Isomers of Human α-Synuclein Stabilized by Disulfide Bonds Exhibit Distinct Structural and Aggregative Properties†

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ABSTRACT: The discovery of three mutants in the α-synuclein (αSyn) gene and the identification of αSyn as the major component of Lewy body have opened a new field for understanding the pathogenesis of Parkinson's disease (PD). αSyn is a natively unfolded protein with unknown function and unspecified conformational heterogeneity. In this study, we introduce four Ser/Ala → Cys mutations at positions 9, 42, 69, and 89 in human wild-type αSyn (wt-αSyn) and two PD-associated αSyn mutants, A30P-αSyn and A53T-αSyn. This allows expression of three αSyn mutants, wt-αSyn(4C), A30P-αSyn(4C), and A53T-αSyn(4C). Subsequent oxidative folding enables each αSyn(4C) mutant to form three partially stabilized two-disulfide isomers, designated as αSyn(2SS), that are amenable to further isolation and characterization. These αSyn mutants exhibit the following properties. (a) A30P-αSyn(4C) exhibits a lower folding flexibility than wt-αSyn(4C) and A53T-αSyn(4C). (b) All three αSyn(4C) mutants, like wt-αSyn, exhibit a predominant structure of random coil. However, wt-αSyn(2SS) adopts an α-helical conformation, whereas A30P-αSyn(2SS) and A53T-αSyn(2SS) take on significant *β*-sheet structure. (c) A30P-αSyn(2SS) shows a stronger tendency to aggregate than A53T-αSyn(2SS) and wt-αSyn(2SS). (d) Three isolated isomers of wt-αSyn(2SS) exhibit a propensity for forming oligomers different yet enhanced versus that for wt-αSyn. These data together substantiate the notion that under physiological conditions, human αSyn exists as diverse conformational isomers which exhibit distinct propensities for aggregation and fibril formation.

α-Synuclein (αSyn) is a 140-amino acid protein, which is enriched in the presynaptic terminals of neurons and is the major fibrillar component of Lewy bodies, a pathological hallmark of Parkinson's disease (PD)[1] (*1*, *2*). Its function has not yet been well established, though it might be involved in the regulation of dopamine neurotransmission (*3*, *4*) and the participation in a number of cell signaling pathways. Two different missense mutations in the αSyn gene, corresponding to A53T and A30P substitutions in αSyn, have been identified as being associated with familial early-onset PD (*5*, *6*). Recently, a new mutation, E46K, has been found to be involved in the pathogenesis of PD and Lewy body dementia (*7*). Furthermore, the production of wild-type αSyn in transgenic mice (*8*) or of the wild type, A30P, and A53T in transgenic flies (*9*) leads to the motor deficits and neuronal inclusions reminiscent of PD. Most recently, Singleton et al. reported that locus triplication in αSyn causes PD (*10*). All these findings indicate that αSyn plays a critical role in the pathogenesis of PD and several other neurodegenerative synucleinopathies.

Structurally, αSyn is intrinsically unstructured in its native state, which means that under physiological conditions it lacks an ordered secondary structure (*11*). Thermodynamically, the natively unfolded wt-αSyn must comprise a mixture of heterogeneous conformational isomers that exist in a state of equilibrium. In this equilibrium model, it is generally believed that any shift of the equilibrium toward isomers of αSyn with a highly aggregative propensity would trigger or initiate the process of synucleinopathies. Therefore, there are two important challenges related to the protein chemistry of synucleinopathies that need to be addressed: (a) elucidation of the conformational heterogeneity of the natively unfolded αSyn and (b) stabilization and isolation of conformational isomers of αSyn for further structural and functional evaluation. We carried out experiments to address these two issues. On the basis of our experience, diverse conformational isomers of a denatured protein can be trapped and isolated using the technique of disulfide scrambling (*12*). This approach, however, is only applicable to proteins containing at least two disulfide bonds. Therefore, we created mutations of human wt-αSyn at Ser^{9}, Ser^{42}, Ala^{69}, and Ala^{89} by site-directed mutagenesis, replacing all the target sites with cysteine (S9C, S42C, A69C, and A89C, respectively) to generate wt-αSyn(4C). Both Ser^{9} and Ser^{42} are not known to be phosphorylated, and both Ala^{69} and Ala^{89} are not reported to be the key amino acids for the structural conformation and protein function. Since the acidic C-terminal tail of αSyn seems to be critical for its chaperone-like activity, which functions to prevent the accumulation

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[1] Abbreviations: wt-αSyn, wild-type α-synuclein; A30P-αSyn, Ala^{30} → Pro α-synuclein; A53T-αSyn, Ala^{53} → Thr α-synuclein; HPLC, high-performance liquid chromatography; GdmCl, guanidine hydrochloride; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption ionization; PD, Parkinson's disease.

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of misfolded proteins (*13*), and NMR spectroscopy studies of αSyn in solution have shown that its C-terminal part remains free and unfolded (*14*), we thus avoid introducing any modification into the C-terminal part to ensure that the chemical nature of wt-αSyn(4C) is as close as possible to that of wt-αSyn.

Subsequent oxidative folding of wt-αSyn(4C) should in theory allow formation of three different two-disulfide isomers of wt-αSyn(2SS), which are amenable to further isolation and characterization of their structural and aggregative properties. These experiments were applied not only to wt-αSyn but also to two PD-associated mutants, A30P-αSyn and A53T-αSyn.

MATERIALS AND METHODS

Nomenclature of Mutants and Isomers of α-Synuclein. To facilitate the description of various isomers and mutants of α-synuclein throughout this paper, we take the following measures. (a) The naturally occurring mutations are added as a prefix. For instance, wt-αSyn, A30P-αSyn, and A53T-αSyn represent wild-type, A30P, and A53T α-synuclein, respectively. (b) The introduced Ala/Ser → Cys mutations are attached as a suffix. For example, wt-αSyn(4C) and wt-αSyn(2SS) correspond to wt-αSyn with four Cys residues and two disulfides, respectively. (c) Among αSyn(2SS), there are three possible disulfide isomers. These isomers are distinguished by a−c as a second suffix. For instance, wt-αSyn(2SS)-a symbolizes isomer a of wt-αSyn(2SS).

Plasmid Construction. A neuroblastoma cell line, SH-SY5Y, was cultured in DMEM/F12 medium (Gibco) containing 10% FBS (Hyclon) as described previously (*15*). αSyn was obtained by PCR amplification of cDNAs that were generated by reverse transcription of total RNA isolated from SH-SY5Y cells using TRIzol reagent (Life Technol). The amplified products were cloned into pGEX5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ) using XmaI and XhoI. The full-length αSyn protein contains 140 amino acid residues. Two αSyn mutants that are involved in the pathogenesis of rare familiar PD are A53T and A30P, which have an Ala-to-Thr mutation at residue 53 and an Aal-to-Pro mutation at 30, respectively. Detailed construct information and the primer sequences are available upon request. The sequence of the constructs was verified by DNA sequencing.

Expression and Purification of GST−αSyn Fusion Proteins. Expression of GST−αSyn fusion proteins in BL21 [F-*ompT hsdSB(rB -mB-) gal dcm*] cells (Stratagene) was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at room temperature. The cultures were collected by centrifugation, and the bacterial pellets were resuspended in Celllytic B bacterial cell lysis/extraction reagent (Sigma, catalog no. B-3553) containing protease inhibitors (Sigma, catalog no. P8849). The GST−αSyn fusion proteins were purified from crude cell lysates under nondenaturing conditions by selectively binding to glutathione−Sepharose 4B beads (Amersham Pharmacia Biotech) as described previously (*16*). The GST−αSyn fusion proteins bound to beads were digested with factor Xa (1/100, w/w, Amersham Pharmacia Biotech) in reaction buffer [100 mM NaCl, 50 mM Tris-HCl, and 1 mM $CaCl_2$ (pH 8.0)] at 25 °C for 16 h with gentle mixing following three washes with PBS and one with reaction buffer. The cleaved proteins were further purified using HPLC. Purified αSyn proteins were lyophilized and stored at −80 °C.

Oxidative Folding of Ala/Ser → Cys Mutants of αSyn. The purified mutant of αSyn was first reduced and denatured in Tris-HCl buffer (0.1 M, pH 8.4) containing 6 M GdmCl and 30 mM dithiothreitol, to generate the starting material of folding, αSyn(4C). The reaction was carried out for 90 min at 23 °C. To initiate folding, the reduced αSyn mutant was passed through a PD-10 column (Sephadex-25, Pharmacia) equilibrated in 0.1 M Tris-HCl buffer (pH 8.4), diluted immediately with the same Tris-HCl buffer to a final protein concentration of 0.5 mg/mL. The oxidative folding was performed in this Tris-HCl buffer (0.1 M, pH 8.4) alone or in the presence of $CuSO_4$ (2 μM). Folding intermediates were trapped in a time course manner by mixing aliquots of the sample with an equal volume of 4% aqueous trifluoroacetic acid and analyzed directly by reverse-phase HPLC.

HPLC Analysis for αSyn. The purified isomers and mutants of αSyn were purified and analyzed by HPLC using the following conditions. The column was a Zorbax 300XB-C18, 250 mm × 4.6 mm, 5 μm model. Buffer A was 0.1% TFA in water. Buffer B was 0.086% TFA in a 9/1 acetonitrile/water mixutre (by volume). The gradient of elution was linear from 10 to 70% B over 30 min. The flow rate was 0.5 mL/min. The column temperature was 23 °C.

Characterization of Disulfide Structures of Two-Disulfide Isomers of wt-αSyn(2SS). HPLC-purified wt-αSyn(2SS)-a−c (15 μg) were digested with 1.5 μg of thermolysin (Sigma, catalog no. P1512) in 30 μL of *N*-ethylmorpholine/acetate buffer (50 mM, pH 6.4), or with Glu-C in 30 μL of ammonium bicarbonate buffer (50 mM, pH 8.0). Digestion was carried out at 37 °C for 16 h. Peptides were then isolated by HPLC and analyzed by both MALDI mass spectrometry and Edman sequencing to identify peptide fragments containing disulfide bonds.

Analysis of the Molecular Mass of Mutants and Isomers of αSyn. The molecular masses of αSyn mutants and isomers, both unmodified and those modified with vinylpyridine and iodoacetic acid, were determined with a MALDI mass spectrometer (Perkin-Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as a matrix. Molecular masses of analyzed peptides were calibrated with the following standards: bradykinin fragment (residues 1−7) (MH^+ 757.3997), synthetic peptide P14R (MH^+ 1533.8582), and ACTH fragment (residues 18−39) (MH^+ 2465.1989).

Assay of the Aggregation Rate of αSyn Isomers by Western Blotting. Aggregation of the αSyn isomer was performed in PBS (pH 7.4) at room temperature or 37 °C. The protein concentration was 20 μM. At different time points (0, 1, and 3 days), the samples were harvested for a Western blot assay. Equal quantities of the proteins from an equal volume of each fraction were boiled in sample buffer for 5 min, separated on a SDS−PAGE gel, and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in PBST for 1 h at room temperature, probed with the primary antibodies in 5% nonfat dry milk overnight at 4 °C, washed with PBS, and incubated with the an appropriate secondary antibody for 1 h at room temperature. The signals were detected using the enhanced chemiluminescence assay (Amersham Life Science Inc.,

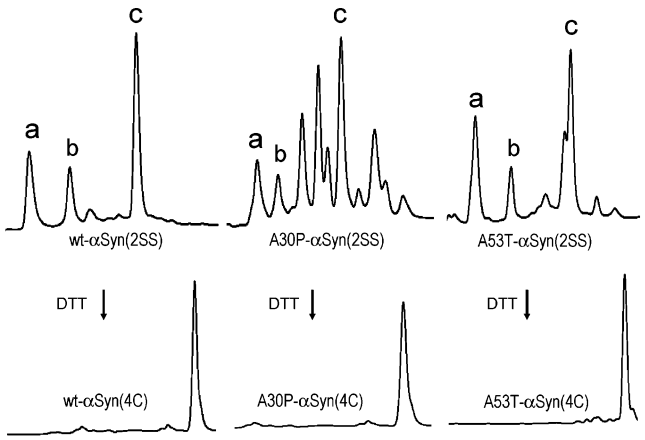

FIGURE 1: HPLC pattern of wt-αSyn(2SS), A30P-αSyn(2SS), and A53T-αSyn(2SS) following the process of expression and purification. The wild type and A53T mutants were isolated as three major two-disulfide isomers. The A30P mutant was recovered as a mixture of one- and two-diuslfide isomers. Reduction converts all three αSyn mutants to form fully reduced wt-αSyn(4C), A30P-αSyn-(4C), and A53T-αSyn(4C). HPLC conditions are described in the text.

Arlington Heights, IL), according to the manufacturer's instructions.

Assay of Fibrillation of α*Syn Isomers by Fluorescence Measurements.* Fluorescence measurements were performed according to the procedures described previously (*17*). In brief, the ThT fluorescence was recorded immediately following the addition of the aliquots of wt-Syn, wt-Syn-(2SS)-a, wt-Syn(2SS)-b, wt-Syn(2SS)-c, and collective isomers of wt-Syn(2SS), A30P-Syn(2SS), and A53T-Syn(2SS) to the ThT mixture at different time points (0, 1, 3, and 7 days). Measurements were performed at 37 °C and pH 7.4. The protein concentration was 1 mg/mL. The ThT fluorescence was excited at 450 nm, and the emission wavelength was 482 nm.

RESULTS AND DISCUSSION

Rapid Purification of α*-Synuclein Proteins.* To develop a simple method for expressing and purifying αSyn proteins for the biochemical and molecular studies in this experiment, we utilized the pGEX expression system. In this expression system, wild-type and mutated αSyn proteins were expressed as GST−αSyn fusion proteins. We found that most of the fusion proteins are present in the soluble state and almost undetectable in insoluble precipitates at room temperature for 6 h. By selectively binding to glutathione−Sepharose beads, GST−αSyn fusion proteins were purified at high levels under nondenaturing conditions. A factor Xa proteolytic cleavage site has been engineered between GST and αSyn, allowing αSyn proteins to be efficiently cleaved for further HPLC purification.

αSyn mutants obtained at this stage are already oxidized, but with different degrees of oxidation (Figure 1). In the cases of wt-αSyn(4C) and A53T-αSyn(4C), oxidation is near completion, and three major two-disulfide isomers (designated a, b, and c) are observed in each mutant. In the case of A30P-αSyn(4C), the oxidation of disulfide bonds is incomplete and the protein was recovered as a mixture of one- and two-disulfide isomers (there are six possible one-disulfide isomers and three possible two-disulfide isomers).

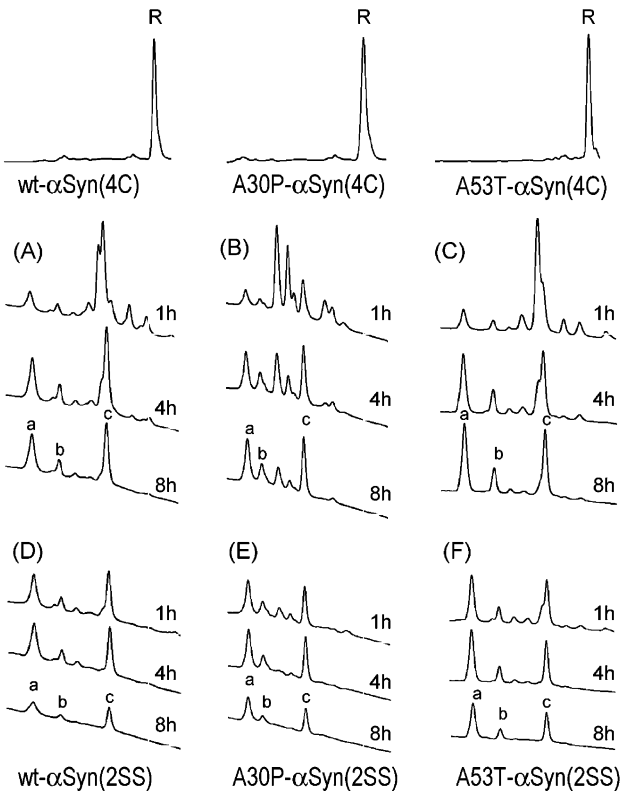

FIGURE 2: Oxidative folding of wt-αSyn(4C), A30P-αSyn(4C), and A53T-αSyn(4C). The three fully reduced αSyn mutants were allowed to refold in Tris-HCl buffer (0.1 M, pH 8.4) (A−C) or in the same Tris-HCl buffer containing $CuSO_4$ (2 μM) (D−F). The process of folding was trapped by sample acidification and analyzed by HPLC.

These results are reproducible and therefore indicate that A30P-αSyn(4C) has a lower folding capability than wt-αSyn(4C) or A53T-αSyn(4C) under identical conditions.

The three αSyn mutants were treated with denaturant and DTT to generate fully reduced species. They were then analyzed by HPLC and MALDI mass spectrometry. On HPLC (Figure 1), wt-αSyn(4C), A30P-αSyn(4C), and A53T-αSyn(4C) exhibit a retention time almost indistinguishable from that of wt-αSyn, evidence of the similarity of their chemical properties despite the insertion of four Ser/Ala → Cys mutations. The observed molecular weights of wt-αSyn [15230 (observed) vs 15229 (experimental)], wt-αSyn(4C) [15325 (observed) vs 15325 (experimental)], A30P-αSyn-(4C) [15350 (observed) vs 15351 (experimental)], and A53T-αSyn(4C) [15355 (observed) vs 15355 (experimental)] are in agreement with their expected molecular weight.

*Oxidative Folding of wt-*α*Syn(4C), A30P-*α*Syn(4C), and A53T-*α*Syn(4C).* To further evaluate the properties of the three mutant proteins, we conducted systematic folding experiments with wt-αSyn(4C), A30P-αSyn(4C), and A53T-αSyn(4C). Oxidative folding was carried out under the following conditions: (a) In Tris-HCl buffer (0.1 M, pH 8.4) alone without redox agents, air oxygen was responsible for the disulfide oxidation. Folding of wt-αSyn(4C) and A53T-αSyn(4C) was complete within ~8 h, and each was shown to generate three isomers of αSyn(2SS) (Figure 2A,C). The folding of A30P-αSyn(4C) is slower than that of wt-αSyn-(4C) or A53T-αSyn(4C) (Figure 2B). (b) In Tris-HCl buffer (0.1 M, pH 8.4) containing $CuSO_4$ (2 μM), copper ions are known to catalyze air oxidation of thiol groups to disulfide

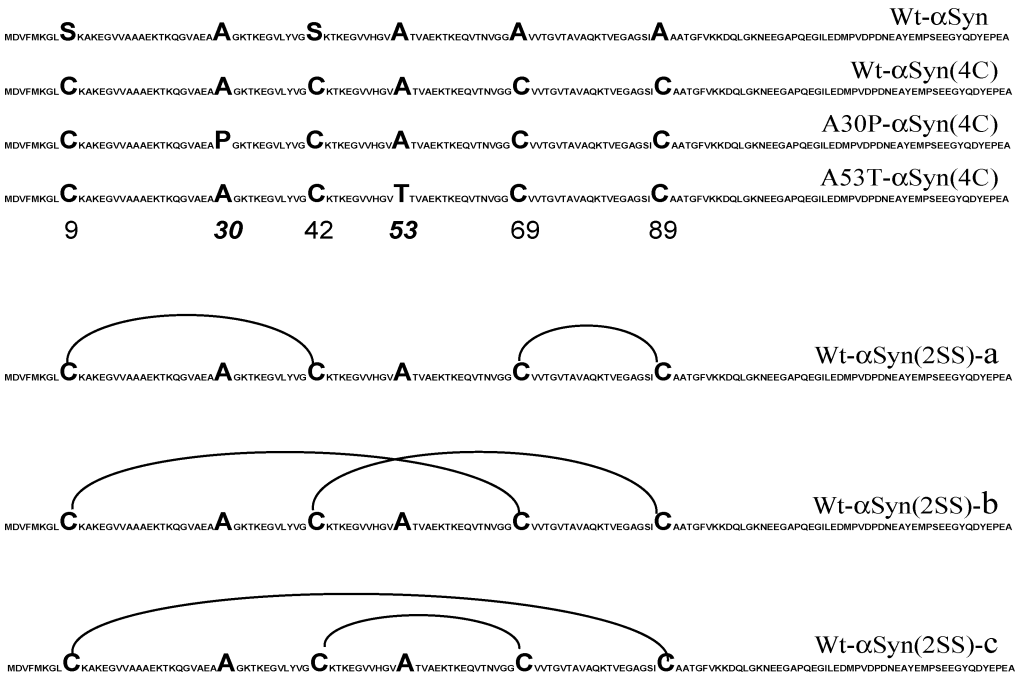

FIGURE 3: Amino acid sequences of αSyn mutants and disulfide structures of wt-αSyn(2SS)-a, wt-αSyn(2SS)-b, and wt-αSyn(2SS)-c. The disulfide structures were derived from analysis of their thermolytic peptides by MALDI mass spectrometry and Edman sequencing. Isomers a−c have $Cys^{9}-Cys^{42}/Cys^{69}-Cys^{89}$, $Cys^{9}-Cys^{69}/Cys^{42}-Cys^{89}$, and $Cys^{9}-Cys^{89}/Cys^{42}-Cys^{69}$ disulfide connectivities, respectively.

bonds (*18*). We found that they are a useful catalyst for promoting oxidative folding of αSyn(4C). The presence of 2 μM $CuSO_4$ increases the folding rate of αSyn(4C) by approximately 4-fold (compare panels A−C to panels D−F of Figure 2). However, the presence of copper ions also appears to accelerate the aggregation of folded αSyn(2SS). This is evident because in the presence of $CuSO_4$, the recovery of αSyn(2SS) isomers decreases significantly during an extended period of folding, probably due to protein aggregation.

The reports that the Cu(II) concentration was elevated in the cerebrospinal fluid of PD patients (*19*) and that Cu(II) is the most effective ion in promoting oligomerization of αSyn (*20*) further suggest that copper ion might be a risk factor for the pathogenesis of PD. However, the copper concentrations most laboratories applied (0.5−5 mM) are far greater than those normally presented in vivo. Most recently, it was reported that copper at the physiologically relevant concentration is sufficient for accelerating αSyn aggregation (*21*). A copper concentration of 2 μM was intended to promote the oxidative folding of reduced αSyn(4C) mutants (*18*). In this study, this concentration also effectively increases the kinetics of aggregation of folded (oxidized) two-disulfide isomers of αSyn(2SS) (Figure 2D,E). Alternatively, the copper-enhanced oligomerization might be simply a consequence of increased folding kinetics.

The end products of three folding experiments all comprise three major fractions of two-disulfide isomers. This conclusion is substantiated by the observation that their modification with vinylpyridine affects neither their molecular mass nor their HPLC retention time, evidence of the absence of free Cys. The three two-disulfide isomers of wt-αSyn(2SS) were isolated and digested with thermolysin and Glu-C. Peptides were then isolated by HPLC and analyzed by MALDI mass spectrometry and Edman sequencing. On the basis of the known sequences of αSyn, the data of mass and sequence analysis (available upon request) allow identification of peptides containing the disulfide bonds and lead to the conclusion of disulfide pairings of wt-αSyn(2SS)-a, wt-αSyn(2SS)-b, and wt-αSyn(2SS)-c as shown in Figure 3. The disulfide structures of three corresponding isomers of A30P-αSyn(2SS) and A53T-αSyn(2SS) have not been characterized but most likely adopt the same disulfide pairing similar to that of wt-αSyn(2SS).

The existence of all three possible two-disulfide isomers in significant and steady proportion in the mutants of wt-αSyn, A30P-αSyn, and A53T-αSyn also supports the credence that αSyn is truly a natively unfolded protein consisting of diverse and defined conformational isomers. It is relevant to notice that wt-αSyn(2SS)-c is the most predominant isomer (Figure 3). Among the disulfide configurations of three isomers (Figure 3), wt-αSyn(2SS)-c represents the most compact state. Its predominance thus implies that more compact isomers account for the major structure in the natively unfolded state of wt-αSyn. In the cases of two αSyn variants, the concentrations of A30P-αSyn(2SS)-c and A53T-αSyn(2SS)-c decrease significantly, most likely due to the increase in the concentrations of less compact isomers.

A30P-αSyn(4C) Has a Lower Folding Flexibility than wt-αSyn(4C) or A53T-αSyn(4C). The findings that single mutations in wt-αSyn are linked to familial early-onset forms of PD point to a central role for the protein in the etiology of the disease. Here we found that A30P-αSyn(4C) has a much lower folding flexibility than wt-αSyn(4C) or A53T-αSyn(4C) under identical folding conditions (Figure 2). The A30P mutant has been reported to exhibit a defective or weakened ability to bind lipid vesicles compared to the wild-type protein (*22*, *23*). The formation of soluble oligomeric intermediates is also accelerated in the A30P mutant (*24*). The A30P mutant favors the protein's self-aggregation capacity, as demonstrated in vitro (*24*, *25*). *Drosophila*

develops adult-onset motor dysfunctions that appear earlier in specimens expressing the A30P mutant than in those expressing wt- or A53T-αSyn (*9*).

These earlier observations may be explained by the unique folding behavior of A30P-αSyn(4C) demonstrated here. From the viewpoint of protein folding, αSyn appears to be a very dynamic molecule whose secondary structure depends on its environment. Therefore, the decreased folding flexibility of A30P-αSyn might weaken its dynamics and its physiological functions. On the other hand, the reduced conformational flexibility could also lead to the exposure of hydrophobic residues and makes A30P-αSyn naturally prone to forming insoluble aggregates.

Alteration of Secondary Structure Accompanied by αSyn(4C) → αSyn(2SS) Conversion. Human wt-αSyn is a natively unfolded protein and exhibits random coil structure under physiological conditions. When lipid binds, wt-αSyn undergoes a conformational change from random coil to α-helical structures (*26*). The A53T mutation appears to have little effect on the αSyn−lipid interaction and subsequent conformational change (*27*). The A30P mutation, however, results in either a defective or weakened ability of αSyn to bind lipid (*28*, *29*).

We have shown here that reduced forms of αSyn mutants wt-αSyn(4C), A30P-αSyn(4C), and A53T-αSyn(4C), similar to that of wt-αSyn, all display a structure of predominantly random coil (Figure 4A). After oxidation and formation of two disulfide bonds, wt-αSyn(2SS) takes on a major portion of α-helical structure. In contrast, A30P-αSyn(2SS) and A53T-αSyn(2SS) exhibit a significant β-sheet structure content (Figure 4B). In essence, this wt-αSyn(4C) → wt-αSyn(2SS) conversion accompanied by random coil → α-helix transformation is akin to the structural change of wt-αSyn induced upon lipid binding. Both involve to some extent stabilization of the natively unfolded structure of wt-αSyn. The puzzle is why a similar conformational transformation does not apply to A53T-αSyn(2SS) or A30P-αSyn(2SS). Perceptibly, one can argue that the mode of stabilization of αSyn via lipid binding and introduced disulfide bonds may be very different. A satisfactory account of this discrepancy should also take into consideration the fact that all two-disulfide isomers of αSyn have four additional Ser/Ala → Cys mutations.

Nonetheless, the β-sheet content of A30P-αSyn(2SS) and A53T-αSyn(2SS) may facilitate their aggregation, and this can well explain their enhanced aggregative propensity as compared to that of wt-αSyn(2SS) (see below).

The Isolated Isomers of wt-αSyn(2SS) Exhibit Dissimilar but Enhanced Aggregation Rates Compared to That of wt-αSyn. The aggregation rate of the a−c isomers of wt-αSyn-(2SS) (Figure 3) was compared to that of wt-αSyn. They were incubated in PBS buffer (pH 7.4) at room temperature (22 °C) and 37 °C for different periods of time (0, 1, and 3 days). Aggregated samples were analyzed by Western blotting. The protein concentration was 20 μM. Rigorous control of the same protein concentration is essential to establishing a convincing aggregation rate measurement, since a higher protein concentration leads to a higher aggregation rate. The results show that under physiological conditions in vitro, all three isomers of wt-αSyn(2SS) display an aggregation rate faster than that of wt-αSyn, both at room temperature and at 37 °C (Figure 5). Among the three

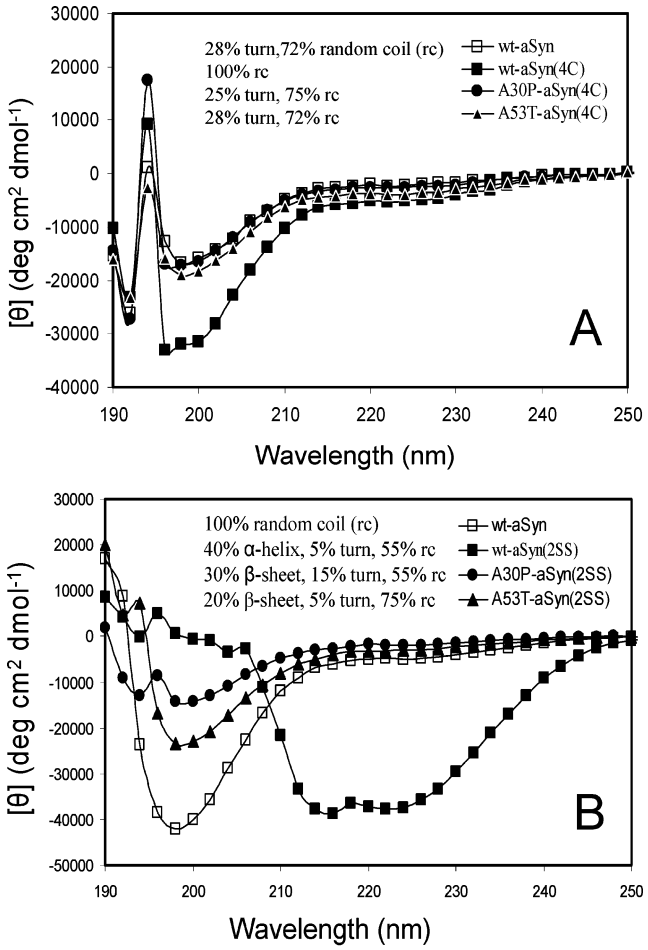

FIGURE 4: Far-UV CD spectra of variants of αSyn. (A) Mutants of αSyn(4C) were measured in acetate buffer (pH 4.5). (B) Mutants of αSyn(2SS) were measured in phosphate buffer (pH 7.4). Each αSyn(2SS) comprises three isomers. The protein concentration was 0.1 mg/mL. wt-αSyn was included as a control. Each spectrum was an average of 10 scans. The content of secondary structure was calculated using the Softsec software provided by Jasco Inc.

isomers of wt-αSyn(2SS), the order of aggregation rate is as follows: c > a > b.

We further verified the differential and enhanced fibrillation by thioflavin T (ThT) fluorescence measurement. The results are shown in Figure 6. The histological dye ThT is widely used for the detection of amyloid fibrils (*17*, *30*). With the presence of fibrils, ThT gives rise to a new excitation maximum at 450 nm and enhanced emission at 482 nm, whereas naked ThT is essentially nonfluorescent at these wavelengths. ThT is supposed to bind specifically to the crossed β-sheet structure, and the binding is independent of the primary structure of the protein. Only the multimeric fibrillar forms give significant fluorescence with ThT. The binding of ThT to α-Syn fibrils is therefore an effective method for the fibril formation assay. Figure 6 shows the differences in the ThT fluorescence at different time points (1, 3, and 7 days) of α-Syn fibril formation. Consistent with the Western blot analysis, isomers a−c all exhibit higher ThT fluorescence, which suggests that the isomers have a propensity to fibrillate different from but enhanced compared to that of wt-α-Syn. Among the isomers, wt-α-Syn(2SS)-c exhibited the highest propensity for fibril formation, which is consistent with the higher aggregation rate described above.

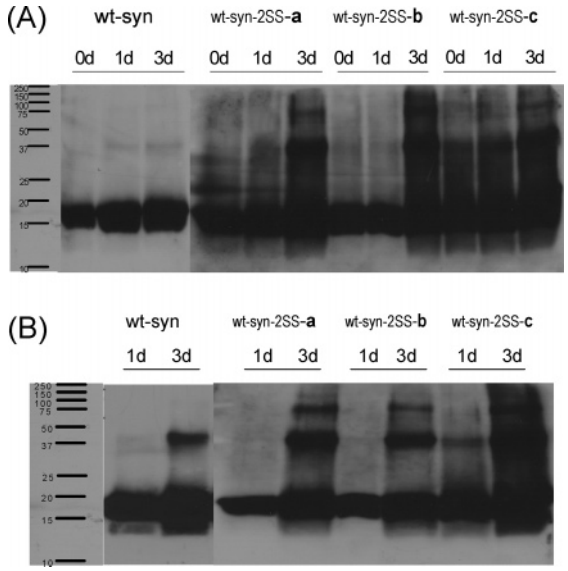

FIGURE 5: Oligomerization of wt-αSyn and three isolated two-disulfide isomers of wt-αSyn(2SS). Aggregation experiments were performed at 22 (A) and 37 °C (B). The protein concentration was 20 μM. Proteins were separated by SDS−PAGE and detected by Western blotting.

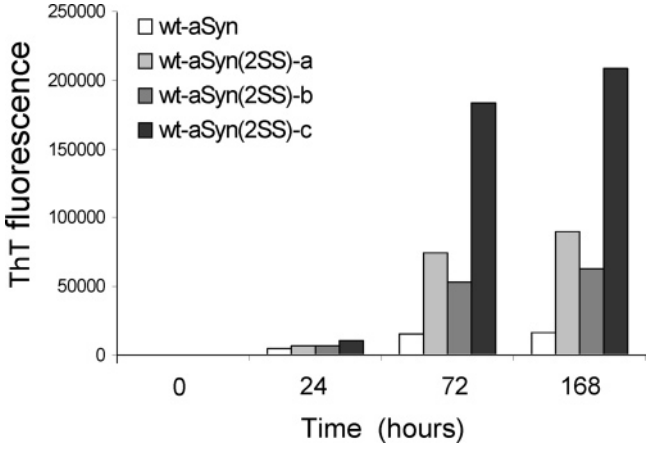

FIGURE 6: Fibrillation (Thio T fluorescence assay) of wt-αSyn and three isolated two-disulfide isomers of wt-αSyn(2SS). Experiments were carried out in PBS at 37 °C. The protein concentration was 65 μM. The ThT fluorescence was excited at 450 nm, and the emission wavelength was 482 nm.

Our results demonstrate that (a) different conformational isomers of wt-αSyn may exhibit different rates of aggregation and fibrillation and (b) partial stabilization of the conformation of wt-αSyn (via two introduced disulfide bonds) may enhance the propensity of wt-αSyn for aggregation and fibrillation. These data are consistent with the notion that under physiological conditions, human α-Syn exists as diverse conformational isomers which exhibit distinct propensities for aggregation and fibril formation.

A30P-αSyn(2SS) Has a Faster Aggregation Rate than A53T-αSyn(2SS) and wt-αSyn(2SS). We further compare the aggregation rates of two-disulfide isomers of wt, A30P, and A53T in mixture form. wt-αSyn(2SS), A30P-αSyn(2SS), and A53T-αSyn(2SS) were incubated for different periods of time (0, 1, and 3 days) for the aggregation rate assay. We found that even in the control (0 days), there are strong bands at around 35 (dimer) and 70 kDa (tetramer) in an A30P-αSyn(2SS) isomer mixture, and after incubation (1 and 3 days) at room temperature, the levels of the oligomers

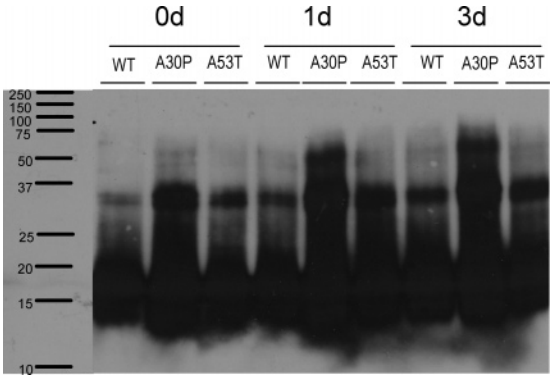

FIGURE 7: Oligomerization of collective isomers of wt-αSyn(2SS), A30P-αSyn(2SS), and A53T-αSyn(2SS). Aggregation experiments were performed at 22 °C. The protein concentration was 20 μM. Proteins were separated by SDS−PAGE and detected by Western blotting.

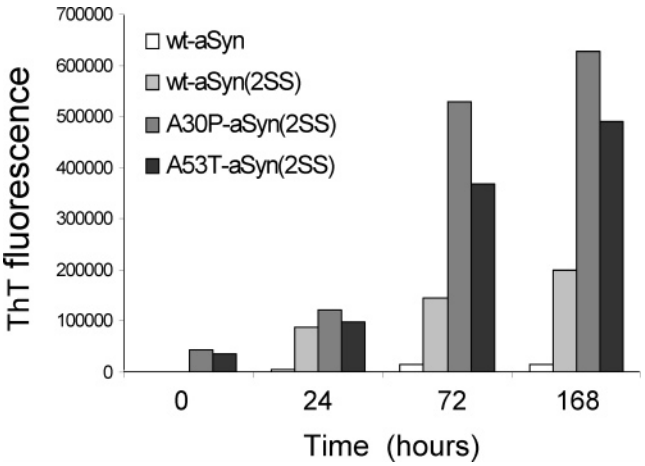

FIGURE 8: Fibrillation (Thio T fluorescence assay) of collective isomers of wt-αSyn(2SS), A30P-αSyn(2SS), and A53T-αSyn(2SS). Experiments were carried out in PBS at 37 °C. The protein concentration was 65 μM.

increased significantly (Figure 7). In contrast, a wt-αSyn-(2SS) isomer mixture has an only insignificant band at around 35 kDa at the time point of control and a comparatively weaker band at around 35 kDa after incubation for 1 or 3 days. The aggregation rate of A53T-αSyn(2SS) is between those of A30P-αSyn(2SS) and wt-αSyn(2SS). The data here suggest A30P-αSyn(2SS) has a much higher aggregation rate than A53T-αSyn(2SS), which in turn has a higher aggregation rate than wt-αSyn(2SS). We also confirmed the increased rate of fibril formation by ThT fluorescence measurement, and the results are shown in Figure 8. A30P-αSyn(2SS) displays a stronger tendency to fibrillate than A53T-αSyn(2SS) or wt-αSyn(2SS).

The faster aggregation and fibrillation rate of the A30P-αSyn(2SS) mixture is probably due to its low folding flexibility described above. The higher aggregation rate of the A30P-αSyn mutant has been consistently reported (*24*, *25*). It was also reported that PD-linked mutations accelerate αSyn aggregation (*31*). However, under their experimental conditions (protein concentration of 7 mg/mL, Tris-buffered saline at 37 °C), A53T-αSyn has a faster aggregation rate than A30P-αSyn and WT-αSyn.

Implications of the Properties of Partially Stabilized αSyn. Protein conformation-dependent toxicity is an emerging theme in neurodegenerative disorders, including the α-sy-

nucleinopathies (*32*). The native proteins are generally not pathogenic; however, they are the pool for providing the alternative conformations (nonnative isomers), which are the real culprit. Under different stressful conditions, the shift of equilibrium from the benign to the malignant isomers is commonly believed to be the underlying cause of these diseases. In this context, the more stable the native state, the lower the risk of the conformational change-induced occurrence of diseases. However, the genetic factors (mutations), the environmental factors (pesticides) (*33*), and the aging might all contribute to the destabilization the native state of αSyn and the initiation of the process of α-synucleinopathies.

Disulfide isomers of αSyn(2SS) described in this study are in essence partially stabilized αSyn isomers that constitute and mimic the structure of natively unfolded αSyn under physiological conditions. The findings that these isomers exhibit distinct folding flexibility, dissimilar secondary structure, and different aggregative propensity are in agreement with the our current understanding that αSyn comprises diverse conformational isomers which exhibit distinct propensities of aggregation and that single-amino acid substitution (A30P or A53T) might significantly alter the conformational properties of αSyn.

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