Residual Structure and Dynamics in Parkinson’s Disease-associated Mutants of α-Synuclein*

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Robert Bussell, Jr.,‡ and David Eliezer§§
From the ‡Department of Physiology, Biophysics, and Molecular Medicine and §Department of Biochemistry and Program in Structural Biology, Weill Medical College of Cornell University, New York, New York 10021

α-Synuclein (αS) is a pre-synaptic protein that has been implicated as a possible causative agent in the pathogenesis of Parkinson’s disease (PD). Two autosomal dominant missense mutations in the αS gene are associated with early onset PD. Because αS is found in an aggregated fibrillar form in the Lewy body deposits characteristic of Parkinson’s patients, aggregation of the protein is believed to be related to its involvement in the disease process. The wild type (WT) and early onset mutants A30P and A53T display diverse in vitro aggregation kinetics even though the gross physicochemical and morphological properties of the mutants are highly similar. We used high resolution solution NMR spectroscopy to compare the structural and dynamic properties of the A53T and A30P mutants with those of WT αS in the free state. We found that the A30P mutation disrupts a region of residual helical structure that exists in the WT protein, whereas the A53T mutation results in a slight enhancement of a small region around the site of mutation with a preference for extended conformations. Based on these results and on the anticipated effects of these mutations on elements of secondary structure, we proposed a model of how these two PD-linked mutations influence αS fibril formation that is consistent with the documented differences in the fibrillization kinetics of the two mutants.

Expression of human αS in transgenic animals produces PD-like symptoms and deposits (11, 12), whereas the αS double knockout mouse exhibits a subtle phenotype with no movement disorder (13). Comparison of these phenotypes suggests that A53T and A30P are gain of function mutations. Additional connections between αS and other neurological diseases continue to accumulate (4). For instance αS reportedly binds to and induces the aggregation of the amyloid-β peptide (Aβ) of Alzheimer’s disease (14, 15) and is also found in inclusions from dementia with Lewy bodies and multiple system atrophy (16). Thus, a preponderance of evidence suggests that αS plays a significant role in human neurological disease.

The fact that aggregated αS is a major component of Lewy bodies in the brains of PD patients suggests that some aspect of αS aggregation may be relevant to PD pathology. The fibrillar morphology of aggregated αS for both the WT and the PD-linked mutants is generally similar to that found in Lewy bodies extracts (17–21), suggesting that the mutations do not exert their pathological effects by changing the structural characteristics of the mature fibril state. In contrast, the rates of oligomer and fibril formation do differ among the αS variants (17, 22, 23), raising the possibility that the kinetics of αS aggregation may be coupled to the toxic event(s) leading to early onset of disease. However, the appealingly simple hypothesis that accelerated fibril formation leads to early onset PD is apparently at odds with in vitro observations demonstrating that A30P fibrils take longer to form than WT fibrils (23). Whereas in vivo fibril formation rates may differ from those observed in vitro, evaluation of this possibility awaits experiments in intact cellular or animal models comparing WT and mutant αS aggregation kinetics.

Because neither differences in mature fibril morphology nor in the rate of formation of mature fibrils provides a ready explanation of how αS mutations are linked to PD etiology, a hypothesis has emerged that some oligomeric state of αS preceding the mature fibril may be the relevant toxic agent (24). In vitro kinetics data indicate that αS probably populates several intermediate conformations, both monomeric and oligomeric (17, 25), on the pathway to the mature fibril form, and pre-fibrillar αS oligomers have also been visualized using atomic force microscopy (23), providing candidates for the hypothetical toxic species. The PD-linked mutations A53T and A30P apparently alter the transition rates between the various intermediates along the fibril formation pathway and may therefore lead to a build up of a potentially toxic oligomeric intermediate, without necessarily accelerating the formation of mature αS fibrils. This model reconciles the apparent link between αS aggregation and disease with the observation that the rate of in vitro mature fibril formation is not itself a good predictor of pathogenicity.

A thorough understanding of αS fibril formation will require the characterization of all states of the reaction process and the

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Program in Structural Biology, Weill Medical College of Cornell University, 1300 York Ave., New York, NY 10021. Tel.: 212-746-6557; Fax: 212-746-4843; E-mail: dae2005@med.cornell.edu.
§ The abbreviations used are: αS, α-synuclein; PD, Parkinson’s disease; WT, wild type; NOE, nuclear Overhauser effect; Aβ, amyloid β peptide of Alzheimer’s disease; NAC, non-amyloid–β component peptide found in Alzheimer’s amyloid plaques.
transitions that link them. In the present work we focus on the starting point of the process, the free monomer state of WT, and the PD-related mutants of αS. The free monomer is largely unstructured (26) and therefore not amenable to classical crystallographic or NMR structure determinations. However, NMR techniques can still be used to ascertain the structural properties of such proteins on a residue-by-residue basis (see for example Ref. 27). Our previous NMR study of the free WT protein revealed interesting secondary structural propensity near the site of the A30P PD mutation (28). We hypothesized that mutations may modulate structural or dynamic properties of the free protein and thereby influence the intermolecular interactions required for the aggregation process. Here we use NMR to study the effects of PD-linked mutations on the residual structure and dynamics of free monomeric αS. Our results reveal that the secondary structure of the free disordered state of WT αS is, in fact, altered by the disease-linked mutations A30P and A53T. Moreover, the effects of the two mutations differ significantly. The A30P mutation strongly attenuates the helical propensity found in the N-terminal region of WT αS, whereas the A53T mutation leaves this region unperturbed, exerting a more modest and local influence on structural propensity. We present an interpretation of these results that suggests how the PD-linked mutations may enhance production of an oligomeric (potentially toxic) pre-fibrillar intermediate. The dynamic properties of the αS backbone reveal several distinct regions of modestly differing flexibility. An increased degree of slow motions in the N-terminal region of the WT protein appears well correlated with the residual helical propensity that was observed previously (28). In the acidic C-terminal region a short segment containing several hydrophobic and proline amino acid residues and preceding a potential tyrosine phosphorylation site also exhibits an increase in slower motions. Somewhat surprisingly, the central hydrophobic region of αS (residues 61–95) corresponding to the non-amyloid β-component (NAC) peptide, which is found in Alzheimer’s amyloid plaques (29) and which interacts with the Aβ peptide in vitro (30, 31), is relatively flexible with respect to the rest of the protein.

MATERIALS AND METHODS
Plasmids containing constructs for WT and mutant αS under control of the T7 promoter were generously provided by Dr. Peter Lansbury (Harvard Medical School). Proteins were expressed and purified, and NMR samples were prepared at 100 μM protein concentration in phosphate-buffered saline, pH 7.4, as described previously (28). All NMR data were collected at 10 °C using a Varian Unity INOVA 600-MHz spectrometer. Triple resonance data for backbone resonance assignments were acquired as for the wild type (28), with typical spectral widths being 5,400-Hz in the direct dimension, 1,600-Hz in the nitrogen dimension, 1,100-Hz in the carbonyl dimension, 10,000-Hz for the Cα, Cβ dimension, and 3,400-Hz for the Cα dimension, and acquisition times ranged from 36 to 96 h, depending on the sensitivity of the experiment in question, with 1,024 complex data points acquired in the direct dimension and 46 complex data points in each indirect dimension. Despite the low protein concentration, adequate signal to noise was achieved because the flexible nature of the αS backbone (see below) leads to narrow, relatively intense resonances. Data were processed using the program nmrPipe (32). Because quantitative comparisons of peak heights and chemical shifts among different spectra were required, the same processing protocol was used for all spectra where possible. We employed a processing scheme that maximizes the number of resolved peaks while retaining adequate sensitivity to resolve broadened peaks. Spectra were apodized with a 54° shifted sine-squared function and then filtered with a top-hat (square) function to sharpen resonances in the directly detected 1H dimension. Following Fourier transformation of the direct dimension, linear prediction of the 15N dimension was used to double the number of 15N points. In the relaxation experiments, a total of 92 resonances (of a possible 135 non-proline residues in αS) was adequately resolved in each of the three measurements (R1, R2, NOE), whereas the remainder were excluded from the analysis.

Processed spectra were analyzed using the program NMRView (33). Assignments and Cα chemical shifts were obtained using the same set of triple resonance experiments used in our previous study of the WT protein (28). Duplicate triple resonance spectra collected on different days for three αS variants, prepared potentially by two separate protocols, showed that the Cα chemical shifts are generally reproducible to better than 0.2 ppm with a few deviations as large as 0.4 ppm. Secondary shift values reported here are the differences between the measured Cα chemical shift and the empirical random coil value for the appropriate amino acid type (34). To estimate the degree of helical propensity in consecutive sections of αS backbone resonance assignments, methods based on secondary shifts were used. In the first method the observed Cα secondary shifts were normalized by the empirically determined secondary shift expected for that residue type in a fully helical conformation (35), summed, and normalized by the number of residues in the segment. In the second method, the Cα secondary shifts were averaged and then normalized by the average (over all residue types) Cα secondary shift for residues in fully helical conformations. The two estimates were in good agreement. NMR 15N relaxation data were acquired with modern versions of pulse sequences based on those described by Farrow (36), incorporating pulsed field gradients to reduce the solvent signal, to correct for imperfections in π pulses and to filter coherence transfer pathways prior to detection. Data were collected using a spectral width of 5,400 Hz with 1,024 complex points in the direct dimension and a spectral width of 1,600-Hz with 64 complex points in the nitrogen dimension. R1 and R2 data were collected for a total of about 36 h each. Spectra used for R1 analysis were collected using relaxation delay times in the following order (in ms): 10, 1800, 20, 1280, 40, 640, 80, 320, 160, 10, 640, and 80. R2 data were measured using a pulse sequence employing a Carr Purcell Meiboom Gill pulse train with the following relaxation delays (in ms): 14.4, 115.2, 28.8, 86.4, 43.2, 57.6, 14.4, 115.2, 460.8, and 230.4. Duplicate spectra were collected at several time points to facilitate error estimation. The delay between π pulses in the Carr Purcell Meiboom Gill pulse train of the R2 measurements was 990 μs. For determination of R1 (spin-lattice) and R2 (spin-spin) relaxation rates resonance heights were extracted and fit as a function of the relaxation delay time using the program fittingnmpipe (33). The relaxation rates were extracted by fitting to a two-parameter exponential model of the data, because adding a third (constant) parameter to the model did not significantly improve the fitting statistics.

Steady state NOE values are reported as the ratio of peak heights in paired spectra collected with and without an initial period (4 s) of proton saturation during the 5-s recycle delay. The acquisition time for each individual spectrum was 12 h, and four pairs of spectra (total of 96 h) were collected for each sample. The variance of the pairwise ratios was used to estimate the experimental uncertainty.

The reduced spectral density formalism (37–39) was used to analyze further the αS relaxation data. This mathematical approach makes no assumptions about the microscopic details of the atomic motions (37) and appears to be more suitable for analyzing unstructured proteins than the model formalism (40, 41) that assumes that the motion of the protein is adequately described by two rotational correlation times, one global and a second describing local motions. Given the lack of stable global structure of free αS (26, 28), the notion of an overall rotational correlation time becomes problematic (39). Spectral densities were calculated using in-house code setting chemical shift anisotropy equal to −170 ppm (42) and the amide bond length equal to 1.02 Å (37).

The input relaxation rates and corresponding uncertainties were determined using NMRview fitting routines and a standard error propagation assuming uncorrelated random errors was used to obtain the final uncertainties.

RESULTS
Residual Secondary Structure—We previously observed (28) that residues 18–51 of free WT αS display positive Cα secondary shifts indicative of a significant preference for helical secondary structure. This contiguous region is long enough to accommodate multiple helical turns signaling the presence of transient complex populates of α-helical conformations. A quantitative analysis of the Cα secondary chemical shifts in this region (see “Material and Methods”) indicates that, on average, helical conformations are populated approximately 10% of the time. Presumably these are in rapid exchange with non-helical conformations. A similar structural analysis of Cα secondary shifts of PD-associated mutants A53T and A30P (Fig. 1) reveals that...
these two mutations have qualitatively different effects on structural preferences in the free state. A striking result is that the helical propensity of residues 18–31 found in the WT is absent in A30P (Fig. 1b), with an average population of α-helical conformation (calculated as above) of zero. In contrast, the A53T mutation (Fig. 1c) leaves the helical character of this region intact. Instead, A53T exhibits a slightly enhanced (but still very weak) local preference for extended, β-sheet-like conformations.

As expected, secondary chemical shifts distant from mutation sites are similar in all variants studied. This observation is consistent with the interpretation that αS is largely disordered in the free state and that mutations have no global effects on the structural properties of the protein.

In addition to residues 18–31, the Ca secondary shifts of the entire N-terminal region of αS that is believed to adopt helical structure upon lipid binding (residues 1–100 (28, 43, 44)) exhibit a slight bias toward the helical (positive) direction (Fig. 1). In acidic C-terminal domain, the secondary Ca shifts are more evenly distributed around zero but with short negative stretches that may be indicative of small regions of extended bond angles, possibly suggesting β-turn-like conformations.

**Dynamics**—The relaxation rates of backbone 15N amide magnetization are a function of backbone motions, particularly those that occur at linear combinations of the Larmor frequencies of the nuclei involved (45). As a result, measurements of 15N relaxation rates can provide information on protein mobility and dynamics, particularly in the ps-ns time scale. The longitudinal (spin-lattice) and transverse (spin-spin) relaxation rates, as well as the steady state NOE, of resolved resonances in the proton–nitrogen correlation spectra of both WT αS and the two mutants are shown in Fig. 2. The steady state NOE is most sensitive to faster motions, and $R_2$ is most sensitive to slower motions on the relevant (ps-ns) time scale, whereas $R_1$ is typically somewhat less informative (46). Typical values of the NOE and $R_2$ parameters for residues in the core of well structured proteins are around 0.8 and 10 s\(^{-1}\). Lower values of the NOE parameter are indicative of a greater degree of fast motions, whereas higher values of $R_2$ indicate a greater degree of slower motions. Clearly, the relaxation parameters observed for all three αS variants indicate a much greater mobility than that found in well structured proteins and are consistent with previous results indicating that all three variants are predominantly unfolded. Nevertheless, some degree of heterogeneity is present in the data. The first noteworthy feature is a peak in the WT $R_2$ data around residue 20 that is reasonably well correlated with the stretch of transient helical structures indicated by the Ca secondary shifts. An additional feature is a peak in the $R_2$ data of all three variants around residue 122. This region includes several proline and hydrophobic residues and immediately precedes a potential tyrosine phosphorylation site at Tyr-125. Finally, the central hydrophobic region of the protein (residues 61–95), which corresponds to the NAC peptide that is found as a minor component of amyloid plaques in Alzheimer’s disease (29), exhibits a slight decrease in both NOE and $R_2$ values. This region of αS exhibits the most pronounced hydrophobic character within the protein, and interestingly the pattern of the $R_2$ values in this region closely matches its hydrophobicity pattern.

The spectral density functions of each αS variant at each of three different frequencies, $J(0)$, $J(\omega N)$, and $J(0.87\omega H)$, are shown in Fig. 3. The spectral density function is the Fourier transform of the time correlation function of bond vector orientation and describes the relative amount of NH bond vector fluctuations that occur at a given frequency. Thus, this treatment of the data allows a more direct evaluation of the relative degree of motions at the three different frequencies or time scales. Typically, the spectral densities show a high degree of fidelity to the relaxation rates from which they are derived, with $J(0)$ closely correlating to $R_2$, $J(\omega N)$ to $R_1$, and $J(0.87\omega H)$ to the heteronuclear NOE. This is the case here as well, and the features observed in the relaxation parameters are also evident in the spectral density data.

**DISCUSSION**

**Structural Effects of A30P and A53T**—The discovery of a genetic link between PD and αS has made it clear that αS can play an important role in the pathogenesis of Parkinson’s disease. Nevertheless, the majority of PD cases occur in individuals who carry the WT αS gene. It seems likely then that the familial early onset mutations emphasize or enhance some property that is already inherent in WT αS. To date, the only observable effect of these mutations in vitro has been to alter the fibrillization kinetics of the protein. When combined with the observation that fibrillar αS is the predominant component of Lewy bodies, this suggests that the relevant inherent property is the propensity of this protein to aggregate. It is therefore of great interest to gain an understanding of the biophysical basis of this property of αS. At the same time, it is important to investigate the connection between the aggregation process and the associated disease. The existence of mutations that exacerbate the disease phenotype provides us with the opportunity to address both of these goals simultaneously. Because the mutations affect αS aggregation, studying their effects on the biophysical properties of αS should shed light on those properties of the protein that are important in the aggregation process. At the same time, because the mutations share a specific common phenotype, they can also help to pinpoint those features of the aggregation process that are relevant to disease. For example we can hope to understand why both mutations increase the rate of αS oligomerization, whereas the rate of mature fibril formation is increased by one (A53T) and decreased by the other (A30P). This question is of specific interest in this current study.
Previous studies employing lower resolution techniques than those used here (17, 22) have invariably found the biophysical properties of the WT and mutant αS to be essentially indistinguishable. Our observations reveal, for the first time, significant differences between the structural properties of WT αS and the PD-linked mutants. The most dramatic of these is that a significant helical propensity found in an N-terminal stretch of the WT protein is abolished by the A30P mutation, which occurs at the periphery of this region. A simple analysis of the Cα chemical shifts of this region suggests that it preferentially adopts a helical conformation ~10% of the time in the WT and A53T protein but not in the A30P mutant. Such residual helical structure has been observed in unfolded states of other proteins such as myoglobin (47) and has been proposed to play a potential role in early intramolecular protein folding events. It is conceivable that this region of transient helical structure could play a role in αS aggregation, which is essentially an intermolecular folding process. In the case of myoglobin, residual helical structure is believed to promote productive long range interactions leading to the formation of native structure. In αS, however, the final product of the intermolecular folding/aggregation process is a β-sheet-rich structure (20), and therefore the formation of transient helical structure does not provide a clear path to the final product. Indeed, our observation that the A30P mutation eliminates this transient helicity combined with the fact that this mutation accelerates early events in the oligomerization process (17, 23) suggests that the residual helical structure may in fact retard or interfere with the initial

![Figure 2: R1, R2, and steady state NOE relaxation parameters for backbone 15N nuclei in WT (a), A30P (b), and A53T αS (c). Error bars are displayed every 3rd data point.](http://www.jbc.org/Downloadedfrom)

![A] WT Relaxation Rates

![B] A30P Relaxation Rates

![C] A53T Relaxation Rates
intermolecular interactions of αS. We are investigating this hypothesis further by engineering new mutants of αS that should enhance the residual helical structure of the WT protein and should therefore further retard αS oligomerization.

A puzzling property of the A30P mutant is that despite slightly accelerating the initial oligomerization of αS, this mutation does not accelerate and in fact significantly retards the formation of mature fibrils (23). This observation was a key factor leading to the theory that oligomeric intermediates of αS, rather than mature fibrils, may in fact be the disease-associated species of the protein (24). Proline is a very poor β-sheet-forming residue (48), and therefore it should not be entirely surprising that this mutation retards the formation of the β-sheet-rich mature fibril structure. Our results here suggest that the acceleration of the initial oligomerization of A30P may be caused by the destabilization of a transient helical propensity that interferes with the requisite intermolecular interactions. However, it is quite likely that this mutation also hinders the formation of β-sheet structure in the same region. Therefore, the steps leading to the initial oligomerization of αS probably do not involve β-sheet structure in the neighborhood of residue 30. Subsequent steps in the process of mature fibril formation, however, may require this region of αS to participate in β-sheet structure, and these steps would likely be hindered by the presence of the proline mutation.

Compared with A30P the structural alterations resulting from the A53T mutation are quite minor. The mutation alters the secondary shift of residue 53 from a positive to a negative

FIG. 3. $J(0)$, $J(ωN)$, and $J(ω_{0.87}ωH)$ spectral density function values for WT (a), A30P (b), and A53T αS (c). Error bars are displayed every 3rd data point. Hydrophilicity, calculated using a Kyte-Doolittle algorithm, is plotted below the wild type $J(0)$ data, with increasing hydrophilicity above and increasing hydrophobicity below the gray horizontal line.
value and thereby creates a short contiguous region of negative shifts around the site of the mutation, indicating a local preference for extended backbone conformations. However, the magnitudes of the negative secondary shifts in this region are too small to conclusively indicate any significant structural preference. Rather, it is more likely that the observed effect simply reflects the fact that threonine has an intrinsically higher preference for β-sheet-like conformations than alanine (48). Despite this very minor alteration of the properties of free monomeric protein, the A53T mutation significantly accelerates the rates of both oligomerization and fibrillization of aS. Apparently, a property of this mutant that is not present, or not readily detectable, in the free monomeric protein comes into play during both the initial oligomerization and the latter steps of fibril formation. It seems most likely that the region near the mutation site must participate in elements of β-sheet structure in both the early oligomers and later fibrillation intermediates, and that the greater propensity of threonine for the β-sheet conformation facilitates the conformational transitions that lead to these elements.

Our data, combined with other results from the literature, can be used to construct a model for events occurring during the oligomerization and fibrillization of aS. The ability of the NAC region of aS (residues 61–95) to form fibrils and to seed fibrillization (30, 31) together with a recent report that resolution of the N terminus in fibril formation (49) suggest that this region of the protein may be first to populate β-sheet conformations and is probably involved in early intermolecular interactions. This region may become partially structured before oligomerization takes place, perhaps in a partially folded monomeric state such as the one reportedly populated by αS at low pH (25). The effect of the A53T mutation may be to extend this region of β-sheet, enhancing both the initial intermolecular contacts, and the latter interactions leading to mature fibril formation. The transient helical segment at the N terminus of the protein may present a hydrophobic surface that interacts with the nascent structure in the hydrophobic NAC region and interferes (albeit weakly) with intermolecular contacts (the N terminus of αS can adopt an amphipathic helical structure that interacts with hydrophobic lipid membranes, in support of this possibility (28, 43)). Disruption of the residual helicity in A30P may relieve the interference and slightly accelerate the formation of αS oligomers. However, the formation of mature fibrils requires the participation of the N terminus in β-sheet structure, and this is hindered by the presence of the proline mutation, leading to a slower rate of mature fibril formation and extending the lifetime of potentially toxic oligomeric intermediates. This model provides a rationale for most of the reported effects of the two PD-linked mutations on αS fibrillization and can be tested through the characterization of new rationally designed aS mutants.

A simple kinetic description of the model above includes four states, a largely unstructured monomer (M), a partially folded monomer (I), an oligomer (O), and the mature fibril (F), with rate constants $k_1$ for the unstructured to partially folded monomer transition (M to I), $k_2$ for the partially folded monomer to oligomer transition (I to O), and $k_3$ for the oligomer to fibril transition (O to F), with $k_{-1}$, $k_{-2}$, and $k_{-3}$ for the respective inverse transitions.

\[
\begin{align*}
  & \quad \quad M \quad \xrightarrow{k_1} \quad I \quad \xrightarrow{k_2} \quad O \quad \xrightarrow{k_3} \quad F \\
  & \quad \quad k_{-1} \quad \quad k_{-2} \quad \quad k_{-3}
\end{align*}
\]

**REACTION 1**

Under our model (Reaction 1), the A30P mutation would slightly increase $k_2$ and would significantly decrease $k_3$. The A53T mutation would primarily increase $k_3$ but could decrease $k_{-3}$ and increase $k_3$ as well. In fact, the fibril assembly process is likely to involve more than one oligomeric intermediate, and the mutations may have effects on more than a single transition. Nevertheless, this simplistic view is consistent with existing measurements of fibrillization kinetics and provides a framework for designing experimental tests of our model. For example, if the poor sheet propensity of proline is in fact responsible for decreasing $k_3$, whereas its very helix propensity leads to a slight increase in $k_3$, then substituting Ala-30 with a side chain that is a poor sheet former, but a good helix former (such as lysine (48)) should eliminate the observed increase in $k_3$, while preserving the decrease in $k_3$. Conversely, substituting a good helix former but a good sheet former, such as tyrosine (48), should preserve the increase in $k_3$ while eliminating the decrease in $k_3$. Similarly, the sheet-forming propensity of different mutations at position 53 should correlate with the degree of increase of $k_3$. Experiments using these and other mutations to test the validity of our model are currently in progress.

It is also worthwhile to consider the potential effects of the two PD-linked mutations of αS on the lipid interactions that are believed to occur during the normal function of this protein (43). There has been conflicting evidence (44, 50, 51) regarding the effect of the A30P mutation on the affinity of αS for lipid membranes, whereas the A53T mutation reportedly does not affect this affinity. Because the structure of lipid-bound αS is highly helical (28, 43), the nascent helical structure we observed in the WT protein may play a role in the folding of the protein during the transition to the lipid-bound state. The disruption of this helical propensity in the A30P mutant may interfere with early folding steps in the presence of lipids, which could result in slower protein-lipid association, and a reduced affinity. This, in turn, could interfere with the normal function of αS, or alternately a reduced affinity for lipid membranes could lead to a higher concentration of free αS and could accelerate initial steps of oligomerization through the law of mass action (50) (this assumes αS oligomerization occurs predominantly from the free protein and not the lipid-associated state).

**Dynamic Properties of αS**—The dynamic behavior of the αS backbone appears largely insensitive to the presence of the PD-linked mutations, within the precision of the current data, indicating that these mutations do not exert their effects by altering the flexibility of the free protein backbone. Despite this lack of differences between the three αS variants, the dynamics are informative about properties of the monomeric protein. The transiently helical segment in the free protein corresponds to a region of increased slower motions, as indicated by higher values in the $R_2$ and $J(0)$ data. Such correlations between residual secondary structure and backbone dynamics have been previously observed, as in the case of apomyoglobin (47). The elimination of the helical propensity of this segment by the A30P mutation is mirrored in the dynamics data, which show that this stretch has a lower propensity for slower motions in the A30P mutant than in the WT. However, the decrease of lower frequency motion is also observed in the A53T mutant, despite the fact that according to the Cα secondary shifts, the transient helical structure is not significantly affected by this mutation. Resolution of this disparity will have to await more precise measurements, which should become feasible even at the current low protein concentrations as new cryogenic probe technology becomes available.

An additional area of increased lower frequency motions is indicated by higher $J(0)$ and $R_2$ values around residue 122 in all three αS variants. This region contains two closely spaced
proline residues (117 and 120), as well as three hydrophobic residues (Met-116, Val-118, and Tyr-125), all of which may contribute to slower backbone motions. Tyrosine 125 is a potential phosphorylation site and may play a role in regulating the normal function of αS. The slower motions of the surrounding region of the protein might be important for recognition of this site by the appropriate kinase or phosphatase. Alternatively, one of several proposed interaction partners (52–55) of αS may recognize this locus in the unfolded and flexible C-terminal tail.

Finally, the NAC region of αS, corresponding to residues 61–95, has a noticeable decrease in slower motions (as indicated by lower R2 values) and an apparent increase of higher frequency motions (as indicated by lower NOE values, especially in the WT data) than the remainder of the protein. As discussed above, this region surrounds residues 71–82, which have been implicated as comprising a critical segment for αS fibrillation (49), and the NAC peptide has been reported to seed the fibrillation of the Aβ peptide of Alzheimer’s disease (30, 31). These observations suggest an important role for the NAC region in the fibrillation process. This region is also the most hydrophobic region in the protein. As such, it might be expected to exhibit a lower degree of backbone flexibility, due to the potential formation of hydrophobic clusters and the generally bulky nature of hydrophobic side chains. Instead, we observe both lower R2 and lower NOE values in this region, indicating a preference for faster over slower motions and suggesting an increased backbone flexibility relative to the remainder of the protein. In addition, the R2 values show a good correlation with sequence hydrophobicity (determined using a Kyte-Doolittle algorithm). An interpretation that reconciles this concurrence of hydrophobicity and flexibility is not readily available. However, the hydrophobic nature of this region of the protein is primarily attributable to a large number of valine and alanine residues. The absence of larger bulky hydrophobes could perhaps explain the lack of hydrophobic clustering and the associated slower backbone motions. A correlation between hydrophobicity and flexibility, in the absence of clustering, has not been reported previously but could in fact be an innate property of peptide sequences with this composition of residue types. The possible relation of the greater flexibility of this hydrophobic region to its apparent importance in the fibril formation process is also unclear but deserves further investigation as a potential property of polypeptides that may make them prone to fibrillation. The identification of any such property is of clear interest in the context of understanding amyloid fibril formation.

Conclusions—A detailed NMR chemical shift-based residue-by-residue characterization of structural propensities in αS and its two PD-linked mutants reveals subtle but potentially important differences between the different variants. These differences, combined with previous studies of the effects of the mutations on the αS fibril formation process, have led us to propose an explanation for how these mutations exert their effects. The A53T mutant is proposed to extend a region of nascent β-sheet structure initially formed in the NAC region of the protein that is involved in the early intermolecular contacts leading to the formation of αS oligomers, and also stabilizes the β-sheet structure formation that occurs during the later steps in mature fibril formation. The A30P mutation destabilizes a region of nascent helical structure that was observed in the WT protein. This region may be important for the folding of αS in the presence of lipid membranes and may also weakly retard the initial oligimerization process of αS by binding to and occluding intermolecular interaction sites. Elimination of this residual helicity therefore leads to a slightly accelerated initial oligomerization phase for the A30P mutant. Because proline is a poor β-sheet former, however, this mutation slows the incorporation of the N-terminal region of the protein into the mature fibril structure and thereby retards the formation of mature fibrils, leading to an accumulation of oligomeric and potentially toxic intermediates. The dynamic properties of the αS appear to reflect some of the features of this model, with both the NAC region and the residural helical segment of the protein showing up as well demarcated areas of either reduced or increased flexibility.

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Robert Bussell, Jr. and David Eliezer

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