Characterization of Conformational and Dynamic Properties of Natively Unfolded Human and Mouse α-Synuclein Ensembles by NMR: Implication for Aggregation

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Conversion of human α-synuclein (αS) from the free soluble state to the insoluble fibrillar state has been implicated in the etiology of Parkinson’s disease. Human αS is highly homologous in amino acid sequence to mouse αS, which contains seven substitutions including the A53T 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 αS is reported to aggregate more rapidly than human αS in vitro. Nuclear magnetic resonance experiments of mouse and human αS at supercooled temperatures (263 K) are 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 both αS are natively unfolded at low temperature with different propensities to secondary structure, backbone dynamics and long-range contacts across the protein. Mouse αS exhibits a higher propensity to helical conformation around the C-terminal substitutions as well as the loss of transient long-range contacts from the C- to the N-terminal end and hydrophobic central regions of the protein relative to human αS. Lack of back-folding from the C-terminal end of mouse αS exposes the N-terminal region, which is shown, by 15N relaxation experiments, to be very restricted in mobility relative to human αS. 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 repeats may serve as initiation sites for aggregation in mouse αS. These transient interchain interactions coupled with a non-Aβ amyloid component (NAC) region that is both more exposed and has a higher propensity to β structure may accelerate the rate of fibril formation of αS.

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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 timescales.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 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-synuclein (αS) is the primary protein component of the Lewy body deposits that are the diagnostic hallmark of PD. Aggregation of αS into fibrils is thought to have an important role in the pathogenesis of PD. The mechanism of conversion from soluble αS to amyloid fibril is proposed to arise via a nucleation-dependent mechanism in which amyloidogenic proteins can access an unstable, partially ordered conformation before self-assembly into insoluble protofibrils followed by the formation of amyloid fibrils. Human αS 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.

Human αS is highly homologous in amino acid sequence to mouse αS, human β-synuclein (βS) and γ-synuclein (γS), and variants involved in early onset disease, including the human αS with point mutations A53T, A30P, and E46K. The amino acid sequence of human αS can be divided into three regions; 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 fibrillation is dependent on the sequence of the protein as well as the solution conditions.

Mouse αS forms fibrils fastest relative to human αS, βS and γS, 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 αS > human αS > wild-type human αS > human A30P αS > gS > βS. Mouse αS with 95% sequence identity to human αS forms fibrils in vitro at a rate faster than that of wild-type human αS. The increased backbonemotional restriction of the N terminal region, 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 αS relative to human αS.

**Results**

**Backbone assignments of human and mouse αS at low temperature**

The 1H-15N resonances of human and mouse αS are well resolved and have narrow chemical shift dispersion typical of natively unfolded proteins (Fig. 2). Backbone assignments of human and mouse αS were performed using a series of triple-resonance experiments. Assignments were originally performed at 288K using standard triple-resonance experiments, including HNCACB, CBCA,

![Fig. 1](image-url)  
**Fig. 1.** Sequence alignment of human and mouse αS. Sequence alignment of human and mouse αS from the program ClustalW. Sequences of human and mouse αS are denoted as human and mouse, respectively. The seven nonidentical residues in the mouse αS sequence are indicated, and identical residues to human αS are denoted by dots. The six KTK(E/Q)GV repeats are displayed in boxes and the NAC region (residues 61–95) is colored in red.
(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) could not be assigned due to overlap. In total, 118 resonances of the 134 assignable residues have been assigned for mouse aS, and 117 residues have been assigned for human aS. Low-temperature (263 K) spectra were assigned by a temperature titration and the assignments confirmed by HNCACB experiments. Comparison of the $^1$H-$^{15}$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 263 K

NMR chemical shifts are frequently used to probe the propensity of natively unfolded proteins to sample different regions of conformational space.7,10,25–28 Kim et al. have reported averaged C' and C' chemical shifts for human aS at $-15$ °C and showed that residues 38–98 as well as the C-terminal residues have predominantly negative chemical shifts.29 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 temperature.26 Although there are only minor differences in the $^1$H-$^{15}$N heteronuclear single quantum correlation spectroscopy (HSQC) spectra between human and mouse aS at low temperature, the SSP profile shows...
certain important differences between the two proteins (Fig. 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 residue 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 100–105, which include substitution sites L100M, N103G and A107Y. In addition, mouse aS shows more propensity to form β conformation, in particular in the NAC region at residues 75–82 and 85–97 and in the N-terminal region at 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 substitution, 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 (Fig. 4) were performed to explore the dynamics of the natively unfolded states of human and mouse aS on the fast pico- to nanosecond 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 (Fig. 4a and c). The $R_2$ data range from 8.0 s$^{-1}$, which is approximately the value expected for the random-coil values, and increase to values as high as 13 s$^{-1}$, 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 of the order of 8 s$^{-1}$ and 14 s$^{-1}$. 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 $R_2$ of the order of 10–11 s$^{-1}$. Steady-state heteronuclear nuclear Overhauser effect ($^{1}H$,$^{15}$N NOE) data are of the order of 0.5 s$^{-1}$ and are relatively uniform across the sequence; there is a small dip around residues 107–116 (Fig. 4e).

$^{13}$N relaxation experiments in mouse aS show features similar to those of human aS in $R_1$ values (average 1.48±0.14 s$^{-1}$ and 1.49±0.12 s$^{-1}$ in human and mouse, respectively) but significantly different patterns and amplitudes in the $R_2$ values with values ranging from 8 s$^{-1}$ to 16 sec$^{-1}$, with average values of 8.74±1.72 s$^{-1}$ and 9.50±1.96 s$^{-1}$ for human and mouse aS, respectively (Fig. 4b and d). Cluster I in the N-terminal region is highly elevated relative to the random-coil values ($R_2$ values of the order of 12–16 s$^{-1}$) and relative to the same cluster in human aS. Cluster II has some restricted mobility and appears to be similar to cluster II in human aS. Cluster III in the NAC region shows values that are very close to those of the random coil, indicating a flexible region in mouse different from the NAC region in human aS, which 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 higher $R_2$ values over a wider of residues (94–100 in mouse versus 95–100 in human aS). The $^{1}H$–$^{15}$N NOEs average values of human and mouse aS are similar at 0.47±0.08 and 0.49±0.08, respectively. $^{1}H$–$^{15}$N NOE values are slightly elevated relative to human aS in the N-terminal region but decreased slightly in the NAC and C-terminal regions, indicating restricted motion in the N terminal region (Fig. 4f).

Elevated $R_2$ relaxation rates indicate increased lower-frequency motions or slower backbone motions that 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 micro- to millisecond timescale. In order to identify whether residues that have
Elevated $R_2$ rates are undergoing conformational exchange on the micro- to millisecond timescale and to derive the chemical exchange rate ($R_{\text{ex}}$) for these residues, the difference of two experiments is required. One experiment, the $\eta_{xy}$ (CSA/dipolar cross-correlation rate) experiment, is designed to quantify the intrinsic $R_2$ ($R_2^0$), which is the condition under which chemical exchange is totally suppressed and the second experiment, the in-phase Hahn echo experiment ($R_2^{\text{HE}}$), is designed to measure $R_2$ under conditions where the full exchange contribution to relaxation is obtained. $R_{\text{ex}}$ values derived from the difference in these two experiments shows that for human aS, $R_{\text{ex}}$ are primarily in the range of $-1 \sim 1.5$ s$^{-1}$, suggesting minimal or no chemical exchange across the protein sequence (Fig. 4g). In both proteins, Ser, Thr and Gly residues tend to have slightly higher $R_{\text{ex}}$ values, closer to 2.5 s$^{-1}$, which is seen for example with residues G41, S42.

**Table 1.** The local clusters defined by $R_2$ profiles of human and mouse aS

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

$^a$ Local 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. $^b$ This cluster was defined according to human aS since mouse aS has near random coil $R_2$ values at this region.
and T44 (Rex values of 2.5–3.5 s$^{-1}$). The higher Rex 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 of the order of 5 s$^{-1}$, 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 Rex values in the two proteins, the difference in the R2 rates in human and mouse is attributed primarily to a clustering effect or to transient secondary structures. The most significant differences between the R2 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.$^{8-10,29}$ PREs are used here to map the tertiary long-range contacts of human and mouse aS at low temperature (Fig. 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 ($I_{\text{para}}/I_{\text{dia}}$) can be observed easily by acquiring HSQC spectra in the oxidized or reduced state of the MTSL spin label.

Spin-labeling of the N terminus at residue A19C (Fig. 5a) leads to weak signal attenuation across most of the protein, except for residues at the edge of the C terminus, which have signal attenuation of the order of $I_{\text{para}}/I_{\text{dia}}$ of 0.8 and signal attenuation in a small region of the N terminus at approximately residue 50. Placing the spin label at position G132C in the C-terminal region (Fig. 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 $I_{\text{para}}/I_{\text{dia}}$ of 0.6–0.7, just C-terminal to the NAC region at residues 92–100 and at the N-terminal region of the protein at residues 37–53. In addition to these strong regions of signal attenuation, there is weaker signal attenuation across the entire protein sequence in the range of $I_{\text{para}}/I_{\text{dia}}$ 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 (Fig. 5d), a comparison of mouse and human aS shows that the strong signal attenuation to the N-terminal region at positions 30–50 is maintained, as well as the strong signal attenuation C-terminal to the NAC. However, human and

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**Fig. 5.** Paramagnetic relaxation experiments of amide protons in human and mouse aS PRE experiments using MTSL spin labels were done at 263 K 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 the top and bottom panels, respectively. N-terminal, NAC and C-terminal regions are colored light blue, orange and green, respectively. Red broken lines in each plot show the broadening expected from a random coil polypeptide (see Materials and Methods) to provide a reference to map long-range contacts. Smoothing curves were calculated by using LOESS with a span of five points and they are shown in thin lines over the colored bars.
mouse aS show a difference in the signal attenuation at the N terminus between residues 10 and 30, at positions 60–90 in the NAC region, and at positions 100–110 in the C-terminal region. In mouse aS, there is no signal attenuation in these three regions, indicating that the C terminus 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 10 just C-terminal to the NAC region. Labeling at the N-terminal end at residue A19C shows more subtle differences between human (Fig. 5a) and mouse aS (Fig. 5c). Overall, there is less signal attenuation in mouse aS than in 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 are different. 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 temperature. Mouse aS has a greater hydrodynamic radius \( R_h = 29.5 \) Å than human aS \( R_h = 28.1 \) Å, 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.

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 (288 K) showed only very small differences between the two proteins (unpublished data) but significant differences were observed at supercooled temperatures (263 K), 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 interconversion towards conformational ensembles that are less averaged and characterized more easily 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 at high temperature.

**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 pico- to nanosecond 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 (Fig. 6). PRE effects are shown by a color gradient, with yellow 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 yellow/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 from the \( R_2 \) data as having restricted mobility (residues represented as surface residues). The residues that are identified as mobile on the pico- to nanosecond timescale (residues shown in stick), such as the edges of the NAC region, which are composed of Gly residue, 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–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 aS. 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 PRE results similar to those reported by Kim et al., with the spin label at the N-terminal region; however, experiments where the PRE probe is placed at the C-terminal 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 used 50 mM phosphate buffer, pH 7.4, 300 mM NaCl; whereas Wu used 10 mM phosphate buffer, pH 7.4, 137 mM 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 conditions. 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 contact 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 contact with the C terminal end of the protein as observed by PRE experiments. Decreased contacts from the C terminus allow 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 terminus 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 association. 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 do not show significant restricted mobility due to chemical exchange on the millisecond timescale in this region. In addition, it is unlikely that the restricted mobility arises from long-range intra-chain interactions, as there is no other region of the protein that exhibits the same magnitude of $R_2$ values. In light of the lack of secondary structure propensity and the lack of a long-range interaction, suggestions for the origin of the N-terminal slower backbone motions of cluster I may be made on the basis of back-folding of the hexamer repeats or from transient protein–protein aggregation at the N-terminal end.

**Fig. 6.** Model representations of structural clusters and long-range contacts of human and mouse aS. A 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 Fig. 4b and d, 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 with PyMOL 0.99 [http://pymol.sourceforge.net].
Implications for aggregation

Conformational and dynamics comparison of human and mouse αS were 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 αS acts like a binding partner and interacts with the repeating fragments of the N terminus and the hydrophobic NAC region. It has 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 formation. In mouse αS, substitutions in the C-terminal end, L100M, N103G and A107Y enhance the propensity to α-helical conformation at residues 100–104 and show restricted mobility. In human αS, the same region is very flexible according to the R<sub>2</sub> data. The extent of broadening due to the C-terminal spin label between residues 100 and 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 αS may be a key determinant in preventing the C-terminal region of the mouse αS from interacting with the N-terminal region.

The loss of long-range interactions with the NAC region in mouse αS at low temperature is similar to what is observed for the familial mutations of human αS, A30P and A53T mutations at high temperature. Because the A53T mutation exists in mouse αS, it is interesting to compare the single mutation variant to mouse αS. 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 aggregate. The same destabilization mechanism may be important for mouse αS but, in addition, the increased rigidity of the “hinge” region 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 terminus. 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 αS. 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 formation. 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. It appears that the sequence of mouse αS 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 αS relative to human αS and A53T αS.

Material and Methods

Chemicals

15N-labeled ammonium sulfate, 13C-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 αS 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 αS followed published protocols. For the preparation of NMR samples, dried proteins were dissolved and subjected to size-exclusion gel-filtration using a HiPrep 26/60 Superdex75 column (GE Healthcare, Piscataway, NJ) if the gel electrophoresis result showed dimers or oligomeric αS. The purity of αS was verified again, running as a single band at 14.5 kDa, by gel electrophoresis.

NMR experiments

For NMR experiments in supercooled water at 263 K, samples were prepared following published protocols. The proteins were dissolved in standard PBS (8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 137 mM NaCl) at pH 7.4 and transferred into four 1.7 mm (outer diameter) capillary tubes (Wilmad Labglass, Buena, NJ), which were all put into a regular 5 mm NMR tube with a cap. All NMR experiments were recorded at 263 K on a Varian 800 MHz spectrometer equipped with a warm probe. NMR data were processed with NMRPipe, with zero-filling and linear prediction if needed. Processed spectra were converted to NMRView or Sparky file format for data analysis. Proton chemical shifts were referenced to DSS as 0.00 ppm, and 15N and 13C chemical shifts were calibrated indirectly by the gyromagnetic ratios.

The assignment of H-15N HSQC spectra and sequential assignments of human and mouse αS were first carried out at higher temperature (288 K). Assignment of low-temperature (263 K) H-15N HSQC spectra of human and mouse αS were done by monitoring the movement of each cross-peak from 288 K to 263 K, and by using HNCACB to confirm the assignment. The SSP program has been developed by Marsh et al., and gives a measure of the secondary structure populated. The SSP scores of

† http://www.cgl.ucsf.edu/home/sparky/
human and mouse aS were calculated using calibrated C\(^{13}\) and C\(^{15}\) chemical shifts at 263 K as inputs.

Samples (150–200 \(\mu\)M) were prepared for each \(^{15}\)N backbone relaxation experiment. \(^1\)H–\(^{15}\)N steady-state heteronuclear NOE (\(^1\)H–\(^{15}\)N NOE), longitudinal relaxation (\(R_1\)) and transverse relaxation (\(R_2\)) experiments of human and mouse aS were recorded. \(^1\)H–\(^{15}\)N steady-state heteronuclear NOE values at 263 K were measured by recording spectra with or without a \(^1\)H saturation period of 3 s. The uncertainties in the measured peak height were set equal to the root-mean-square baseline noise in the spectra, and the errors in \(^1\)H–\(^{15}\)N NOE were the standard deviation of the two sets.\(^{51}\) The \(R_1\) 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, and 1.80. The \(R_2\) experiments were collected using the CPMG (Carr–Purcell–Meiboom–Gill) pulse train,\(^{51}\) with an inter-pulse delay of 625 ms and a recycle delay of 2 s. Each \(R_2\) experiment was acquired using the 1H-15N NOE experiments at 263 K were measured by recording spectra with or without a 1H saturation period of 3 s. The DOSY pulse sequence (Dbppste_cc)\(^{38}\) in the Varian BioPac library was used to obtain the translational diffusion coefficients (\(D_{\text{trans}}\)) of 0.2 mM human and mouse aS at 263 K. Data were processed and analyzed by VNMRI 2.1B. Convection compensation\(^{52}\) was applied to correct the temperature effects on \(D_{\text{trans}}\). \(D_{\text{trans}}\) of 1,4-dioxane (1 mM in PBS) was also measured at both temperatures as a reference molecule for calculating the \(R_0\) 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 \(R_0\) from the Stokes–Einstein equation. \(R_0\) of human and mouse aS can be calculated from the relationship:\(^{53}\)

\[
R_0^{\text{protein}} = \frac{(D_{\text{dioxane}}^{\text{trans}}/D_{\text{protein}}^{\text{trans}})R_{\text{dioxane}}^{\text{trans}} - 2.12 \, \text{Å}}{\text{D}_{\text{trans}}^{\text{protein}}} \tag{3}
\]

where \(R_0^{\text{dioxane}}\) is 2.12 Å and \(D_{\text{dioxane}}^{\text{trans}}\) 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 cysteine residues (A19C and G132C) for each aS. DNA sequences were verified and proteins were expressed and purified as described for wild type aS. A 5 mg sample of lyophilized protein was dissolved in 1 ml of PBS with 10 mM DTT and incubated at room temperature for at least 4 h 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 of MTSL solution dissolved in acetone. The spin-labeling reaction proceeded in the dark at 4 °C for at least 16 h and then the sample was concentrated to 250 \(\mu\)M for NMR experiments. The molecular masses of the four cysteine mutants and the four spin-labeled samples were verified by electrospray ionization mass spectrometry.

PRE experiments were performed by acquiring \(^1\)H–\(^{15}\)N HSQC spectra in the absence and in the presence of MTSL spin labeling of each cysteine mutant. PRE experiments were first acquired at 288 K to confirm the consistency of the published data,\(^{51}\) 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 \(^1\)H–\(^{15}\)N HSQC acquisition. It should be noted that solutions containing DTT were kept at room temperature for 2–3 h for complete removal of 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 in the absence of MTSL attachment.

Theoretical PRE curves were calculated by using XPLOR-NIH to generate MTSL-attached fully extended structures.\(^{54}\) 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 interaction.\(^{55}\) To aid the eye, the PRE data were smoothed using the LOESS approach similar to the way it was used for the \(R_2\) data. Since
LOESS takes every five points to calculate and smooth the curve, residues in the predecessor or the successor positions of the A19C or G132C data were not included in the fitting.

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References


