fig. 5, D–F). For all three variants, monomer loss showed a good correlation with both aggregation monitored by ThT and intrinsic fluorescence (Table 1). Our data are consistent with a model of aggregation, in which monomer conversion into the growing fibril is characterized by a structural rearrangement of the Josephin domain. During this first stage of aggregation, the aggregates are soluble, based upon their ability to run before or within the void volume of the SEC column (data not shown), and the lack of any increase in light scatter (data not shown). The second stage of aggregation is accompanied by a dramatic loss of solubility of the aggregates as they visibly precipitate from solution. This stage predominantly occurs after the loss of monomer from solution, suggesting that SDS-insoluble aggregates are formed through the summation of smaller aggregates, rather than by monomer addition to the end of growing fibrils. More detailed analysis of these stages of aggregation is currently underway to further kinetically characterize the stages of ataxin-3 aggregation.

Aggregate Formation Is Associated with an Increase in β-Sheet Secondary Structure—The changes in secondary structure of the ataxin-3 variants during aggregation were monitored using far-UV CD spectroscopy. At 0 h all variants display CD spectra similar to those previously reported (19), indicative of a predominantly α-helical protein (Fig. 6). After completion of aggregation at 96 h, both at3(QHQ) (Fig. 6A) and at3(Q15) (Fig. 6B) have undergone similar changes, with a gain of signal at 220 nm causing a flattening of the CD spectra. These spectral changes are indicative of an increase in β-sheet content, and a loss of α-helix. This is similar to that recently reported for fibril formation of the Josephin domain and non-pathological lengths of ataxin-3 under thermal denaturation (29, 31).

The secondary structural changes of at3(Q64) during the same incubation are more complex (Fig. 6C). An initial change in secondary structure, comparable to that of the non-pathological length variants, is seen after 24 h during the first phase of aggregation. Secondary structural changes are also evident upon the formation of SDS-stable aggregates, however during this phase the CD spectrum undergoes a significant loss of signal, thought to be caused by a large increase in light scatter during the second phase of aggregation (data not shown). This increase in light scatter unfortunately precludes us from obtaining detailed information on the secondary structure of end-point at3(Q64) fibrils.

Poly(Q) Tract Length Affects End-stage Aggregate Morphology—TEM has been used in a range of studies to observe both intermediate assemblies formed prior to fibril formation and end-stage aggregates (43–45). Here, we have undertaken a detailed analysis of the intermediate species and fibril morphologies formed during aggregation of all ataxin-3 variants at 30 μM. At 0 h, for all variants, the TEM grid has no prominent observable structures consistent with the ataxin-3 monomer as the predominant species (data not shown).

The first structures we observe, for all three variants, are a combination of small spherical-like aggregates and short curvilinear oligomeric aggregates (Fig. 7, A–C). Consistent with the ThT kinetics, these structures are formed at a faster rate in at3(Q64) than the other two shorter variants. The diameters of the spheroids vary between 5 and 20 nm,
which are similar to those observed recently with non-expanded ataxin-3 (33), other poly(Q) proteins/peptides (39, 43, 45), and amyloidogenic proteins (43, 46–48). The oligomeric aggregates also mirror those previously observed for non-expanded ataxin-3 (33), having a consistent diameter 10–12 nm and a length that increases with time. These short curvilinear aggregates also strongly resemble the protofibrils observed during amyloid-β aggregation (44, 49), α-synuclein aggregation (48, 50), and previous poly(Q) studies (45, 51). As aggregation continues these oligomers elongate, up to many hundreds of nanometers in length, while their diameter remains the same (Fig. 7, D–F). These banded aggregates appear quite flexible and have a very similar morphology to that of thermally derived Josephin aggregates (29). For both at3(QHQ) and at3(Q15) this represents the end-stage morphology of the aggregation pathway with no change in structure observed at later time points (Fig. 7, G and H).

At3(Q64) aggregates further into a second type of morphology whose occurrence is concurrent with the second phase of aggregation (Fig. 7I). These aggregates are larger and more fibrous than the banded aggregates formed during the previous stage, resembling those previously reported for at3(Q50) (19). Upon close analysis, some individual aggregates also resemble the beaded morphology previously reported for at3(Q78) (28). These fibrils tend to self-associate and form large insoluble aggregates, making their characterization by TEM difficult. These large filamentous aggregates are usually 50–100 nm in width and can be up to many microns in length. The banded aggregates that represent the end-stage morphology of the shorter variants no longer appear to be present.

Cross-seeding Reveals a Model Pathway of Ataxin-3 Aggregation—Our data suggest that the conformation of the final aggregates of at3(Q15) and at3(QHQ) are structurally different from those of at3(Q64). One of the properties of fibrillizing proteins, including the poly(Q) family of proteins, is that propagation of the aggregates can be accomplished by the presence of “seed,” which catalyzes the aggregation of free protein. We next determined whether the different aggregates produced by the different length repeat proteins could catalyze the aggregation of each other or there is a structural barrier within this family of aggregates that limits its propagation. Cross-seeding of each of the three variants was analyzed initially by ThT assay (Fig. 8).

All of the aggregation assays monitored display no observable lag phase and follow a linear gradient, indicative of the abolishment of the rate-limiting nucleation stage (Fig. 8, A–C). This confirms that ataxin-3 aggregation is nucleation-dependent, in line with previous studies of ataxin-3 (33), and kinetically is indicative of amyloid-like fibril formation (17). Furthermore, as each of the variants are able to cross-seed the aggregation pathways of the other, these data suggest that the aggregation pathways of the three proteins are also structurally similar, with each variant able to act as a template for the elongation of the other variants.

We then examined the same cross-seeding assays by the membrane filter-trap assay, which reports the formation of SDS-stable aggregates. In Fig. 8 (D and E), we can see that none of the variants are able to cross-seed the shorter variants into forming SDS-stable aggregates. This is most likely because the poly(Q) tracts in each of the variants are either non-existent or too short to readily form poly(Q)-dependent aggregates over the timeframe analyzed. Only at3(Q64) aggregates can noticeably accelerate the formation of poly(Q)-dependent fibrils from free at3(Q64) (Fig. 8F).

DISCUSSION

The poly(Q) family represents a biomedically important class of proteins whose aggregation is intrinsically linked to the disease process. A number of reports have analyzed the aggregation properties of poly(Q) peptides (15, 18), fragments of poly(Q) proteins (16, 39, 45), or fusion proteins containing poly(Q) tracts (38, 52). However, little is known about the aggregation of intact naturally occurring poly(Q) proteins. This has become increasingly important as numerous studies have recently highlighted the importance of protein context in the aggregation of some poly(Q) proteins (34, 35). In this present study, we report the kinetic and morphological changes of both non-expanded and pathologically expanded ataxin-3 upon aggregation. In taking this comparative approach, we have been able to explore both the role of protein context, and the poly(Q) tract in defining the pathway of ataxin-3 aggregation.

The major findings of this study arise from our ability to directly compare the aggregation of at3(QHQ), at3(Q15), and at3(Q64). Our detailed analysis of ataxin-3 aggregation showed that, independent of
the poly-Q tract length, the protein has an inherent propensity to aggregate. Aggregation of the shorter ataxin-3 variants (at3(QHQ) and at3(Q15)) occurred through a nucleation-dependent process that displayed simultaneous changes in ThT fluorescence, intrinsic tryptophan fluorescence, and SEC. Furthermore, morphological analysis revealed the formation of amyloid-like fibrils, which closely resemble those formed by the isolated Josephin domain (29). These data are consistent with previous studies of non-expanded ataxin-3, under physiological and denaturing conditions, which have also suggested that this protein has an intrinsic tendency to aggregate (32, 33). The biological relevance of this in-built aggregation propensity, displayed by both non-expanded ataxin-3 and the Josephin domain (29), to the disease process is unclear. It has been suggested that intracellular binding partners may strongly limit the conformational flexibility of ataxin-3 and in turn its susceptibility to aggregation (29).

Consistent with the poly(Q) tract length dependence of SCA3 (2) at3(Q64) has an increased rate of aggregation. The first stage of at3(Q64) aggregation occurs faster in expanded ataxin-3 but appears not to be structurally dependent on the poly(Q) tract. During this stage of aggregation the conformation of the Josephin domain has been shown to be altered. Only expanded ataxin-3 is capable of forming highly stable, SDS-resistant aggregates. This stage of aggregation is directly reliant on poly(Q) tract interactions.
These aggregates are dependent upon the presence of an expanded poly(Q) tract for their formation. The aggregates are highly stable and morphologically resemble those formed in previous studies of expanded poly(Q) proteins and peptides (16, 39, 45, 53).

On the basis of our data we propose a minimal two-stage model of ataxin-3 aggregation, which takes into account both protein context and the poly(Q) expansion (Fig. 9). The first stage involves a conformational change and self-association in regions other than the poly(Q) tract that results in the formation of SDS-soluble aggregates; the second step that occurred solely in the protein with an expanded poly(Q) tract results in formation of an SDS-insoluble state. Previous aggregation analysis of poly(Q) peptides and huntingtin exon 1 identified a concerted single stage of aggregation, which is characterized by the formation of SDS-insoluble aggregates and an increase in β-sheet secondary structure (15–18, 53). Our data clearly demonstrate that for ataxin-3 this single stage model is not sufficient, due to the added complexity provided by regions other than the poly(Q) tract.

The early aggregate species formed during this first stage of aggregation by all variants resemble the morphologies of pre-fibrillar aggregates implicated in the toxicity of numerous neurodegenerative diseases (43–45, 49, 54). Similar spheroids and oligomers have previously been shown to form from non-expanded ataxin-3 and poly(Q) peptides, with antibody analysis indicating that such pre-fibrillar aggregates may share both a common structure and toxic mechanism (33, 43, 54). Therefore, the neuronal toxicity observed in SCA3 is potentially caused by the ability of ataxin-3 to form these toxic structures. In keeping with this, recent studies showed that overexpression of non-expanded poly(Q) proteins and other neurodegenerative-associated proteins in animal models can lead to toxicity (36, 55). Our observations suggest a toxic mechanism in agreement with this theory, in which expansion of the poly(Q) tract accelerates, but is not structurally involved in, the misfolding of ataxin-3 into toxic intermediate structures. However, in contrast with such a theory overexpression of wild-type ataxin-3 in a variety of animal models has failed to reveal patterns of neural toxicity (56, 57). Furthermore, overexpression of wild-type ataxin-3 in a Drosophila model has recently been shown to be neuroprotective (57). Therefore, whether such a proposed mechanism is relevant to disease-associated toxicity requires further exploration. As such, current attempts are underway to analyze in vivo relevance of these structures to cellular toxicity in ataxin-3.

Alternatively, a wealth of data suggests that aggregates formed through poly(Q) tract interactions, whether they are spheroids, oligomers, or mature fibrils, are themselves the toxic species (58, 59). For example, aggregates derived from poly(Q) peptides were found to be toxic to mammalian cells when delivered to the nucleus (59). Furthermore, a mutagenesis approach has linked aggregation of the poly(Q) tract in huntingtin exon 1 to enhanced toxicity in vivo (58). In this current study, the observation that only pathological length ataxin-3 is able to form poly(Q)-based fibrils is indeed compatible with such a mechanism. The increased stability of these poly(Q) aggregates would most likely only serve to enhance their lifetime within the cell, and therefore their chance of causing toxicity.

In this study, we have proposed a model of ataxin-3 aggregation in vitro that takes into account both the role of protein context and the poly(Q) tract in modulating the aggregation pathway. In doing so, we have shown the importance of taking a comparative approach to understand the complexity of poly(Q) aggregation. The aim now is to characterize these structural changes and determine strategies to prevent them.

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