Supporting Information for:

Diverse metastable structures formed by small oligomers of α-synuclein probed by force spectroscopy

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CONTENTS

Supporting Experimental Procedures

Supporting References

Supporting Figures

Figure S1. SDS-PAGE of protein constructs
Figure S2. Tetramer aggregation into amyloid fibers
Figure S3. Control FEC of DNA handles only
Figure S4. Circular dichroism of protein constructs
Figure S5. Examples of unfolding transitions from different structural models
Figure S6. Refolding FECs of the tetramer
Figure S7. Size-dependent folding rate comparison with different wait times
Figure S8. Size-dependent folding rate comparison to other native proteins

Supporting Table

Table S1. Summary of potential unfolding distances estimated from structural models in literature
Supporting Experimental Procedures

Protein purification

Cell pellets expressing tetrameric (or dimeric) α-synuclein were resuspended in lysis buffer A (50 mM NaH₂PO₄, 0.5 M NaCl, 6 M GdmCl, 0.5 mM PMSF, 20 mM imidazole, pH 7.4), 10 mM β-mercaptoethanol and 0.5% v/v Tween20, and sonicated for 40 s. The lysates were then centrifuged and filtered prior to loading onto a 5 mL Ni-NTA Superflow Cartridge (Qiagen). The column was previously equilibrated with 50mM NaH₂PO₄, 0.5 M NaCl, 20 mM imidazole, 6 M GdmCl, pH 7.4 using FPLC (GE Healthcare). All purification steps were performed at 4°C. Unbound proteins were washed out with the equilibration buffer, and α-synuclein was then eluted with 50 mM NaH₂PO₄, 0.5 M NaCl, 6 M GdmCl, 500 mM imidazole pH 7.4. The 32-kDa dimer protein was expressed and purified following the same protocol.

Cell pellets expressing monomeric α-synuclein were resuspended in lysis buffer B (PBS, pH 7.4 with 5 mM TCEP, 1 mM EDTA, 0.5 mM PMSF), sonicated for 60 s, centrifuged briefly before loading onto a GST column (Qiagen), and purified using affinity chromatography by FPLC. Again, all purification steps were performed at 4°C. The monomer was cleaved on-column using enterokinase protease (Novagen) and eluted into PBS, pH 7.4.

Aggregation of tandem-repeat constructs

The ability of the tandem-repeat α-synuclein constructs to aggregate into amyloid fibers was tested by measuring ThT fluorescence similar to standard methods[73]. Briefly, 50 μM tetrameric α-synuclein and 40 μM ThT dye were incubated at 37°C and shaken linearly at 20 and 30 Hz for 10 days. The ThT fluorescence, excited at 430 nm, was measured at 485 nm in a microplate reader (Gemini EM, Molecular Devices). As seen in Figure S2, the ThT fluorescence displayed the typical sigmoidal rise that occurs during amyloid formation after a lag time of approximately two days.

CD spectra of monomer, dimer, and tetramer

To assess the average structural content of the tandem-repeat oligomers, we measured the CD spectra of the three protein constructs (Jasco J–810 CD/ORD spectrometer) at a concentration of 10 μM in 10 mM phosphate buffer pH 7.0, using a 1-mm path length and subtracting the baseline spectrum of the buffer. All three spectra, shown in Figure S4, were characteristic of a largely disordered protein and similar to results found previously for α-synuclein[73].

Interpretation of unfolding distances

It is difficult to assign specific structures to particular ΔLc values without additional information, especially given the large number of transitions observed here. However, we have catalogued some of the various different structures that have been observed or proposed for α-synuclein and listed the associated contour length changes expected for unfolding (Table S1). Contour length estimates are based on the number of amino acids involved in the structures and the distance between the points at which force is applied to the protein (as estimated from the structural models).

Monomers of α-synuclein have been observed to form α-helical structures at the N-terminus under certain conditions[74-76]: an extended α-helix of ~90 aa, or two broken α-helices – one ~35 aa long and the other ~48 aa, folded into a helix-turn-helix “hairpin”. Since helices have a length of 0.15 nm/aa[76], unfolding these α-helices would give rise to ΔLc ~20 nm for the extended helix, ~7 and 10 nm for the short helices individually if not folded into a hairpin, and ~30 nm for the full hairpin. Suggestively, transitions with ΔLc ~10, 20, and 30 nm are all seen for each of the constructs (monomer, dimer, and tetramer). Interestingly, the unfolding transitions with ΔLc ~30 nm seen in FECs of the monomer match the results of a previous AFM study (ΔLc ~28 nm), which the authors suggested arose from β-sheet structures in the N-terminus [77].

The structure of the amyloid fibril formed by α-synuclein[78] involves each monomer forming a 5-strand β-sandwich, with individual sandwiches then aligned side by side. Many different ΔLc values could be expected from unfolding different components of such β-sandwiches, whether the sandwiches are in isolation or stacked in multimers. For example, unfolding of different numbers of β-strands in one or more sandwiches would lead to ΔLc ~8–10 nm for 2–3 strands or ~16–19 nm for 4–5 strands from one or two sandwiches, separation of neighboring sandwiches would produce ΔLc ~33–34 nm, and unfolding of one complete monomer from a stack of sandwiches would produce ΔLc ~50 nm. Some of these unfolding transitions are illustrated in Figure S5. Several different transitions could in principle occur in combination, depending on the size of the construct (dimer or tetramer), giving rise to the large
number of $\Delta L_c$ values listed in Table S1. Intriguingly, many of these values coincide with transitions observed in the FECs, suggesting that amyloid-like structural motifs may form even in the smallest oligomers.

A tetrameric native structure for $\alpha$-synuclein was recently reported [79,80]. The structural model involves monomers folded into helix-hairpins similar to the micelle-bound structure, stacked in parallel. As for the $\beta$-sandwich structure, multiple $\Delta L_c$ values could be expected from unfolding different combinations of structural components. Many of these values would be degenerate with the $\Delta L_c$ values expected from other structures (e.g. 50 nm for removing a complete monomer, $\sim$ 30 nm for unfolding a single helix-hairpin, $\sim$ 18–19 nm for separating two neighboring helix-hairpins). $\Delta L_c$ for complete unfolding of a tetramer, trimer, or dimer are quite distinct, however, being $\sim$ 185 nm, $\sim$ 135 nm, and $\sim$ 85 nm, respectively.

The ability to assign specific structures to the unfolding transitions would provide deeper insight into the conformational changes of $\alpha$-synuclein. One approach that may prove fruitful for such identifications is comparison to molecular dynamics simulations. Recent work showed how such simulations can be used to help interpret single-molecule fluorescence studies of intrinsically-disordered proteins [81]. However, simulations of oligomers have not yet been done, and may present challenges owing to the large size of the system being simulated.

As a final note, $\alpha$-synuclein has been shown to bind non-specifically to dsDNA [82,83]. Hence it is possible that the sawtooth patterns in the FECs reflect dissociation of $\alpha$-synuclein bound to the DNA handles rather than cooperative unfolding transitions. If this were the case, however, we would expect to observe a smooth distribution of $\Delta L_c$ values, since non-specific interactions will not give rise repeatedly to the same discrete values. The fact that the actual distribution is highly peaked indicates that the transitions do indeed arise from protein structures, rather than protein-handle interactions.

**Supporting References**

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Supporting Figures

Figure S1. 12% SDS-PAGE gel of α-synuclein samples after purification. Lane 1: monomer; lane 2: dimer; lane 3: tetramer; lane 4: protein ladder.

Figure S2. Aggregation into amyloid fibers. α-Synuclein tetramers aggregated over the course of several days to form amyloid fibrils, as seen by the increase in ThT fluorescence.
**Figure S3. FEC of DNA handles only.** A FEC measured on DNA handles only, without protein (black), is well-fit with a WLC model (red). Handle FECs never showed any discrete unfolding transitions like those that occurred when α-synuclein is present.

**Figure S4. Comparison of CD spectra of monomer, dimer, and tetramer.** CD measurements of the α-synuclein monomer (A), dimer (B), and tetramer (C) all show spectra characteristic of unstructured proteins.
Figure S5. Examples of unfolding transitions from different structural models. (A) Possible unfolding transitions in a monomer of the β-sandwich model. Unfolding of two β-strands (e.g. β4→ β5) produces $\Delta L_s \sim 8$–10 nm. Unfolding all the β-strands in a monomer produces $\Delta L_s \sim 18$ nm. (B) Possible unfolding transitions in a stacked β-sandwich. Unfolding one β-sandwich completely from the tetramer produces $\Delta L_s \sim 50$ nm (upper), while unstacking two β-sandwiches produces $\Delta L_s \sim 33$ nm (lower). (C) Possible unfolding transitions in the α-helical tetramer: unfolding of the N-terminal helix produces $\Delta L_s \sim 12$ nm (top), unfolding the C-terminal monomer produces $\Delta L_s \sim 33$ nm (middle), and unstacking neighboring helix-hairpins produces $\Delta L_s \sim 18$ nm (bottom).
Figure S6. Refolding FECs. FECs measured while relaxing the force continuously from the fully-unfolded state did not show any discrete refolding transitions, in contrast to the behavior during unfolding curves, indicating that the structure formation occurs at or near zero force.

Figure S7. Effect of waiting time on size-dependent apparent folding rates. The frequency which structures of different size formed in the dimer and tetramer was not noticeably affected when the waiting time at zero force between each pull was changed. In addition to the data shown in Figure 7 are the results for dimers with 0 s waiting time (blue crosses) or over 20 s (blue diamonds) and tetramers with a 10 s waiting time (red stars). Apparent rates were estimated from the occurrence frequency of specific $\Delta L_c$ values, binned in 15-nm increments to improve the statistics. No clear trend in the number of intermediates formed was observed as a function of the waiting time.

Figure S8. Size-dependent folding rate comparison. The apparent rate of formation of structures of different contour lengths in $\alpha$-synuclein is compared to the folding rate of multi-state natively-folded proteins having different sizes taken from Ivankov et al., 2003. $\Delta L_c$ values were binned in 5-nm increments for comparison to the rates for natively-folded proteins.
Supporting Table

**Table S1.** Summary of potential unfolding distances estimated from structural models in literature.

| Length changes for unfolding β-sandwich | Length changes for unfolding helical tetramer |
|----------------------------------------|-----------------------------------------------|
| Part of the structure unfolded          | Δ$L_c$ (nm)$^a$                               |
| 2 β-strands in a monomer (β1→ β2, β4→ β5) | 8–10                                          |
| 3 β-strands in a monomer (β1→ β3, β3→ β5) | 8–10                                          |
| 4 or 5 β-strands in a monomer (β1→ β4, β2→ β5, β1→ β5) | 16–18                                         |
| Unstacking of two β-sandwiches          | 33                                            |
| Complete unfolding monomer              | 51                                            |
| Complete unfolding of dimer              | 68                                            |
| Complete unfolding of trimer             | 128                                           |
| Complete unfolding of tetramer           | 168                                           |
| Part of the structure unfolded          | Δ$L_c$ (nm)$^b$                               |
| N-terminal helix                        | 11–12                                         |
| C-terminal helix                        | 17–18                                         |
| Helix-hairpin (both N- and C-terminal helices) | 33                                           |
| Unstacking of two helix-hairpins        | 18                                            |
| Complete unfolding of a monomer         | 51                                            |
| Complete unfolding of a dimer            | 85                                            |
| Complete unfolding of a trimer           | 135                                           |
| Complete unfolding of a tetramer         | 185                                           |

$^a$Contour length changes expected from unfolding different structural components of the stacked, 5-stranded β-sandwich structure in amyloid fibrils of α-synuclein, and the proposed α-helical tetramer of native α-synuclein. For the β-sandwich, Δ$L_c$ values include unfolding of various β-strands, unstacking of β-sandwiches, and unfolding of complete monomers. $^b$For the helical tetramer, Δ$L_c$ values include unfolding individual helices, unfolding hairpins consisting of the N- and C-terminal helices, unstacking of neighboring helix-hairpins, and unfolding of complete monomers. Different permutations of these transitions are also possible.