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# Detection of Transient Interchain Interactions in the Intrinsically Disordered Protein α-Synuclein by NMR Paramagnetic Relaxation Enhancement

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Protein aggregation and amyloid fibril formation are associated with a wide range of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Prion disease.[1] Despite numerous studies on protein aggregation, the mechanism by which proteins are converted from their normally soluble form to insoluble amyloid fibrils is still not well understood. Amyloid fibril formation for α-synuclein (αSyn), the primary protein component in Parkinson's disease,[2] begins from an ensemble of heterogeneous intrinsically disordered conformations. To date, solution NMR spectroscopy has played an important role in characterizing the monomeric intrinsically disordered αSyn, thereby beginning to define the starting point for aggregation.[3] However, the next steps in the mechanism of αSyn aggregation have not yet been characterized experimentally as these early self-associated species are transient and exist at very low populations. Here we report the use of NMR paramagnetic relaxation enhancement (PRE) experiments to provide a direct visualization of transient interchain contacts and describe the earliest events in the self-assembly of these intrinsically disordered proteins (IDPs).

αSyn, like other IDPs, is characterized by low sequence complexity, low overall hydrophobicity, and high net charge.[4] The charged residues are unevenly distributed within the sequence (Figure 1a) and result in a net charge of −9 at neutral pH. Aggregation rates are very sensitive to pH and have been shown to be lower at neutral pH than at low pH.[5] We compared the transient interchain interactions observed at neutral and low pH to understand the role of the distribution of hydrophobicity and charge in driving the aggregation process.

It has recently been shown that NMR PRE experiments can be used to detect transient lowly populated encounter complexes for native state protein−protein and protein−DNA interactions.[6] Here we applied these experiments for the first time to IDP to determine transient long-range interchain contacts in αSyn at neutral and low pH.[5b] When samples containing a 1:1 mixture of $^{15}$N-labeled αSyn and $^{14}$N-labeled αSyn with the MTSL spin label are mixed, the broadening of the signal in the $^{15}$N-labeled chain will be limited only to the residues that interact with the MTSL on the $^{14}$N-labeled chain, thereby allowing detection of interchain interactions only (Figures S1 and S2 in the Supporting Information).

Comparison of the PRE experiments at neutral (6.0) and low (2.5) pH (Figure 1) indicates that the locations of the transient interchain contacts are very different under conditions where the total number of charged residues changes from 39 to 15. In addition, the PRE effects are significantly weaker at neutral pH (Figure 1b), despite the fact that the concentration is 1.6 times greater than at low pH (Figure 1c). αSyn can be divided into three regions:[3d−f] an N-terminal region (residues 1−60) with a net charge of +4 at pH 7, a central hydrophobic region (NAC) (residues 61−95), which is proposed to be primarily responsible for aggregation and forms the core of the amyloid fibrils, and a C-terminal region (residues 96−140) that is highly acidic (net charge of −12) (Figure 1a). At neutral pH, when the paramagnetic spin label is placed at the negatively charged C-terminus (G132C), there are interchain interactions between G132C and N-terminal residues 3−15 and 35−50; when the spin label is placed at the N-terminus, there are interactions between A19C and a broad region of the C-terminus, including residues 110−140, as well as a narrower range of residues at the N-terminus (residues 3−12). This profile suggests that the charged C- to N-terminal interchain interactions dominate. Interestingly, when the spin label is placed in the more hydrophobic NAC region (residue A90C), the interactions between the NAC and the rest of the protein are minimal, despite the fact that the NAC is thought to play a key role in initiating aggregation.

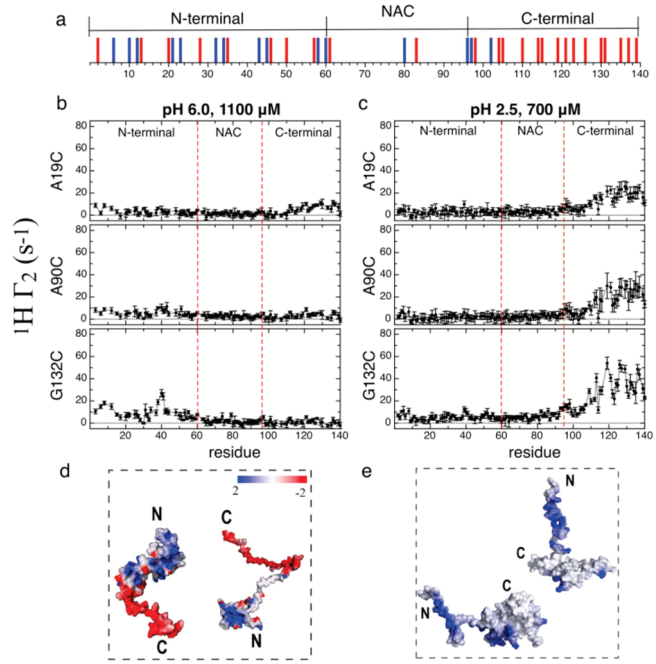


***Figure 1.*** Transient encounter complexes in the intrinsically disordered protein αSyn. (a) Distributions of positively and negatively charged residues (blue and red bars, respectively). (b, c) Interchain NMR PRE profiles for intrinsically disordered αSyn at (b) pH 6.0 and (c) pH 2.5. $^1$H $\Gamma_2$ values with MTSL labels at A19C, A90C, and G132C are shown for both sets of conditions. (d, e) Models illustrating the interchain interactions observed in the PRE experiments at (d) neutral and (e) low pH. Monomer conformations were selected from the REMD ensembles generated at neutral and low pH in ref 5b and are shown as electrostatic surface potentials. The surface potential was calculated using Delphi,[7] and the color gradient from red to blue indicates surface potentials from −2 to 2 *kT*/*e*.

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The interchain PRE profile at low pH shows stronger PRE effects than at neutral pH, consistent with stronger and possibly shorter-range interchain interactions (Figure 1c). At low pH, the strongest interactions are between the C-terminal G132C and the essentially neutralized C-terminal end of another chain. When the spin label is placed at A90C in the NAC region, the interactions with the C-terminal end are weaker; when the spin label is placed at A19C, the interactions with the C-terminal are still weaker (Figure 1c). The lack of symmetry between the stronger N-(A19C) to C-terminal interactions and the extremely weak C-(G132C) to N-terminal interactions may arise from differences in the relative orientations of the spin labels at these sites due to differences in conformation. As in the neutral pH state, the NAC regions do not interact with one another.

The NMR PRE profiles represent an ensemble of encounter complexes that arise from interactions between disordered monomer ensembles in solution. Changes in solution conditions, including concentration, viscosity of the solvent, and ionic strength, alter the magnitude of self-association of αSyn rather than the location of the interactions, as monitored by the strength of the PRE interaction (Figures S3−S5). Solvent PRE effects[6b,c] were ruled out for low and neutral pH on the basis of concentration-dependent experiments (Figure S4). At neutral pH, when the ionic strength of the solution is decreased, the strength of the interactions increases significantly, supporting the fact that the interaction between the interchain N- and C- terminal ends is electrostatic in nature (Figure S4). These data under different solution conditions suggest that despite the heterogeneity of the monomer conformational ensembles in the IDP, the encounter complex ensembles appear to have a non-random distribution of interactions.

We present cartoon representations of a possible set of transient encounter complexes within the ensembles at neutral and low pH that highlight the strongest interactions in the PRE experiment (Figure 1d,e). The weaker interactions seen in Figure 1b,c exist in the encounter complex ensembles but are not depicted here, as their populations may be low. To portray the encounter complexes, we selected representative structures from the heterogeneous conformational ensembles of the monomer that were calculated previously.[5b] The conformations of the monomer have been shown to be more compact than expected for a random coil at both neutral and low pH.[3c−f,5b−d] At neutral pH, the monomer conformation highlights the largely extended, highly negatively charged C-terminal tail and the more self-interacting N-terminal and NAC regions.[3c−f] The picture of two monomers interacting in a head-to-tail arrangement and having noninteracting NAC regions is most consistent with the weak interchain interactions between the complementary charged N- and C-terminal regions (Figure 1d). The weakness of these interactions may be due to the fact that the highly charged sequence would prefer to be solvated rather than interact with another highly charged monomer. These preferences would suggest that αSyn has evolved as a relatively poor aggregator at pH 6.0.

In contrast to the highly charged neutral pH sequence, the percentage of charged residues at low pH decreases from 40 to 6.7. We[5b] and others[5c,d] have previously reported that at low pH, the C-terminus is primarily collapsed onto itself and the NAC region rather than being extended and solvated as seen at neutral pH. Through the use of a representative conformation from the low-pH REMD conformational ensemble as a starting point,[5b] the strong interchain C- to C-terminal contacts along with the lack of N- to N-terminal interactions in the PRE experiment can be represented by two monomers arranged with the C-terminal ends in contact while the N-terminal ends remain, on average, farther apart (Figure 1e). The suggested configuration optimizes both hydrophobic interactions in the C-terminal ends and charge repulsion between the N-terminal ends. In addition, the lack of NAC-to-NAC contacts is consistent with the optimized configuration, as charge repulsion between the N-terminal ends may make it difficult for the NAC regions to interact. The weak head-to-tail contacts driven by electrostatics at neutral pH versus the stronger tail-to-tail contacts driven by hydrophobicity at low pH highlight the importance of the distribution of charge and hydrophobicity in directing interchain interactions. The difference in the nature of the interchain interactions at the two pHs may be related to the faster fibril formation at low pH.[5b−d]

There is increasing evidence that the early intermediates in the misfolding process may be more toxic than the final aggregates,[1] and therefore, characterizing the interactions that define the initial steps of amyloid formation in αSyn are of particular importance. At neutral pH, where the charged residues dominate the αSyn sequence at both the N- and C- terminal ends, the antiparallel head-to-tail interchain interactions under physiological conditions in vitro are very weak. In living cells, antiparallel contacts similar to those seen *in vitro* have been observed using biomolecular fluorescence complementation.[8] The results presented here show that $^1$H NMR paramagnetic relaxation enhancement experiments are a powerful tool for visualizing transient low-population initial encounter complexes in intrinsically disordered proteins. These experiments can be extended to other IDPs such A$\beta$ in Alzheimer's disease or Tau in Parkinson's disease to understand the basic principles of self-assembly in aggregation or amyloid formation.

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**Supporting Information Available:** Experimental details and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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