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Familial Mutants of α-Synuclein with Increased Neurotoxicity Have a Destabilized Conformation*[S]

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Carlos W. Bertoncini‡§¶, Claudio O. Fernandez‡‖, Christian Griesinger§, Thomas M. Jovin‡, and Markus Zweckstetter§‡‡**

From the ‡Department of Molecular Biology and the §Department of NMR-based Structural Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany and the ‖Instituto de Biología Molecular y Celular de Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina

A30P and A53T mutations of the presynaptic protein α-synuclein are associated with familial forms of Parkinson disease. NMR spectroscopy demonstrates that Parkinsonism-linked mutations greatly perturb specific tertiary interactions essential for the native state of α-synuclein. However, α-synuclein is not completely unfolded but exhibits structural fluctuations on the time scale of secondary structure formation and loses its native conformation gradually when protein stability decreases. The redistribution of the ensemble of α-synuclein conformers may underlie toxic gain-of-function by fostering self-association and altered binding affinity to ligands and receptors.

Parkinson disease (PD)[1] is the most common neurodegenerative movement disorder and is characterized by the loss of dopaminergic neurons in the substantia nigra and the deposition of eosinophilic fibrillar proteinaceous aggregates known as Lewy bodies (1). A central role in this neurological disorder is played by the abundant, presynaptic 140-residue protein α-synuclein (αS). αS is the major component of Lewy bodies (2), and locus triplication causing an increased dosage of the wild type (*wt*) *αS* gene potentiates PD (3). Two autosomal dominantly inherited forms of PD are caused by Ala^{53} to Thr (A53T) and Ala^{30} to Pro (A30P) mutations in the αS gene, which were identified in Italian (4) and German kindreds (5), respectively. Both αS mutants display an impaired degradation by chaperone-mediated autophagy (6) and share an increased tendency to form soluble oligomeric intermediates, with the A53T mutant fibrillating even faster than the *wt* protein (7). Recent reports suggest that these soluble oligomers, or protofibrils, may constitute the major toxic species, whereas mature fibrils may account for a protective function (8). As the loss of αS appears to have minimal effects on development (9), the pathogenic effects of mutant αS are attributed to a toxic gain-of-function. These findings are intriguing inasmuch as αS has generally been considered as an intrinsically unstructured protein (10). Why and how single missense mutations in disordered αS accelerate its oligomerization and contribute to the degeneration of dopamine nerve cells are questions fundamental to the understanding and treatment of PD.

Recently, it was shown that native αS adopts an ensemble of conformations that are stabilized by long range interactions (11–14). The latter form an intricate network involving the N (residues 1–60) and C termini (residues 109–140), which shields the highly amyloidogenic, non-Aβ component of Alzheimer disease amyloid (NAC) (residues 61–95) from the solvent (Fig. 1*A*). Polyamine binding and temperature increase release the intrinsic tertiary structure of αS, leading to a completely unfolded conformation (13). The relevance of these conditions, which cause aggregation *in vitro*, for the development of PD is, however, unclear. On the other hand, very strong support for the importance of αS in PD comes from the A30P and A53T mutations in the αS gene linked to familial forms of the disease. Using NMR residual dipolar couplings (RDCs) and paramagnetic relaxation enhancement (PRE) we demonstrate here that Parkinsonism-linked mutations greatly perturb the ensemble of α-synuclein conformers. This conformationally altered αS may constitute a common mediator in the induction of PD by both environmental and genetic conditions.

EXPERIMENTAL PROCEDURES

Protein Preparation—PT7 plasmids containing *wt*, A30P, and A53T αS sequences were a gift from the Lansbury Laboratory, Harvard Medical School, Cambridge, MA. The following αS cysteine-containing mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) on the above mentioned plasmids: Ala18Cys/A30P, Ala90Cys/A30P, Ala140Cys/A30P, Ala18Cys/A53T, Ala90Cys/A53T, and Ala140Cys/A53T. The introduced modifications were further verified by DNA sequencing. The positions of the cysteine replacements was distributed along the sequence of αS and their introduction did not cause chemical shift changes in residues other than those surrounding the point of mutation. ^{15}N-labeled αS, *wt*, and mutants were expressed in *Escherichia coli* grown in M9 minimal medium supplemented with $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) and purified as described previously (15). The final protein working solutions were dialyzed against buffer A (20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl).

Spin Labeling of αS—The nitroxide spin label chosen for reaction with the cysteine-containing mutants was MTSL (1-oxy-2,2,5,5-tetramethyl-D-pyrroline-3-methyl)-methanethiosulfonate (Toronto Research Chemicals, Toronto, Ontario, Canada). MTSL had already proven to efficiently react with αS cysteine mutants, and the reaction was carried out as described previously (13). Paramagnetic samples were measured at 100 μM MTSL-labeled protein concentration, and diamagnetic exper-

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‡‡ To whom correspondence may be addressed. Tel.: 49-551-201-2220; Fax: 49-551-201-2202; E-mail: mzwecks@gwdg.de.

[1] The abbreviations used are: PD, Parkinson disease; αS, α-synuclein; RDC, residual dipolar coupling; wt, wild type; NAC, non-Aβ component of Alzheimer disease amyloid; PRE, paramagnetic relaxation enhancement; MTSL, 1-oxy-2,2,5,5-tetramethyl-D-pyrroline-3-methyl methanethiosulfonate; HSQC, heteronuclear single quantum coherence.

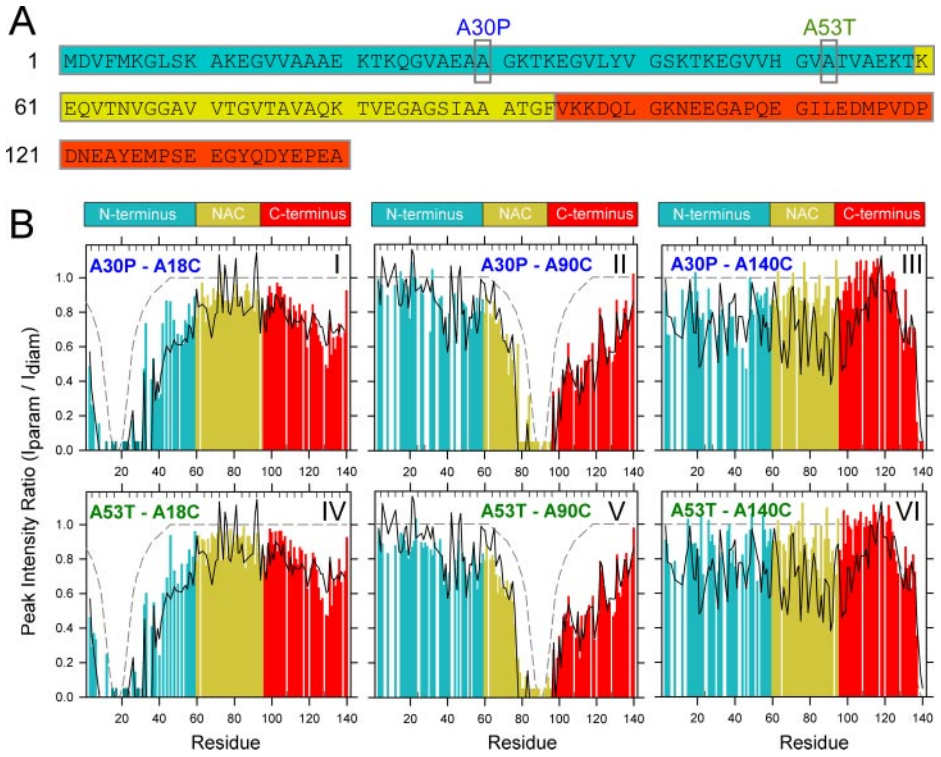

FIG. 1. *A*, primary sequence of αS. *Cyan*, *brown*, and *red* denote the basic N-terminal domain, the highly amyloidogenic non-Aβ component of Alzheimer disease amyloid region, and the acidic C-terminal domain. The two Parkinsonism-linked mutations of αS studied in this work are highlighted. *B*, NMR PRE of amide protons in Parkinsonism-linked mutant αS. Intensity ratios (I_{param}/I_{diam}) of the resonance peaks in ^{1}H-^{15}N HSQC spectra in the presence (paramagnetic) and absence (diamagnetic) of the spin label MTSL at 15 °C. *Dashed lines* indicate intensity ratios simulated for a random coil polypeptide. Decreases in peak intensity ratios that occur far from the site of spin labeling (>10 residues) are indicative of intramolecular long range contacts between the spin label and distant areas of sequence. Intensity ratios for A30P/A18C (*panel I*), A30P/A90C (*panel II*), A30P/A140C (*panel III*), A53T/A18C (*panel IV*), A53T/A90C (*panel V*), and A53T/A140C αS (*panel VI*). *Solid lines* indicate the intensity ratios observed for *wt* αS (13).

iments were performed with 100 μM protein in the presence of 0.5 mM dithiotreithol (Sigma) to counteract undesirable intensity changes in certain amide resonances of αS caused by addition of the oxidant ascorbic acid.

Alignment of αS in Anisotropic Media—RDCs were measured in αS variants aligned in 5% w/v *n*-octyl-penta(ethylene glycol)/octanol (C_8E_5) (Sigma) (16). Formation of the anisotropic, dilute liquid crystalline phase was monitored by the splitting of the deuterium signal, which was 20 ± 2 Hz. Alternatively, alignment was achieved by addition of 10 mg/ml of Pf1 bacteriophage (Asla, Riga, Latvia) with deuterium splittings of 9 ± 1 Hz (17).

NMR Measurements—NMR spectra were acquired at 15 °C on Bruker Avance 600 and 700 NMR spectrometers on a 100 μM sample of αS variants in buffer A. Measurement conditions of low temperature and absence of stirring were chosen to suppress oligomerization. PRE profiles were derived from the measurement of the peak intensity ratios from two two-dimensional ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) NMR spectra aquiered in the presence and absence of the nitroxide radical. Spectra were recorded using 256 × 1024 complex data points in F1 and F2 dimensions with 16 scans per increment and a relaxation delay of 1.2 s. The spectral widths were 1650 and 5500 Hz in the ^{15}N and ^{1}H dimensions, respectively. One-bond N-H residual dipolar couplings (D_{NH}) were acquired using the two-dimensional inphase-antiphase-HSQC sequence (18) under both isotropic and anisotropic conditions. Spectra were processed and analyzed using nmrPipe (19) and NMRView (20). D_{NH} values were calculated as the difference between splittings measured in an aligned sample and those measured in the isotropic phase (RDCs were not corrected for the negative gyromagnetic ratio of ^{15}N). RDCs observed under different conditions were normalized based on the size of the splitting of the deuterium signal (relative to RDCs for *wt* αS in buffer A).

RESULTS AND DISCUSSION

Familial Mutations Do Not Completely Unfold αS—Paramagnetic enhancement of nuclear spin relaxation by nitroxide spin labels is a useful technique for deriving distance restrains for unfolded states of proteins (21). A nitroxide radical causes broadening of the NMR signals of nearby protons, an effect that extends as far as 20 Å from modified cysteine residues. As αS lacks cysteine, three different double mutants containing variable single Ala to Cys replacements were constructed for both the A30P and A53T mutant αS. Attachment of the spin label MTSL to residue 90 greatly attenuated NMR signals of amino acids in the C terminus of A30P mutant αS with peak intensity ratios between the paramagnetic and the diamagnetic states (I_{param}/I_{diam}) in the range of 0.5 to 0.9 (Fig. 1*B*, *panel II*). This result indicates that the C-terminal domain of A30P αS is compact, as had been observed previously for *wt* αS (13) and also holds for the A53T mutant (Fig. 1*B*, *panel V*). When the spin label was attached to residue 140 in the *wt* protein, the NMR signals of residues located in the NAC region were considerably broadened, providing support for a back-folding of the C terminus on this region in *wt* αS (13). There was no evidence for such a long range interaction in either the A30P or the A53T mutants (Fig. 1*B*, *panels III* and *VI*). In the A18C spin-labeled mutant αS, intensity ratios as low as 0.5 were observed in the C terminus, similar to those of *wt* αS (13). In contrast, the relaxation enhancement of residues 40–60 was decreased by about 20% for both missense mutants compared with the *wt* protein (Fig. 1*B*, *panels I* and *IV*). The reduced paramagnetic broadening of residues 40–60 suggests that the compaction of the N-terminal region is reduced in mutant αS, in agreement with recent tryptophan fluorescence energy transfer measurements (22).

Parkinsonism-linked Mutations Greatly Perturb Specific Tertiary Interactions Essential for the Native State of αS—Residual dipolar couplings are exquisitely sensitive to bond vector orientation and can be measured in a weakly aligned protein, for which the large internuclear dipolar interactions no longer average to zero (23). Recently, we reported that RDCs identify five different domains in *wt* αS (domain I, residues 1–28; domain II, residues 33–65; domain III, residues 70–88; domain IV, residues 95–105; and domain V, the C terminus) and probe a hydrophobic core comprising the C terminus and domain III and IV (13). RDCs in PD-linked mutant αS were observed with the A30P and A53T αS mutants dissolved in a dilute liquid crystalline phase of 5% C_8E_5/octanol. No significant differences in the chemical shifts of spectra in C_8E_5/octanol and free in solution were observed, suggesting that the anisotropic phase did not appreciably perturb the ensemble of αS conformations. The RDCs in A30P and A53T mutant αS were predominantly positive, with regions displaying relatively large RDCs sepa-

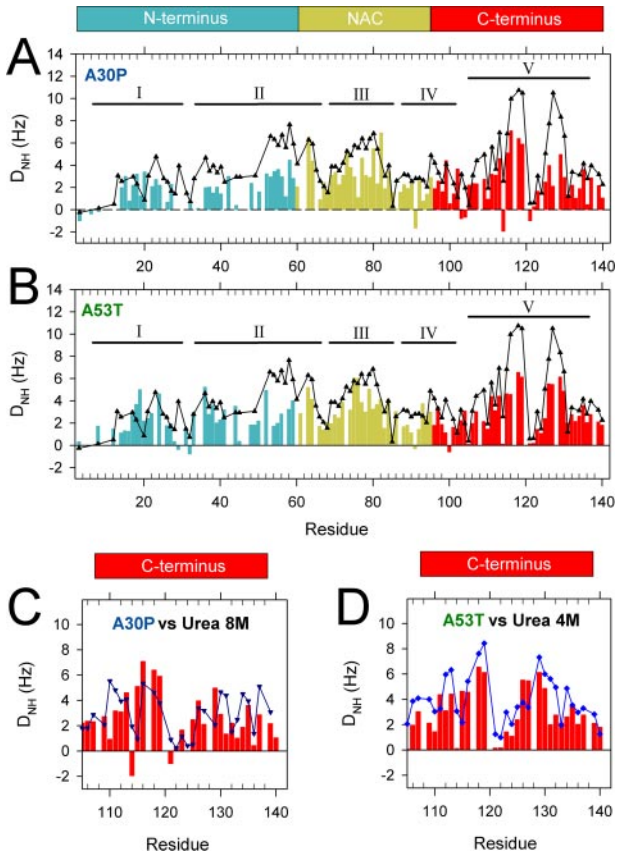

FIG. 2. **RDCs in mutant αS.** Backbone N-H dipolar couplings, D_{NH}, were measured in A30P and A53T mutant αS oriented in a 5% C8E5/octanol mixture at 15 °C. Non-zero dipolar couplings are indicative of structural and motional restrictions of the protein backbone. *A*, D_{NH} profile for A30P αS (*bars*) and *wt* αS (*line*) in buffer A (20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl). *B*, D_{NH} profile for A53T αS (*bars*) and *wt* αS (*line*) in buffer A. *C*, D_{NH} couplings in the C terminus of A30P αS in buffer A (*bars*) and of *wt* αS in buffer A + 8 M urea (*line*). *D*, D_{NH} couplings in the C terminus of A53T αS in buffer A (*bars*) and of *wt* αS in buffer A + 4 M urea (*line*). RDC domains identified in *wt* αS (domain I, residues 1–28; domain II, residues 33–65; domain III, residues 70–88; domain IV, residues 95–105; and domain V, the C terminus) (13) are indicated.

rated by residues with RDCs close to zero (Fig. 2, *A* and *B*). Slightly negative couplings were observed for some residues. The mutant dipolar couplings correlate well with the RDC domain pattern of the *wt* protein. In contrast to *wt* αS, however, the RDCs for domain II (residues 50–62) and domain III (residues 70–94) were strongly reduced. Moreover, residues 20–44 displayed much smaller RDCs in the A30P mutant than in both *wt* and A53T mutant αS, likely due to the missense mutation in this region. In the C-terminal domain, the magnitudes of RDCs were comparable with those in the rest of the protein, in contrast to the RDC profile of *wt* αS, in which the effect was higher by ~ 100%. The dipolar couplings of A30P αS were similar to those of *wt* αS in the presence of 8 M urea (Fig. 2*C*), whereas the magnitude of RDCs of A53T αS was comparable with that of *wt* αS in the presence of 4 M urea (Fig. 2*D*). In addition, the RDC profile of A18C αS, a non-amyloidogenic mutant of αS with an unaltered time course of aggregation (data not shown), was nearly identical to that of *wt* αS (supplemental Fig. S1). The results presented in Fig. 2 were reproduced in a second alignment medium and at 37 °C (supplemental Fig. S2).

We previously showed that polyamine binding induces a complete unfolding of wt αS. In the A30P and A53T mutants, however, the strong paramagnetic broadening exerted by a spin label attached to either residue 18 or 90 on the C-terminal domain (Fig. 1*B*, *panels I*, *II*, *IV*, and *V*) indicate that long range interactions are not completely abolished. This interpretation is compatible with measurements of the radius of gyration, which did not differ significantly between *wt* and mutant αS, but were smaller than for *wt* αS in 8 M urea (24, 25). Moreover, ^{15}N relaxation time measurements did not indicate any major long range effects of A30P and A53T replacements on the backbone flexibility of αS (26). Residual dipolar couplings, as reported in this study, were considerably reduced in the vicinity of both missense mutations. For the A30P mutation, the increased flexibility is in agreement with the reduced heteronuclear ^{15}N R_1^ρ relaxation rates of backbone amide groups and a reduced helical propensity for residues 18–31 (26). Besides these local changes, however, a very strong reduction of dipolar couplings in the C-terminal domain of αS was observed (Fig. 2). As only RDCs and not ^{15}N relaxation time measurements are sensitive to motions from nano- to microseconds, this suggests that the PD-linked mutations strongly increase structural fluctuations on this time scale, one in which secondary structures form during protein folding (27). Additional long range effects were observed for the hydrophobic NAC region, in which the magnitude of RDCs was considerably decreased (Figs. 2, *A* and *B*) and in which no paramagnetic broadening was detected by a spin label attached to residue 140 (Fig. 1*B*, *panels III* and *VI*). These results point toward a reduced shielding of the hydrophobic NAC region in the A30P and A53T mutant αS. In summary, the dipolar couplings demonstrate that the intricate network of long-range interactions is perturbed in PD-linked mutant αS. The protein is more flexible and able to sample a large range of conformations. Thus, both A30P and A53T mutant αS can overcome more easily the energetic barrier for self-association, leading to an increased tendency to oligomerize (7). Formation of mature fibrils, on the other hand, requires an incorporation of the N terminus in a β-sheet structure, a process hindered by the presence of proline and thus potentially accounting for a slower rate of fibril formation by the A30P αS mutant (7).

αS Gradually Loses Its Native Conformation—RDCs and PREs probe different structural features of αS. Backbone RDCs report on local, backbone conformational propensities (such as transient elements of secondary structure) in unfolded states of proteins (28), whereas PREs are sensitive to average, long range intramolecular distances (21). Therefore, NMR dipolar couplings and paramagnetic relaxation enhancement by nearby spin labels correspond to two other techniques commonly used to characterize unfolding of proteins: far-ultraviolet CD to monitor secondary structure content, and fluorescence resonance energy transfer to evaluate intramolecular distances. Although differential scanning calorimetry did not reveal any thermal transition for either *wt* or mutant αS in the range 15–110 °C (29), backbone RDCs in the C terminus of αS were greatly reduced upon heating to 37 °C (13) or by the introduction of missense mutations (Fig. 2). As the sites of mutation are far from the C terminus, the detrimental effects of the mutations must be transmitted through the network of transient long range interactions to the C-terminal domain of αS, such that conformational restrictions of the backbone are reduced. However, the retention of strong PRE broadening at 37 °C or in the presence of missense mutations indicates that dipolar couplings and PREs have different thermal transition midpoints.

Redistribution of the Ensemble of αS Conformers May Underlie Toxic Gain-of-Function—Several biological and biochemical properties underlying the toxic gain-of-function of the familial mutants of αS can now be rationalized in terms of the different intrinsic structural features of these proteins (Table I). Recently, it was shown that C-terminal truncation of αS is a

TABLE I
Distinctive features of αS-mediated pathogenesis correlate with the properties of A30P and A53T mutants

Characteristics associated with the increased neurotoxicity of A30P and A53T	
Biological features	Reduced cell viability in cell culture models (33)
	Increased inhibition of proteasomal activity (34, 35)
	Impaired protein degradation via chaperone-mediated autophagy (6)
	Enhanced *in vivo* C-terminal truncation (30)
Biochemical features	Facilitated formation of dimers (31)
	Acceleration of oligomerization (7)
	Presence of annular protofibrills (32)
	Rapid fibrillation (only A53T) (7)
	Higher vesicle permeabilization activity (36)
Structural features	Perturbed autoinhibitory long range interactions (this work)
	Increased backbone flexibility in the nano- to microsecond time scale (this work)
	Increased exposure of NAC region (this work)

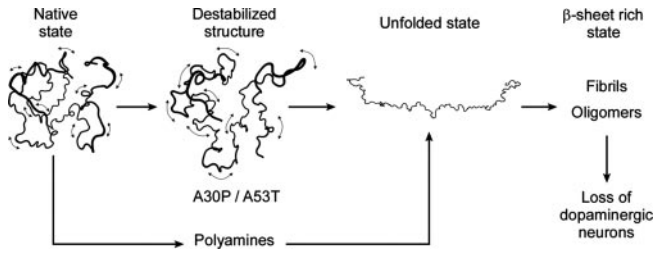

FIG. 3. **Proposed scheme for the increased neurotoxicity of αS genetic mutants.** In this study we demonstrated that the two familial Parkinson's disease associated mutants A30P and A53T perturb the long range interactions essential for the native state of αS. Long range interactions characterize the auto-inhibited native state of αS, maintaining the protein in a soluble innocuous state (13). Mutant αS can overcome more easily the energetic barrier for self-association, leading to an increased tendency to oligomerize. Polyamine binding and high temperature, conditions that *in vitro* enhance αS aggregation, induce a completely unfolded structure (13). Therefore we propose that redistribution of the ensemble of αS conformers, such that the highly amyloidgenic NAC region becomes exposed to the solvent, and β-sheet-rich conformations are adopted more easily, represents the common mechanism for both genetically linked and environmentally induced αS oligomerization in Parkinson disease.

normal cellular process and that the generation and accumulation of truncated αS may be involved in the initiation and progression of αS aggregation *in vivo*. Residues 119–123 were identified as potential cleavage sites and the accumulation of truncated αS was enhanced by the expression of mutant αS (30). These findings may be explained by the results of the present study. Although the truncation site is far from the A30P and A53T missense mutations, the destabilization of αS tertiary structure may allow proteases to degrade the αS C terminus more efficiently. This destabilization may also be the source for the increased affinity of lysosomal chaperone-mediated autophagy receptors for mutant αS, recognizing the sequence ^{95}VKKDQ^{99} present in the C terminus of αS and thereby causing an impairment in protein catabolism (6). Regarding self-association, an increased tendency to form dimers, oligomers, and annular protofibrills has been evidenced for both PD-linked mutants (7, 31, 32), which may as well be explained in terms of our findings. The decreased shielding of the highly hydrophobic NAC region due to the reduction of long range interactions allows A30P and A53T mutant αS to preferentially expose this region to the solvent, facilitating self-association.

We conclude that the redistribution of the structural ensemble of the A30P and A53T αS mutants potentiates oligomerization and modulates binding to ligands, proteases, and receptors. This effect may account for the toxic gain-of-function of familial Parkinsonism-linked mutant αS. Conformationally altered αS may constitute a general molecular mechanism underlying the induction of PD by environmental and genetic conditions (Fig. 3). Thus, agents specifically designed to stabilize the native state of αS may prove useful in impeding or reversing its pathologic aggregation in both idiopathic and familial forms of Parkinson disease.

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