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Characterization of conformational and dynamic properties of natively unfolded human and mouse α -synuclein ensembles by NMR: implication for aggregation

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Summary

Conversion of human α -synuclein (aS) from the free soluble state to the insoluble fibrillar state has been implicated in the etiology of Parkinson's disease. Human aS is highly homologous in amino acid sequence to mouse aS which contains seven substitutions including the A53T substitution that is the same as the one that has been linked to familial Parkinson's disease, and including five substitutions in the C terminal region. It has been shown that the rate of fibrillation is highly dependent on the exact sequence of the protein and mouse aS is reported to aggregate more rapidly than human aS *in vitro*. Nuclear magnetic resonance of mouse aS and human aS at supercooled temperatures (263K) is used to understand the effect of sequence on conformational fluctuations in the disordered ensembles and to relate these to differences in propensities to aggregate. We show that human and mouse aS are natively unfolded at low temperature but that they exhibit different propensities to secondary structure, backbone dynamics and long range contacts across the protein. Mouse aS exhibits a higher propensity to helical conformation at the C terminal substitution sites as well as the loss of transient long range contacts from the C terminal end to the N terminal and hydrophobic central region of the protein relative to human aS. Lack of back-folding from the C terminal end of mouse aS exposes the N terminal region which is shown, by ^{15}N relaxation experiments, to be very restricted in mobility relative to human aS. We propose that the restricted mobility in the N terminal region may arise from transient interchain interactions suggesting that the N-terminal KTK(E/Q)GV hexamer repeats may serve as initiation sites for aggregation in mouse aS. These transient interchain interactions of the N terminal region coupled with a non-A β amyloid component (NAC) region that is both more exposed and has a higher propensity to β structure may explain the increased rate of fibril formation of mouse aS relative to human aS and A53T aS.

Keywords

Parkinson's disease; α -synuclein; mouse; NMR; dynamics

Introduction

Natively unfolded proteins can adopt a variety of conformational states and interconvert between them on a wide range of time scales ^{1; 2}. Identifying the range of conformations and timescales that can be accessed is important to understanding how natively unfolded proteins can be converted from their normally soluble form to insoluble fibers or plaques

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found in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease (PD), Huntington's disease and type II diabetes. Fluctuations within the ensemble of unfolded states are likely to be important in determining the pathway of folding to the aggregated form as conformational fluctuations provide a mechanism for different residues to come into contact with one another by exposing or protecting different regions of the protein to other proteins or to solvent.

Human α S is the primary protein component of the Lewy body deposits that are the diagnostic hallmark of PD³. Aggregation of α S into fibrils is thought to play an important role in the pathogenesis of PD⁴. The mechanism of conversion from soluble α -synuclein protein to amyloid fibril is proposed to arise via a nucleation dependent mechanism in which amyloidogenic proteins can access an unstable, partially ordered conformation prior to self assembly into insoluble protofibrils followed by the formation of amyloid fibrils^{5; 6}. Human α S is natively unfolded in solution but is composed of conformational ensembles that are on average more compact than those expected for a random coil protein^{7; 8; 9; 10; 11}.

Human α S is highly homologous in amino acid sequence to mouse α S, human β -synuclein (bS) and γ -synuclein (gS), and variants involved in early onset disease including the human α S with point mutations A53T¹², A30P¹³ and E46K¹⁴. The amino acid sequence of human α S can be divided into three regions including the N and C-terminal regions and the central NAC. The N-terminal region (residues 1-60) consists of 11-residue repeats with a highly conserved hexamer motif KTK(E/Q)GV. The charged C-terminal region (96-140) has a high percentage of aspartate, glutamate, and proline residues and the NAC region is a hydrophobic region. It has been shown that the rate of fibrillization is highly dependent on the sequence of the protein as well as the solution conditions^{15; 16; 17; 18}. Mouse α S forms fibrils fastest relative to human α S, bS and gS as well as relative to the variants A53T and A30P involved in early onset of PD. Overall the rates of oligomer or fibril formation can be ordered from fastest to slowest in the order of mouse α S > human A53T α S > *wt* human α S > human A30P α S > gS > bS^{6; 16; 19; 20}.

Mouse α S with 95% sequence identity to human α S and faster aggregation kinetics *in vitro* provides a good system to understand the sequence determinants of the conformation and dynamics of the disordered ensembles and their relationship to aggregation. There are seven substitutions between human and mouse α S including the A53T substitution that is the same as the one that has been linked to familial PD (figure 1). There is a single S87N substitution in the NAC region and the remaining five substitutions are found in the C terminal region. One of C terminal substitutions, D121G, is at a position characterized as a key metal ion binding site in human α S²¹. Structural characterization of mouse α S by CD shows a natively unfolded state in solution¹⁶ but little is known about the nature of the conformational ensembles that populate mouse α S.

Comparison of the conformational fluctuations of human and mouse α S by nuclear magnetic resonance (NMR) at supercooled temperatures (263K) are reported here in order to understand their sequence dependence and to relate these to the kinetics of fibril formation. Low temperatures were selected in order to freeze out motions and more clearly define the extent of restricted motion and nonrandom interactions in both proteins. ¹⁵N backbone relaxation experiments which are sensitive indicators of local conformational restriction on the ps to ms timescale and paramagnetic relaxation enhancement (PRE) experiments which allow the determination of long range conformational contacts were correlated and show that mouse α S populates more extended conformations in solution than human α S. The NMR data indicate that regions of restricted mobility in human α S are correlated with regions that have transient long range contacts to the C terminal end of the protein whereas restricted mobility in mouse α S, which is prominent in the N terminal region, does not show this

correlation. The increased backbone motional restriction of the N terminal region coupled with the flexible NAC region with a higher propensity of β conformation may explain the increased rate of fibril formation of mouse aS relative to human aS.

Results

Backbone assignments of human and mouse aS at low temperature

The ^1H - ^{15}N resonances of human and mouse aS are well resolved and have narrow chemical shift dispersion typical of natively unfolded proteins (figure 2). Backbone assignments of human and mouse aS were performed using a series of triple resonance experiments. Assignments were originally performed at 288K using standard triple resonance experiments²² including HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO, and C(CO)NH as well as (5,3D) HACACOCANH and (5,3D) HACACONH GFT-NMR^{23; 24} in order to overcome ambiguities that arise in the standard experiments. Certain segments of the KTK(E/Q)GV repeats and several double repeats (AA, VV, GG, and EE) were not able to be assigned due to overlap. In total, 118 resonances of the 134 assignable residues have been assigned for mouse aS and 117 residues assigned for human aS. Low temperature (263K) spectra were assigned by a temperature titration and the assignments confirmed by HNCACB experiments. Comparison of the ^1H - ^{15}N chemical shifts of human and mouse aS indicates only small differences near the substitution sites indicating that there is no large global conformational change that takes place between the two proteins.

Secondary structure propensities of human and mouse aS at 263K

NMR chemical shifts are frequently used to probe the propensity of natively unfolded proteins to sample different regions of conformational space^{7; 10; 25; 26; 27; 28}. Kim et al have reported averaged C^α and C' chemical shifts for human aS at -15°C and show that residues 38-98 as well as the C terminal residues have predominantly negative chemical shifts²⁹. Here we use the secondary structure propensity score (SSP) that has been used for the comparison of human aS and gS at 5°C to compare the propensity to secondary structure of human and mouse aS at low temperature²⁶.

Although there are only minor differences in the ^1H - ^{15}N HSQC spectra between human and mouse aS at low temperature, the SSP profile shows certain important differences between the two proteins (figure 3). The SSP profile of human aS shows very little propensity to helical conformation along the protein sequence, but does show a mild propensity of β conformation from residue 30 to 140. In contrast, mouse aS shows more propensity overall of secondary structure with some helical propensity at positions 33–37, at the substitution site A53T and at residues 100 to 105 which include substitutions sites L100M, N103G and A107Y. In addition, mouse aS shows more propensity to form β conformation in particular in the NAC region at residues 75 to 82 and 85 to 97 and in the N terminal region from residues 25-30. Many of the SSP changes occur in the regions around the substitution sites indicating that local conformational propensities are sensitive to the substitutions. However there are also changes in SSP in regions that contain no substitutions such as the changes for residues 33-37 in the N terminal region.

Mouse aS exhibits restricted backbone dynamics in the N terminal region and increased flexibility in the NAC region relative to human aS

^{15}N relaxation experiments (figure 4) are performed to explore the dynamics of the natively unfolded states of human and mouse aS on the fast ps-ns timescales as well as on the slower millisecond timescale. In human aS the R_1 data are extremely uniform across the sequence whereas the R_2 data show distinct regions of variability (figure 4a, 4c). The R_2 data range from 8.0 sec^{-1} which are approximately the values expected for the random coil values³⁰

and increase to values as high as 13 sec^{-1} suggesting that human aS has significant heterogeneity in backbone dynamics at low temperature. In order to define the variable regions in the R_2 data, locally weighted scatterplot smoothing fitting (LOESS)³¹ has been performed to smooth the R_2 data and five qualitative clusters of deviations from the random coil R_2 have been assigned to the sequence (Table 1). Clusters I and II in the N terminal end from residues 11 to 31 and 37 to 64 respectively display increased R_2 values on the order of 8 sec^{-1} and 14 sec^{-1} . Cluster III which is in the NAC region is clearly defined by low R_2 values at the two edges and increased values in the center. Cluster IV at the C terminal edge of the NAC region displays relatively flat R_2 values consistent with random coil like R_2 values and the final cluster V shows increased values for R_2 on the order of 10 sec^{-1} to 11 sec^{-1} . ^1H - ^{15}N NOE data are in the range of 0.5 and are relatively uniform across the sequence; there is a small dip around residues 107 to 116 (figure 4e).

^{15}N relaxation experiments in mouse aS show similar features to human aS in R_1 values (average is $1.48 \pm 0.14 \text{ sec}^{-1}$ and $1.49 \pm 0.12 \text{ sec}^{-1}$ in human and mouse respectively) but significantly different patterns and amplitudes in the R_2 values with values ranging from 8 to 16 sec^{-1} and average values of $8.74 \pm 1.72 \text{ sec}^{-1}$ and $9.50 \pm 1.96 \text{ sec}^{-1}$ for human and mouse aS respectively (figure 4b, 4d). Cluster I in the N terminal region is highly elevated relative to the random coil values (R_2 values on the order of 12 sec^{-1} to 16 sec^{-1}) and relative to the same cluster in human aS. Cluster II with some restricted mobility appears to be similar to cluster II in human aS. Cluster III in the NAC region shows values that are very close to the random coil indicating a flexible region in mouse different from the NAC region in human aS that has more restricted mobility. Cluster IV in mouse aS just C terminal to the NAC region has increased R_2 values relative to the same region in human and cluster V in the charged C terminal has slightly larger R_2 values over a wider of residues (from 94 to 100 in mouse versus 95 to 100 in human aS). The ^1H - ^{15}N NOEs average values of human and mouse aS are similar with 0.47 ± 0.08 and 0.49 ± 0.08 respectively. ^1H - ^{15}N NOE values are slightly elevated relative to human aS in the N terminal region but slightly decreased in the NAC and C terminal regions indicating restricted motion in the N terminal region (figure 4f).

Elevated R_2 relaxation rates indicate increased lower frequency motions or slower backbone motions which could arise from a number of factors including restricted motion due to transient secondary structure, local clustering effects, clustering effects due to long-range contacts, or conformational exchange in the μs to ms timescale^{32; 33}. In order to identify whether residues that have elevated R_2 rates are undergoing conformational exchange on the μs - ms timescale and to derive the chemical exchange rate (R_{ex}) for these residues, the difference of two experiments is required. One experiment, the η_{xy} (CSA/dipolar cross-correlation rate) experiment^{34; 35} is designed to quantify the intrinsic (R_2^0), which is the condition under which chemical exchange is totally suppressed and the second experiment, the in-phase Hahn echo experiment (R_2^{HE})^{36; 37} is designed to measure R_2 under conditions where the full exchange contribution to relaxation is obtained. R_{ex} values derived from the difference in these two experiments shows that for human aS R_{ex} are primarily in the range of $-1 \sim 1.5 (\text{sec}^{-1})$ suggesting minimal or no chemical exchange across the protein sequence (figure 4g). The R_{ex} values of human and mouse aS are very similar suggesting that differences in the R_2 values do not arise from differences in chemical exchange in the μs - ms timescale for these proteins at low temperature (figure 4h). In both proteins, Ser, Thr and Gly residues tend to have slightly higher R_{ex} values closer to 2.5 sec^{-1} which is seen for example with residues G41, S42 and T44 (R_{ex} values of 2.5 to 3.5 sec^{-1}). The higher R_{ex} values of Gly, Ser and Thr may be related to solvent water exchange and experiments are underway to investigate this relationship. There are a very small number of residues that have larger exchange rates on the order of 5 sec^{-1} , in particular residues A27 and T59 in human and mouse aS.

Because of the minimal nature of the chemical exchange and the similarity of the R_{ex} values in the two proteins, the difference in the R_2 rates in human and mouse is attributed primarily to a clustering effect or to transient secondary structures. The most significant differences between the R_2 values of human and mouse aS are seen in the more rigid N terminal region and more rigid cluster IV just C terminal to the NAC region and the more flexible NAC region of mouse aS.

Long-range interactions from the C terminal to the N terminal and hydrophobic NAC region are eliminated in mouse aS

Paramagnetic relaxation enhancement (PRE) experiments have been used to locate residual long range interactions in natively unfolded human aS, bS and gS ^{8; 9; 10, 29}. PREs are employed here to map the tertiary long range contacts of human and mouse aS at low temperature (figure 5). Two positions, A19 and G132, were chosen to construct a cysteine mutation in human and mouse aS. The spin label nitroxide, (1-oxy-2,2,5,5-tetra-methyl-3-pyrroline-3-methyl)-methanesulfonate (MTSL), was conjugated to the thiol group of Cys by forming a disulfide bond. The peak linewidth of the amide proton becomes broadened when the distance of the HN-to-MTSL is within 25 Å of another residue and PRE effects (I_{para}/I_{dia}) can be easily observed by acquiring HSQC spectra in the oxidized or reduced state of the MTSL spin label.

Spin labeling of the N terminus at residue A19C (figure 5a) leads to weak signal attenuation across most of the protein except for residues at the edge of the C terminal which have signal attenuation on the order of I_{para}/I_{dia} of 0.8 and signal attenuation in a small region of the N terminal at approximately residue 50. Placing the spin label at position G132C in the C terminal region (figure 5b) shows that human aS exhibits a wide range of transient long range contacts from the C terminal end to the rest of the protein. In particular, there is strong signal attenuation, in the range of I_{para}/I_{dia} of 0.6–0.7, just C terminal to the NAC region at residues 92 to 100 and at the N terminal region of the protein at residues 37 to 53. In addition to these strong regions of signal attenuation there is also weaker signal attenuation across the entire protein sequence in the range of I_{para}/I_{dia} of 0.8 suggesting that the C terminal end of the protein is making transient contact with many regions of the rest of the protein.

Although human and mouse aS have some PRE effects in common, they have very different long range interactions between the C terminus and the rest of the protein. With the spin label attached at residue 132 (figure 5d), comparison of mouse and human aS shows that the strong signal attenuation to the N terminal region at positions 30 to 50 is maintained as well as the strong signal attenuation C terminal to the NAC. However human and mouse aS show a difference in the signal attenuation at the N terminal between residues 10 and 30, at positions 60 to 90 in the NAC region and at positions 100 to 110 in the C terminal region. In mouse aS, there is no signal attenuation in these three regions indicating that the C terminal is not interacting with the first two hexamer repeats of the N terminal region, the majority of the NAC region or the small region at position 100 just C terminal to the NAC region. Labeling at the N terminal end at residue A19C shows more subtle differences between human (figure 5a) and mouse aS (figure 5c). Overall there is less signal attenuation in mouse aS than human aS with exposure of the NAC region. The differences in the signal attenuation of the C terminal spin label in mouse and human aS suggests that the long range contacts within the two proteins differ from one another. In particular, there is elimination of long range interactions from the C terminal end to the hydrophobic NAC region and the N terminal region in mouse and the transient interactions from the C-terminal end to these regions are eliminated in mouse aS relative to human aS.

Pulse field gradient NMR translational diffusion experiments are performed on human and mouse aS in order to obtain the hydrodynamic radius of the proteins at low temperature³⁸. Mouse aS has a greater hydrodynamic radius ($R_h = 29.5 \text{ \AA}$) than human aS ($R_h = 28.1 \text{ \AA}$) indicating that mouse protein is less compact than human aS. These results are consistent with the PRE experiments that show fewer long range contacts from the C terminal end of mouse aS to the NAC and N terminal region relative to human aS.

Discussion

There is significant interest in the conformation and dynamics of human aS, its homologs bS and gS as well as the variants that contain single substitutions that result in early onset disease. Understanding the role and sensitivity of sequence changes to the nature of the conformational ensembles of these natively unfolded proteins has provided insight into the propensity of different synucleins to aggregate and form fibrils^{8; 9; 10; 11; 25; 26; 29}. Mouse aS with only seven substitutions relative to human aS is a good system to identify the role of a small number of residues in redistributing the conformational fluctuations relative to human aS. Backbone relaxation experiments and PRE experiments at high temperature (288K) showed only very small differences between the two proteins (unpublished data) but significant differences were observed at supercooled temperatures (263K) allowing a more detailed investigation of the differences between the conformational ensembles of human and mouse aS.

Working at low temperature may shift the thermodynamics or the kinetics of the system or both. This will result in changes in distributions of populations and/or changes in the kinetics of inter-conversion towards conformational ensembles that are less averaged and more easily characterized by NMR relaxation and PRE methods. In the kinetic limit, the effect of the low temperature will be to induce changes in inter-conversion rates between the conformers allowing the characterization of the same states that are present at physiological temperatures. In the thermodynamic limit, there may be a shift in the population balance toward a more easily characterized state. While that state may not be the major conformation at physiological temperatures undoubtedly it still has some significant population and is energetically accessible at high temperature. Therefore, these low temperature conformations may provide novel insight into the types of interactions that can exist in aS conformers but that may be difficult or impossible to detect under high temperature conditions.

Correlation of long range transient contacts and restricted mobility in human aS

Despite the similar amino acid sequences of human and mouse aS, NMR measurements indicate that restricted mobility on the ps-ns timescale and long range contacts within the proteins are significantly different from one another. A schematic illustration of the combined R_2 and PRE effects of human and mouse aS illustrates the differences between them (figure 6). PRE effects are shown by a color gradient with dark blue indicating a strong loss of signal intensity and red indicating no loss of signal intensity from the PRE probe. Human aS has a wide range of C terminal contacts along the protein as evidenced by the primarily blue/light pink tone of the human sequence. Regions of most contact to the C terminal probe include cluster II and cluster IV, with other regions showing lighter colors suggestive of interactions that are not as strong but that still exist transiently. In human aS there is a strong correlation between regions of restricted mobility and the PRE from the C-terminal label to the rest of the protein. The PRE effects are primarily in regions that have been identified as having restricted mobility from the R_2 data (residues represented as surface residues). The residues that are identified as mobile on the ps-ns timescale (residues shown in stick), such as the edges of the NAC region which are composed of Gly residues and the residues in the C terminal end, have weaker PRE contacts from the C terminal end

(pinker color). This correlation between the regions of restricted mobility and the strong signal attenuation from the spin label suggests that the restriction of motion in clusters I, II and III and IV arises predominantly from the long range contacts with the C terminal end and that restriction of motion arises primarily from transient clustering of residues within the protein.

The picture that emerges from our data for human aS at low temperature is one for which the C terminal end of the protein makes contact with many different parts of the protein suggesting that aS is composed of a large number of different compact structures that populate the ensemble averaged conformation. Previous studies by Kim et al have suggested that the effect of low temperature is to remove the transient long range interactions in human aS²⁹. This is based on the measurement of the hydrodynamic radius of human aS at -15 °C which is increased relative to high temperature, and PRE experiments from the N terminal labels that show minimal long range interactions. Our measurements at -10 °C have shown similar PRE results to Kim's with the spin label at the N terminal region, however additional experiments where the PRE probe is placed at the C terminal end extend the data and show that human aS exhibits a wide range of transient long range contacts from the C terminal end to the rest of the protein. At this point we propose that the discrepancy between the two laboratories may be due to measurement under different solution conditions; in particular measurement under very different buffer/salt concentrations (Kim 50mM phosphate buffer, pH 7.4, 300 mM NaCl; Wu 10mM phosphate buffer, pH 7.4, 137mM NaCl). Fink et al. have shown that the rate of fibrillation of human aS is extremely sensitive to salt concentration suggesting that the two laboratories may be investigating distinct conformational ensembles due to the dissimilar salt conditions³⁹. Further work is required to clarify these issues.

Loss of long range contacts in mouse aS relative to human aS

In contrast to human aS, mouse aS does not exhibit a strong correlation between regions of restricted mobility as defined by increased R_2 values and the PRE effects from the C terminal end. The large cluster at the N terminal end, cluster I, shows the most restricted backbone mobility and yet has no PRE contacts with the C terminal end. A second region that exhibits markedly different behavior from human aS is the flexible NAC region which also does not have any long range contacts with the C terminal end of the protein as observed by PRE experiments. Decreased contacts from the C terminus allows both the NAC and the N terminus to be exposed; however these two regions show very different degrees of restricted motion with the NAC region flexible, while the N terminus is highly restricted in motion. In addition, the NAC region shows increased propensity to β conformation while cluster I of the N terminal shows little propensity to secondary structure. It has been suggested that residual helical propensity in the N terminal region in human aS may stabilize long range interactions and interfere with intermolecular association¹⁰. In mouse aS, the converse may be true where the destabilization of the N terminal helical propensity along with the absence of long range contacts to the C-terminal end may increase aggregation rates. In contrast to human aS the picture that emerges for mouse aS is one that has fewer C terminal contacts across the protein, may have missing elements of the ensemble relative to human aS and fewer compact structures that populate the ensemble averaged conformation. The larger hydrodynamic radius of mouse aS relative to human aS is consistent with this picture.

The lack of correlation between regions of restricted mobility and C terminal long range contacts suggests that the restricted mobility of the N terminal region in mouse does not arise through a clustering effect from transient long range intra-chain interactions but rather may arise from transient secondary structure, a local clustering effect or transient aggregation. Examination of the SSP data does not indicate a high propensity to secondary structure in cluster I and R_{ex} data does not show significant restricted mobility due to

chemical exchange on the ms timescale in this region. In addition, it is unlikely that the restricted mobility arises from long range intra-chain interactions as there are no other regions of the protein that exhibit the same magnitude of R_2 values. In light of the lack of secondary structure propensity and the lack of a long range interactions, suggestions for the origin of the N-terminal slower backbone motions of cluster I may be from back folding of the hexamer repeats or from transient protein-protein aggregation at the N terminal end.

Implications for aggregation

Conformational and dynamics comparison of human and mouse aS are undertaken in order to determine the differences between the natively unfolded ensembles of the proteins and to relate these to the aggregation propensities of the proteins. It has been hypothesized that the C-terminal region of aS acts like a binding partner and interacts with the repeating fragments of the N terminal and the hydrophobic NAC region⁴⁰. It has also been shown that C-terminal truncation variants aggregate much faster than the wild type confirming that the C terminal end is important for directing fibril formation⁴¹. In mouse aS, substitutions in the C terminal end, L100M, N103G and A107Y enhance the propensity to α -helical conformation at residues 100 to 104 and show restricted mobility. In human aS, the same region is very flexible based on R_2 data. The extent of broadening due to the C terminal spin label between residues 100-104 is greater in human versus mouse synuclein suggesting that the sequence changes in mouse are responsible for the altered secondary structure and increased rigidity. The limited flexibility and the secondary structure propensity of residues 100-104 (the N-terminal end of cluster V) in mouse versus human aS may be a key determinant in preventing the C-terminal region of the mouse aS from interacting with the N terminal region.

The loss of long-range interactions to the NAC region in mouse aS at low temperature is similar to what is observed for the familial mutations of human aS, A30P or A53T mutations at high temperature⁴². Because the A53T mutation exists in mouse aS it is interesting to compare the single mutation variant to mouse aS. It has been proposed that the A53T mutation may stabilize alternative structures relative to the folded over structures and shift the population of conformers away from the folded over structures towards conformational ensembles that are more likely to aggregate⁴². The same destabilization mechanism may be important for mouse aS but in addition, the increased rigidity of the “hinge” region (cluster IV) between the C terminal and the rest of the protein that arises from the L100M, N103G and A107Y substitutions further enhances the exposure of the NAC and N terminal. We propose that the restricted mobility in cluster I of the N terminal region may arise from transient interchain interactions suggesting that the N-terminal KTK(E/Q)GV hexamer repeats may serve as initiation sites for aggregation in mouse aS. Increased interaction between the N terminal regions suggests a lower barrier to aggregation. These N terminal repeats have been shown to be very important as deletion or removal of N terminal repeats impacts dramatically on the rate of fibril formation⁴³. Another factor that has been suggested to be important for the rate of fibril formation is the extent of secondary structure propensity in the NAC region^{11; 26}. It appears that the sequence of mouse aS optimizes the effects that are important for rapid fibril formation; transient interchain interactions of the N terminal region coupled with a NAC region that is both more exposed and has a higher propensity to β structure may explain the increased rate of fibril formation of mouse aS relative to human aS and A53T aS.

Material and methods

Chemicals

^{15}N labeled ammonium sulfate, ^{13}C labeled glucose and deuterium dioxide were purchased from Cambridge Isotope Laboratory (Andover, MA). MTSL (1-oxy-2,2,5,5-tetra-methyl-3-pyrroline-3-methyl-methanesulfonate) was purchased from Toronto Research Chemicals (Toronto, On, Canada) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Protein preparation

Plasmids (pT7-7) encoded human and mouse aS cDNA sequences were gifts from Dr. Peter Lansbury (Harvard medical school, Cambridge, MA) and were transformed into *Escherichia coli* BL21 DE3 strain (Invitrogen Inc.). Expression and purification of aS followed the published protocols⁴⁴. For the preparation of NMR samples, dried proteins were dissolved to run size-exclusion gel filtration using a HiPrep 26/60 superdex 75 column (GE healthcare, Piscataway, NJ) if the gel electrophoresis result showed dimers or oligomeric aS. The purity of aS was verified again by gel electrophoresis as a single band at 14.5 kDa.

NMR experiments

For NMR experiments in supercooled water at 263K, samples were prepared following the published protocols^{45; 46}. The proteins were dissolved in standard phosphate buffered saline (PBS: 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, and 137 mM NaCl) at pH 7.4 and transferred into four 1.7 mm (outer diameter) capillary tubes (Wilmad labglass, Buena, NJ) and all capillary tubes were put into a regular 5 mm NMR tube with a cap. All NMR experiments were recorded at 263K on a Varian 800MHz spectrometer equipped with a warm probe. NMR data were processed with NMRPipe⁴⁷ with zero-filling and linear prediction if needed. Processed spectra were converted to NMRView⁴⁸ or Sparky⁴⁹ file format for data analysis. Proton chemical shifts were referenced to DSS as 0.00 ppm and ^{15}N and ^{13}C chemical shifts were indirectly calibrated by the gyromagnetic ratios⁵⁰.

The assignment of ^1H - ^{15}N HSQC spectra and sequential assignments of human and mouse aS were first carried out at higher temperature (288K). Assignment of low temperature (263K) ^1H - ^{15}N HSQC spectra of human and mouse aS were done by monitoring the movement of each cross-peak from 288K to 263K and also by using HNCACB to confirm the assignment. The secondary structure propensity (SSP) program has been developed by Marsh et al and gives a measure of the secondary structure populated²⁶. The SSP scores of human and mouse aS were calculated using calibrated C^α and C^β chemical shifts at 263K as inputs.

150–200 μM samples were prepared for each ^{15}N backbone relaxation experiment. ^1H - ^{15}N steady state heteronuclear NOE (^1H - ^{15}N NOE), longitudinal relaxation (R_1) and transverse relaxation (R_2) experiments of human and mouse aS were recorded. ^1H - ^{15}N steady-state heteronuclear NOE values at 263K were measured by recording spectra with or without a ^1H saturation period of 3 seconds. The uncertainties in the measured peak-height were set equal to the root-mean square baseline noise in the spectra, and the errors in ^1H - ^{15}N NOE were the standard deviation of the two sets⁵¹. R_1 experiments were collected using the following relaxation delay times (in seconds) in a random order: 0.01, 0.05, 0.09, 0.17, 0.36, 0.49, 0.73, 1.01, 1.31, 1.80. R_2 experiments were collected used the CPMG (Carr-Purcell-Meiboom-Gill) pulse train⁵¹, with an inter-pulse delay of 625 μs and the relaxation delay times used were 0.01, 0.03, 0.05, 0.07, 0.09, 0.13, 0.17, 0.21, 0.25 seconds. Two points were duplicated to have the proper error ranges for the R_1 and R_2 experiments. Recycle delays of R_1 and R_2 experiments are 2 seconds.

Chemical exchange R_{ex} was measured by taking the difference of two experiments; the in-phase Hahn echo experiment (R_2^{HE})^{36; 37} and the η_{xy} (CSA/dipolar cross-correlation rate) experiment^{34; 35}. Similarly to the R_2 experiment, R_2^{HE} experiments were performed by using several relaxation delays ranging from 15 ms to 150 ms. The relaxation rates were extracted by fitting the peak intensities to the equation $I(t) = I_0 \exp(-R_2 t)$ using the fitting function of Sparky. Relaxation delays and the recycle delay of the η_{xy} experiments at 263K were 46.08 ms and 2 seconds, respectively. Each η_{xy} experiment was divided into two 1H - ^{15}N HSQC type spectra and the η_{xy} values was determined by following equation:

$$\frac{I_{cross}}{I_{auto}} = \tanh(\eta_{xy} \tau_{HE}) \quad (1)$$

I_{cross} and I_{auto} are the peak intensity of cross- and auto- relaxation, respectively. $\tau_{HE} = n/J_{NH}$, where n is a positive integer (6 for 263K experiments) and J_{NH} is the scalar coupling constant of amide bond (93 Hz). Two more η_{xy} experiments were performed to get the average and standard error of the mean of each residue.

R_{ex} was defined as $R_{ex} = R_2^{HE} - R_2^0$, where $R_2^0 = \kappa \eta_{xy}$, and κ is an average value of the ratio of R_2^{HE}/η_{xy} for those residues not subjected to chemical exchange. To obtain the κ value for human and mouse aS, weighted correlation fittings of R_2^{HE} and η_{xy} were calculated. Residues with differences of R_2^{HE} and η_{xy} greater than 2.5 were not included in the correlation fitting. The calculated κ values for both human and mouse aS are 1.22 ± 0.01 .

The model of the theoretical R_2 values of a random coil like polypeptide chain was calculated as previously proposed³⁰. R_2 values were fitted to the following equation:

$$R_2(i) = R_2^{int} \sum_{j=1}^n \exp\left(-\frac{|(i-j)|}{\lambda}\right) \quad (2)$$

where R_2^{int} is the intrinsic R_2 , n is the residue numbers of the protein (140 for aS), λ is the widow size of the persistence of the protein. For fitting the 263K R_2 data, R_2^{int} and λ are 0.33 and 12, respectively to provide a nice baseline.

Locally weighted scatterplot smoothing fitting (LOESS)³¹ was used to smooth the R_2 data of human and mouse aS in order to define local deviations from the random coil R_2 values. Calculations of LOESS were performed by the curvefit toolbox of Matlab (The Mathworks Inc.) with a 5-point span.

Samples for measuring hydrodynamic radius (R_h , in angstroms) were dissolved in 90% D_2O (v/v) PBS at pH 7.4. The DOSY pulse sequence (Dbppste_cc)³⁸ in Varian BioPack library was used to obtain the translational diffusion coefficients (D_{trans}) of 0.2 mM human and mouse aS at 288K and 263K. Data was processed and analyzed by VNMRJ 2.12B. Convection compensation⁵² was applied to correct the temperature effects on D_{trans} . D_{trans} of 1,4-dioxane (1 mM in PBS) was also measured at both temperatures as a reference molecule for calculating the R_h of human and mouse aS. Since both aS and 1,4-dioxane are dissolved in the same solution, it is not necessary to determine the viscosity to obtain R_h from the Stoke-Einstein equation. R_h of human and mouse aS can be calculated from the relationship:

$$R_h^{protein} = (D_{trans}^{dioxane} / D_{trans}^{protein}) R_h^{dioxane} \quad (3)$$

where R_h^{dioxane} is 2.12 Å and $D_{\text{trans}}^{\text{dioxane}}$ and $D_{\text{trans}}^{\text{protein}}$ are measurable parameters.

Site directed spin labeling and paramagnetic relaxation enhancement (PRE) experiments

Two mutants were successfully constructed to replace the residues to cysteine (A19C and G132C) for each aS. DNA sequences were verified and proteins were expressed and purified as described for wild type aS. 5 mg lyophilized protein was dissolved in 1 ml PBS with 10 mM DTT and at least 4 hours incubation at room temperature to remove the disulfide bonds. The solution was transferred to a desalting column (GE Healthcare, Piscataway, NJ) to remove DTT. The DTT-free solution was immediately added to a 20-fold molar excess MTSL solution which was dissolved in acetone. The spin labeling reaction ran in the dark at 4 °C for 16 hours or more and then the sample was concentrated to 250 μM for NMR experiments. The molecular weights of the four cysteine mutants and the four spin labeled samples were verified by ESI Mass spectroscopy.

PRE experiments were performed by acquiring ^1H - ^{15}N HSQC spectra in the absence and presence of MTSL spin labeling of each cysteine mutant. PRE experiments were first acquired at 288K to confirm the consistency of the published data^{8; 10}, then samples were quickly cooled to run data collection at low temperature. To control the reference more accurately, reference samples were prepared by adding DTT to the MTSL spin labeled samples after the MTSL-labeled ^1H - ^{15}N HSQC acquisition. It should be noted that DTT-added solutions were kept at room temperature for 2–3 hours for complete reaction of removing the conjugated MTSL. Assigned peak intensities were extracted by Sparky to calculate the PRE ratios ($I_{\text{para}}/I_{\text{dia}}$) in the presence or absence MTSL attachment.

Theoretical PRE curves were calculated by using XPLOR-NIH⁵³ to generate MTSL-attached fully extended structures. From these, $I_{\text{para}}/I_{\text{dia}}$ PRE values were calculated and used as a reference for an unfolded protein with no long-range interactions⁵⁴. To aid the eye the PRE data was smoothed using the LOESS approach similarly to the R_2 data. Since LOESS takes every 5 points to calculate and smooth the curve, residues in the predecessor or the successor positions of the A19C or G132C data were not included to perform fitting, respectively.

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Abbreviations

NMR	nuclear magnetic resonance
PD	Parkinson's disease
aS	α-synuclein
bS	β-synuclein
gS	γ-synuclein
NAC	non-Aβ amyloid component
SSP	secondary structure propensities
HSQC	heteronuclear single quantum correlation spectroscopy
R_1	longitudinal relaxation rate

R₂	transverse relaxation rate
¹H-¹⁵N NOE	steady-state heteronuclear NOE
PRE	paramagnetic relaxation enhancement
MTSL	(1-oxy-2,2,5,5-tetra-methyl-3-pyrroline-3-methyl)-methanesulfonate
LOESS	locally weighted scatterplot smoothing method

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Figure 1. Sequence alignment of human and mouse aS

Sequence alignment of human and mouse aS from the program ClustalW. Sequences of human and mouse aS are denoted as human and mouse, respectively. The seven non-identical residues in the mouse aS sequence are indicated, and identical residues to human aS are denoted by dots. The six KTK(E/Q)GV repeats are displayed in boxes and the NAC region (residues 61 to 95) is colored in red.

Figure 2. ^1H - ^{15}N HSQC spectra of human and mouse aS at 263K

^1H - ^{15}N HSQC spectra of human aS (a) and mouse aS (b) at 263K. Both aS were dissolved in PBS buffer (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, and 137 mM NaCl) at pH 7.4. Assignments are indicated on the figure.

Figure 3. Secondary structure propensity (SSP) of human and mouse aS at 263K

C^α and C^β chemical shifts were used to calculate the residue-specific SSP scores of human aS (a) and mouse aS (b). Positive values ranging from 0 to 1 and negative values from 0 to -1 represent the propensities of α and β structures, respectively. A schematic of the sequence containing the positions of the repeats and the point substitutions are indicated on top with repeats shown with green boxes and point substitutions shown by blue lines. The red dashed lines indicate the separation of the N-terminal, NAC and C-terminal regions.

Figure 4. ^{15}N Relaxation parameters of human and mouse aS at 263K

The relaxation data R_1 (a), R_2 (c), ^1H - ^{15}N NOE (e) and R_{ex} (g) of human aS and the relaxation data R_1 (b), R_2 (d), ^1H - ^{15}N NOE (f) and R_{ex} (h) of mouse aS at 263K are shown in the left and right panels, respectively. 150~200 μM samples were freshly prepared to record the four relaxation experiments for each aS on a Varian 800MHz NMR spectrometer. The schematic of the sequence is placed above the data to show the positions of KTK(E/Q)GV repeats and the seven non-identical point substitutions. R_1 plots of human and mouse aS are presented in panels (a) and (b), correspondingly. Relaxation rates calculated for a random coil ³⁰ are presented in both human and mouse aS R_2 plots (c, d) to provide a baseline. Smoothing curves (red) of human and mouse aS were calculated by using a locally weighted scatterplot smoothing method (LOESS) with a span of 5 points. Clusters derived from the deviation of the smoothed curves relative to the calculated random coil values are denoted beneath the experimental R_2 values. ^1H - ^{15}N NOE plots of human and mouse aS are shown in (e) and (f) respectively. R_{ex} plots (g,h) were obtained from the difference of $R_2^{\text{HE}} - \kappa\eta_{\text{xy}}$ experiments where κ is 1.22 ± 0.01 for both human and mouse aS (see Methods). Error bars are shown for every three points and for any point with R_{ex} values greater than 2.5 for clarity.

Figure 5. Paramagnetic relaxation experiments of amide protons in human and mouse aS
PRE experiments using MTSL spin labels were done at 263K for cysteine mutants human A19C aS (a), human G132C aS (b), mouse A19C aS (c) and mouse G132C aS (d). Measured PRE intensity ratios ($I_{\text{para}}/I_{\text{dia}}$) of human and mouse aS are presented in top and bottom panels, respectively. N-terminal, NAC and C-terminal regions are colored light blue, orange and green respectively. Red dashed lines in each plot show the broadening expected from a random coil polypeptide (see Methods) to provide a reference to map long-range contacts. Smoothing curves were calculated by using LOESS with a span of 5 points and they are shown in thin lines over the colored bars.

Figure 6. Model representations of structural clusters and long-range contacts of human and mouse aS

Schematic illustration along the protein sequence of the combined R_2 and C-terminal PRE data for human (top) and mouse aS (bottom). Residues assigned to clusters derived from R_2 data (clusters are shown in figure 4b and 4d, and defined in table 1) are illustrated in surface and residues that do not belong to clusters are shown in stick. Experimental PRE values from 1.0 to 0.5 are represented by a color gradient of red to white to yellow. This model was made by PyMOL 0.99 (<http://pymol.sourceforge.net>).

Table 1

The local clusters defined by R_2 profiles of human and mouse aS^a.

No. of clusters	Human α -synuclein	Mouse α -synuclein
I	A11 ~ G31	L8 ~ Q33
II	V37 ~ T64	V37 ~ T64
III	G73 ~ G86	N/A ^b
IV	V95 ~ L100	F94 ~ M100
V	G111 ~ M127	E105 ~ Y133

^aLocal nonrandom structural clusters are characterized based on the R_2 values greater than 8.5 sec^{-1} at supercooled temperatures. The first and last residues of each cluster are indicated by types of amino acid and position numbers for each aS.

^bThis cluster was defined according to human aS since mouse aS has near random coil R_2 values at this region.