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The H50Q Mutation Induces a 10-fold Decrease in the Solubility of α-Synuclein*

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Background: The basis of the pathogenicity of the H50Q variant α-synuclein is unknown.

Results: The critical concentration of α-synuclein is decreased by 10-fold by the H50Q mutation, and its aggregation is modulated by the wild-type isoform.

Conclusion: Key effects of the H50Q mutation on the aggregation of α-synuclein can be quantified.

Significance: Our data provide insights into the mechanism of Lewy body formation in vivo.

The conversion of α-synuclein from its intrinsically disordered monomeric state into the fibrillar cross-β aggregates characteristically present in Lewy bodies is largely unknown. The investigation of α-synuclein variants causative of familial forms of Parkinson disease can provide unique insights into the conditions that promote or inhibit aggregate formation. It has been shown recently that a newly identified pathogenic mutation of α-synuclein, H50Q, aggregates faster than the wild-type. We investigate here its aggregation propensity by using a sequence-based prediction algorithm, NMR chemical shift analysis of secondary structure populations in the monomeric state, and determination of thermodynamic stability of the fibrils. Our data show that the H50Q mutation induces only a small increment in polyproline II structure around the site of the mutation and a slight increase in the overall aggregation propensity. We also find, however, that the H50Q mutation strongly stabilizes α-synuclein fibrils by 5.0 ± 1.0 kJ mol⁻¹, thus increasing the supersaturation of monomeric α-synuclein within the cell, and strongly favors its aggregation process. We further show that wild-type α-synuclein can decelerate the aggregation kinetics of the H50Q variant in a dose-dependent manner when coaggregating with it. These last findings suggest that the precise balance of α-synuclein synthesized from the wild-type and mutant alleles may influence the natural history and heterogeneous clinical phenotype of Parkinson disease.

α-Synuclein (α-Syn) 4 is central to the pathogenesis of Parkinson disease (1, 2). Mutations and multiplications of the encoding SNCA gene are associated with familial Parkinson disease and polymorphisms with an increased risk of developing sporadic disease (3–7). Monomers of α-Syn have been shown to be capable of forming soluble oligomers and amyloid fibrils (8–10), which are the major component of intraneuronal Lewy bodies, the pathological hallmark of Parkinson disease (11, 12). The molecular basis of the transition of α-Syn from its intrinsically disordered monomeric state (13–15) into cross-β fibrillar assemblies is largely unknown. Future progress is expected from the complementary synergy of two approaches: continuous optimization of algorithms suitable for predicting the aggregation propensity of the protein and the production of experimental models of α-Syn fibrillogenesis mimicking the physiological environment. Particularly informative is the investigation of natural pathogenic mutations of α-Syn associated with misfolding and aggregation.

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4 The abbreviations used are: α-Syn, α-Synuclein; ThT, thioflavin T; EM, electron microscopy; AFM, atomic force microscopy; BEST, band-selective excitation short transient.
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The recently identified H50Q α-Syn mutation (16, 17) was shown to aggregate faster in vitro compared with the WT counterpart (18–21). Here we compare sequence-based predictions of aggregation propensity (22, 23) with further experimental observations of aggregation behavior in the WT and H50Q α-Syn. It is worth noting that in heterozygous carriers, as in the patients with the H50Q mutation, half of the expressed α-Syn is expected to be WT. Although current aggregation propensity algorithms are unable to predict the effect of mixtures of α-Syn variants upon the aggregation process, our experimental data show that the WT protein attenuates the aggregation kinetics of the variant in a dose-dependent manner. However, the molecular explanation for the effect of H50Q and other mutations on the aggregation propensity remains an unresolved issue.

To investigate further the changes in the increased aggregation propensity of the H50Q variant, we characterize the monomeric ensembles of both WT and H50Q α-Syn using NMR spectroscopy. In previous reports, comparative spectroscopic analyses have revealed broadly similar structural ensembles in the WT and H50Q variants, with a small number of chemical shift changes around the site of the mutation (18–20) and, in some cases, additional perturbations in the C-terminal region (18, 19). Here we carry out a comprehensive chemical shift-based analysis of WT and H50Q α-Syn, using the δ2D algorithm (24) to scrutinize changes in the residual secondary structures of the variant.

By recognizing that to understand the effect of a mutation it is important to consider the potential effects on the fibrillar end products of the aggregation process, we use chemical denaturation of fibrils to determine the free energy of elongation. These measurements provide access to the critical concentration, i.e. the concentration above which fibrils are thermodynamically the most stable state (25–27). Therefore, we characterize the relative stabilities of fibrils formed from WT α-Syn and the H50Q disease-associated variant, finding that the mutation has a major impact on the solubility of the protein.

**EXPERIMENTAL PROCEDURES**

**Aggregation Propensities with Solvent Exposure Corrections**—The intrinsic aggregation propensity profile is calculated using the position-dependent score $P_i^{agg}$. For a given residue $i$, the score is calculated as follows,

$$ P_i^{agg} = \alpha_0 p_i^h + \alpha_i p_i^s + \alpha_{hyd} p_i^{hyd} $$

(Eq. 1)

where $p_i^h$ and $p_i^s$ are the propensities for α-helix and β-sheet formation, respectively, and $p_i^{hyd}$ is the hydrophobicity (23). The values are combined to provide a score, $Z_i^{agg}$, that describes the intrinsic propensity for aggregation for the whole amino acid sequence (23, 28),

$$ Z_i^{agg} = \sum_j \alpha_{agg} l_{ij}^{agg} + \alpha_{pat} l_{ij}^{pat} + \alpha_{gel} l_{ij}^{gel} + \alpha_{agg} l_{ij}^{agg} $$

(Eq. 2)

where $l_{ij}^{agg}$ is the term that takes into account the presence of specific patterns of alternating hydrophobic and hydrophilic residues (29), and $l_{ij}^{pat}$ is the term that takes into account the gatekeeping effect of individual charges (23).

By combining the predictions of the intrinsic aggregation propensity profiles with those for the solvent exposure of protein regions, it is possible to account for the influence of transient structure formation on the aggregation propensities. The aggregation propensity profile is defined by modulating the intrinsic aggregation propensity profile with the CamP score, denoted as $P_i$, predicting the local structural stability at that position (30). The propensity of exposed regions to promote aggregation can be expressed as follows,

$$ Z_i^{agg} = \frac{Z_i^{agg}1 - P_i^{max}}{Z_i^{agg}} \text{ if } Z_i^{agg} > 0 $$

$$ Z_i^{agg} = \frac{Z_i^{agg}}{Z_i^{agg}} \text{ if } Z_i^{agg} < 0 $$

(Eq. 4)

where $P_{max} = 15$ is a normalization constant (23). The number of Fourier coefficients employed to obtain the results in Fig. 1 is 7 (23, 30).

**Protein Expression and Purification**—Recombinant WT and H50Q variant α-Syn were expressed and purified as previously described (31). For H50Q α-Syn, the QuikChange site-directed mutagenesis kit (Stratagene) was used with the primer sequence GAGGGAGTGGTGCAAAGGTTGGCAACAGTG containing the underlined codon for glutamine at position 50.

**Kinetics of Fibrillogenesis**—Samples of recombinant WT and H50Q α-Syn, 100 μM at different concentrations (5, 10, 30, 50, and 70, and 100 μM, respectively) in PBS, pH 7.4, containing 10 μM thioflavin T (ThT) (32), were incubated at 37 °C in Costar 96-well black wall plates sealed with sealing film (Atidue Gas Permeable Moisture Barrier Seal) and subjected to 900 rpm double orbital shaking. Bottom fluorescence was recorded at 15-min intervals (FLUOstar Omega, BMG LABTECH). Time courses of aggregation were fitted to a sigmoidal model, as $y = y_0 + (y_{max} - y_0)/(1 + \exp[-k_{app}(t - t_0)])$ using KaleidaGraph 4.0 (Synergy Software, Reading, PA), where $y_0$ and $y_{max}$ are the initial and maximum ThT fluorescence, respectively; $k_{app}$ is the apparent rate constant, and lag time was defined as $t_0 - 2/k_{app}$ (18). Experiments were conducted in triplicate in three independent experiments. Samples containing the ThT positive material were further analyzed by electron microscopy. Further aggregation time courses were performed using mixtures of WT/H50Q at 0:1, 1:5, 1:2, 1:1, 2:1, 5:1, and 1:0 molar ratios, fibrillar aggregates were quantified by assessment of the initial and maximum ThT fluorescence, respectively; $k_{app}$ is the apparent rate constant, and lag time was defined as $t_0 - 2/k_{app}$ (18). Experiments were conducted in triplicate in three independent experiments. Samples containing the ThT positive material were further analyzed by electron microscopy. Further aggregation time courses were performed using mixtures of WT/H50Q at 0:1, 1:5, 1:2, 1:1, 2:1, 5:1, and 1:0 molar ratios, respectively, keeping the total protein concentration at 70 μM.

**Electron Microscopy**—Formvar-coated copper EM grids were placed coated side down onto each sample and incubated for 2 min before blotting with filter paper to remove excess solvent and staining with 2% (w/v) uranyl acetate for 2 min. After further blotting and drying in air, transmission electron microscope (CM120) images were obtained at 80 keV.

**Amyloid Fibril Preparation**—A scaled up method was developed to prepare larger quantities of fibrils for further characterization. Briefly, solutions of WT and H50Q α-Syn at 5 mg/ml in PBS, pH 7.4, were stirred at 1500 rpm for 72 h at 37 °C. Finally, fibrillar aggregates were quantified by assessment of the molar absorptivity is 5960 M$^{-1}$ cm$^{-1}$ for both WT and variant α-Syn.

**Equilibrium Unfolding of WT and H50Q α-Syn Fibrils**—Fibrils (0.5 mg/ml) in PBS, pH 7.4, were incubated with increasing concentrations of guanidine HCl (Merck) from 0 to 5.5 M. Samples were thoroughly mixed by vortexing and incubated at
room temperature for 72 h prior to centrifugation in a Beckman
Optima TL ultracentrifuge at 135,000 × g for 45 min. The incu-
bation time was experimentally verified to be sufficient for the
samples to reach equilibrium, and the monomer concentration
in the supernatant was quantified as previously described (25).
Experiments were conducted in triplicate. Size exclusion chro-
matography of WT and H50Q fibrils after denaturation and
ultracentrifugation was performed using a Superdex 200 col-
umn on the ÄKTA Explorer apparatus (GE Healthcare). The
column was equilibrated and eluted at 0.5 ml/min with PBS
buffer, pH 7.4. WT and H50Q α-Syn at 0.5 mg/ml in PBS were
also run as control. The fraction of soluble monomeric α-Syn
over the total concentration was plotted with denaturant con-
centration for further analysis.

Determination of Thermodynamic Stability Parameters—
The equilibrium unfolding curves of α-Syn fibrils were ana-
yzed using a linear polymerization model (25, 33, 34) [Fel/0.5
+ [M] ↔ [F], in which [M] and [F] represent the concentra-
tion of monomers and fibrillar aggregates of size i, respectively, with
the equilibrium constant $K = c^i[F]/[F]_{el}[M]$, where $c^i$ is the
standard concentration 1 mol liter−1. Based on this model the
fraction of monomeric α-Syn over the total protein concentra-
tion, [M]/[Mₜ], can be expressed as follows.

$$\frac{[M]}{[Mₜ]} = \frac{[M]_i K + 1/2 - \sqrt{[M]_i K + 1/4}}{[M]_i K^2} \quad \text{(Eq. 5)}$$

The equilibrium constant $K$ can also be expressed as $K = \exp(-\Delta G_{el}/RT)$, in which $\Delta G_{el}$ is the free energy of elongation,
$R$ is the gas constant, and $T$ is the absolute temperature. In the
presence of chemical denaturants, i.e. guanidine HCl, $\Delta G_{el}$ is
linearly dependent on the concentration of denaturant, [D],
according to $\Delta G_{el} = m[D] + \Delta G_{el}^0$, where $m$ is a cooperativity
coefficient, and $\Delta G_{el}^0$ is the free energy of elongation in the
absence of denaturants (25). The experimental data of the equi-
librium unfolding of WT and H50Q α-Syn fibrils were fitted to
Equation 5 to obtain the main thermodynamic parameters using
 KaleidaGraph 4.0 (Synergy Software). Values of midpoint
denaturant concentration, [D]ₜ₅₀, and the critical concentra-
tion, cₜ₅₀ = $c^0 \exp(\Delta G_{el}/RT)$, were also calculated. All measure-
ments are reported as means ± S.D. of three independent
experiments.

Atomic Force Microscopy—After a 500-fold dilution, 10 μl of
 α-Syn fibrils were finally deposited on freshly cleaved mica and
dried under mild vacuum. Tapping mode AFM images were
acquired in air using a Dimension 3100 Scanning Probe Micro-
scope and a Multimode Scanning Probe Microscope (Digital
Instruments, Bruker). Single beam uncoated silicon cantilevers
(type OMCL-AC160TS; Olympus) were used. The drive fre-
frequency was between 290 and 310 kHz; the scan rate was
between 0.4 and 0.5 Hz. Fibril height was measured from the
cross-section height of topographic AFM images.

NMR Spectroscopy—NMR data were acquired at 283 K using
a 700 MHz Bruker Avance III NMR spectrometer equipped
with a TXI cryoprobe. Uniformly 15N/13C-labeled samples of
WT and H50Q α-Syn were prepared as previously described
(35), at concentrations of 500 and 700 μM, respectively, in 10
mM sodium phosphate buffer, 100 mM NaCl, pH 7.5, 5% D₂O,
0.01% NaN₃, 0.001% dimethyl-silapentane-sulfonate. 1H,15N
heteronuclear single quantum coherence spectroscopy, BEST-
HNCO, BEST-iHNCO, BEST-HNCOCACB, BEST-HNCACB,
HA(CO)NH, and HNHA experiments were recorded to assign
the backbone HN, N, C, CA, CB, and HA chemical shifts of both
WT and H50Q α-Syn (36, 37). All spectra were processed using
nmrPipe (38) and Collaborative Computing Project for NMR
analysis (39) and were referenced using the internal dimethyl-
silapentane-sulfonate chemical shift (40). The measured chemi-
cal shift values of WT and H50Q α-Syn have been deposited in
the Biological Magnetic Resonance Data Bank (41). Secondary
structure populations were calculated from chemical shifts
using the δ²D web server (version 1.2) (24).

RESULTS

Prediction of the Aggregation Propensity—We studied the
aggregation propensity of H50Q α-Syn using the Zyggregator
method (23, 42). The effects of the His to Gln mutation on the
conformational fluctuations of α-Syn were considered via the
CamP algorithm (30), which provides a prediction of transient
structure formation and solvent accessibility in different
regions of the protein. By combining solvent exposure contri-
butions and aggregation profiles (23), we found that the overall
aggregation propensity of the H50Q variant is increased by
~5% compared with WT α-Syn, from 0.84 to 0.88 (Fig. 1). More
specifically, we observe an increase in aggregation propensity
for residues 35–47 (as the area below the curve increases from
12 to 15) and residues 61–96 (as the area below the curve
increases from 33 to 34). Importantly, glutamine 50 is predicted
to allow exposure of the amino acid stretches around this posi-
tion, which increases the ability to establish long range interac-
tions and promote aggregation. This analysis is in agreement
with predictions of residue burial (43) and accessibility (44)
calculated by independent methods.

Kinetic Analysis of the Aggregation Process—Both WT and
H50Q α-Syn aggregation time courses were measured under
physiological conditions and constant double orbital agitation
over the concentration range of 5–100 μM. Analysis of normal-
ized ThT fluorescence data (Fig. 2) shows that both proteins
form amyloid fibrils following a concentration-independent lag
phase (Fig. 2B), which is consistently shorter for the H50Q var-
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Apparent growth rates ($k_{app}$) were quantified from the same ThT data (Fig. 2A) and confirm that H50Q α-Syn aggregates faster than WT (20) with the highest differences at 70 and 100 µM where H50Q α-Syn $k_{app}$ exhibits a rate of $-0.6$ h$^{-1}$ compared with 0.1 h$^{-1}$ for WT α-Syn.

Because patients carrying the H50Q mutation are expected to express both WT and variant protein, we investigated further the aggregation kinetics of a mixture of the two species. Different proportions of WT and H50Q α-Syn were prepared, keeping constant the total concentration of α-Syn at 70 µM (Fig. 3). A progressive prolongation of the lag phase is directly dependent on the relative proportion of the WT over the variant (Fig. 3B), and on the contrary, the WT aggregates faster by increasing the concentration of the variant (Fig. 3C).

Thermodynamic Analysis of α-Syn Fibrils—Although when examined under the electron microscope, our fibrillar aggregates do not exhibit major differences (Fig. 3D), we found clear evidence that H50Q α-Syn fibrils are more resistant to denaturation than WT. We titrated WT and H50Q α-Syn fibrils with guanidine HCl and analyzed the material in solution after 72 h of incubation at each concentration of denaturant. Size exclusion chromatography showed a single peak eluting from the column at the same retention time as the native monomer (Fig. 4A). The fractions of the quantified soluble monomer over the total protein concentration were fitted with the linear polymerization model, as described under "Experimental Procedures." These results clearly show that the fibrils formed by WT α-Syn are significantly less stable than those formed by H50Q α-Syn (Fig. 4B), with a midpoint concentration of guanidine $Gdn$-HCl of approximately 2.5 M.

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![Figure 2. Kinetics of fibrillogenesis.](image)

**A** normalized fluorescence data of WT (dashed lines) and H50Q (solid lines) α-Syn at six different concentrations: 100 µM (gray), 70 µM (purple), 50 µM (red), 30 µM (green), 10 µM (blue), and 5 µM (black), respectively. **B** effect of initial protein concentration on the duration of the lag phase in the aggregation of WT (open bars) and H50Q α-Syn (solid bars), respectively. **C** dependence of $k_{app}$, apparent growth rate of fibrils, over initial monomer concentration for WT and H50Q α-Syn. The bars represent means ± S.D. of four independent experiments.

![Figure 3. Mutual effect of WT and H50Q α-Syn on the aggregation kinetics.](image)

**A** normalized data of mixtures of WT/H50Q α-Syn at 1:5 (orange), 1:2 (green), 1:1 (red), 2:1 (blue), and 5:1 (black) molar ratios, respectively. Curves at 70 µM of WT (gray) and H50Q (purple) were also included. **B** effect of increasing concentration of H50Q (or WT) α-Syn on the lag time of aggregation. **C** effect of increasing concentration of H50Q (or WT) α-Syn on the $k_{app}$ of aggregation. Means ± S.D. of at least three independent experiments are shown. **D** microscopic analysis of in vitro fibrils of WT and H50Q α-Syn. Scale bars, 100 nm.

![Figure 4. Thermodynamic stability of in vitro fibrils formed by WT and H50Q α-Syn.](image)

**A** size exclusion profile of ultracentrifuged samples of WT and H50Q α-Syn fibrils after denaturation with guanidine HCl. Representative curves at 5, 2.5, and 1.25 M guanidine HCl, respectively, show a single peak eluting at the same retention time as the native monomer (either WT or H50Q α-Syn) in PBS. Both the two isoforms show the same pattern when applied to a Superdex 200 column equilibrated and eluted with PBS at 0.5 ml/min. mAU, milli absorbance units. **B** the proportion of monomer released from WT (circles) and H50Q (squares) α-Syn fibrils over the total protein concentration at increasing guanidine HCl (Gdn-HCl) concentrations was analyzed with Equation 1 following the linear polymerization model as described under “Experimental Procedures.” Curves shown as mean (S.D.) of three independent experiments.
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HCl decreasing from 2.4 ± 0.1 M for the variant to 1.2 ± 0.1 M for WT and a difference in the free energy of elongation in the absence of denaturant, \( \Delta \Delta G_{el}^0 = \Delta G_{el}^0_{H50Q} - \Delta G_{el}^0_{WT} \), of \(-5.0 \pm 1.0 \text{ kJ mol}^{-1}\) (Table 1). This difference corresponds to a significant reduction in the critical concentration, i.e. in the solubility, from 4.1 ± 0.1 \( \mu \text{m}\) for WT \( \alpha\)-Syn to 0.6 ± 0.2 \( \mu \text{m}\) for the H50Q variant.

Accurate AFM measurements reveal that WT and H50Q fibrils do in fact exhibit different structural details (Fig. 5). Although in both cases fibrils are short and straight, with typi-

| TABLE 1 |
| --- |
| Thermodynamic parameters of guanidine HCl induced unfolding of α-synuclein fibrils |
| All of the values in the table are means ± S.D. of three independent experiments. The values are as follows: \( [D]_{208} \) (\( M \)), midpoint concentration of guanidine HCl; \( \Delta G_{el}^0 (\text{kJ mol}^{-1}) \), free energy of association in absence of denaturant; \( m (\text{kJ mol}^{-1} \text{ M}^{-1}) \), dependence of \( \Delta G_{el}^0 \) on denaturant concentration; \( c_s \), critical concentration (\( \mu \text{M}\)). |
| α-Synuclein | \( [D]_{208} \) | \( \Delta G_{el}^0 \) | \( m \) | \( c_s \) |
| WT | 1.2 ± 0.1 | 30.8 ± 0.1 | 5.6 ± 0.5 | 4.1 ± 0.1 |
| H50Q | 2.4 ± 0.1 | 35.4 ± 0.9 | 4.5 ± 0.3 | 0.6 ± 0.2 |

DISCUSSION

The aggregation kinetics data discussed above are consistent with previous reports that the H50Q mutation in the \( \alpha\)-Syn sequence is associated with an increased aggregation propensity (18–21). This mutation was independently reported in an apparently sporadic British case and in a familial Canadian case with British ancestry, with evidence of a founder effect (16, 17).

Oligomerization and aggregation of \( \alpha\)-Syn have long been considered to be crucial in the pathogenesis of Parkinson dis-

FIGURE 5. AFM analysis of in vitro fibrils formed by WT and H50Q α-Syn. A–C, surface plots of topographic AFM images of H50Q α-Syn fibrils. D–F, WT α-Syn fibrils.
ease, although the precise toxic species remains a matter of debate (45). Previously reported missense mutations have accordingly generally been shown to enhance this process. However, the picture is far from clear, and the mechanism by which mutations modulate aggregation kinetics remains unsolved. In this study, we have shown that the WT protein, also expected to be expressed in all heterozygous patients, can modulate the aggregation kinetics of the mixture of WT and H50Q/H9251-Syn. These findings suggest that availability of data on the intracellular molar ratio between variant and WT protein in these patients would be crucial to elucidate the natural history of the disease and interpret some unexplained heterogeneous clinical features.

In patients with the A53T mutation, there is evidence that the expression levels of the WT and mutant alleles may not always be equal (46, 47). It is unknown whether the H50Q mutation is expressed at the same levels as WT/H9251-Syn in vivo. Interestingly, H50Q falls into a region that has been proposed to regulate α-Syn expression through a negative feedback loop between the protein and its own mRNA (48). H50Q is predicted to reduce the regulatory potential of α-Syn, which suggests an increase in protein expression and an enhanced tendency to aggregate.

Although the effect of the mutation on the aggregation kinetics is generally observed (18–21), the structural basis of this pathogenic property has hitherto not been fully explained because of the intrinsic difficulty of singling out abnormally structured species in an ensemble of different conformers. A comparison of WT, H50Q, and H50R mutations indicated that a positive charge at residue 50 suppresses aggregation; however, the aggregation of H50A and H50D variants was slower than H50Q, indicating that aggregation behavior is not solely determined by electrostatic effects (19). A powerful technique to investigate the conformation of α-Syn monomers in solution is NMR spectroscopy, and at least three studies of the H50Q variant have recently been reported (18–20). The extent and

![NMR characterization of residual structure in monomeric H50Q](image)
magnitude of amide chemical shift perturbations (Δδ_{\text{NH}} up to 0.15) that we observe here around the site of the mutation (Fig. 7B) are very similar to these other reports. However, in one case very large chemical shift perturbations (Δδ_{\text{NH}} ~ 0.5) were also observed for residues Asp^{135}, Tyr^{136}, and Glu^{137} in the C terminus of the protein (19). We have not observed evidence of these chemical shift changes in any of our experiments, despite identical experimental conditions (10 mM sodium phosphate, 100 mM NaCl, pH 7.4, 283 K). Khalaf et al. (20) suggested that the C-terminal chemical shift perturbations observed by Chi et al. (19) may arise from metal ion contamination; we also note that multiple cross-peaks can be observed for some C-terminal resonances (e.g. Ala^{140}) in the spectra of Chi et al. (19), raising the additional possibility of degradation or other covalent modification. We have not observed such additional resonances in any of our own spectra.

Ghosh et al. (18) also reported small chemical shift perturbations in the C-terminal region between residues 113 and 135, at pH 6 in the absence of salt. As noted by Khalaf et al. (20), chemical shifts in this region are highly sensitive to the electrostatic environment and the ionic strength. NMR titration experiments have shown that His^{50} has a pK_a of 6.5–6.8 (dependent on ionic strength) (49). Therefore, at pH 6, the H50Q mutation eliminates a positive charge, whereas at pH 6.8 (20) or physiological pH 7.4, as in our study, the charge on His^{50} is reduced and perturbations in the C terminus arising from the H50Q mutation are also diminished. Khalaf et al. (20) investigated the effect of the H50Q mutation on paramagnetic relaxation enhancement effects arising from a nitroxide spin-label positioned at residue 20, using an engineered E20C variant. PREs provide a sensitive probe of transient long range tertiary structure in the disordered state and previously uncovered a weak interaction between the C-terminal region of α-Syn with the N-terminal and the 61–95 non-amyloid-β component regions (50, 51). However, the H50Q variant was not observed to perturb these long range PREs, indicating that the residual tertiary structure of the variant is indistinguishable from that of the WT (20). Khalaf et al. (20) also conducted a preliminary investigation of the residual secondary structure of WT and H50Q α-Syn by comparison of Ca secondary chemical shifts in a solution of bacterial lysate and, as measured in detail in the present study, no significant differences were detected.

NMR chemical shift analysis represents an effective and sensitive tool for understanding residual secondary structure in disordered proteins, with the 8D method having an accuracy of ~2% for the detection of changes in secondary structure populations of disordered proteins (24). We have previously applied this method (24) to characterize the secondary structure populations of α-Syn expressed within living cells, using in-cell NMR spectroscopy (52, 53), and small decreases in β and polyproline II populations (<5%) were observed across the sequence, but otherwise the protein was found to have the same disordered structure as in dilute aqueous solutions. The comprehensive analysis of secondary structure populations in WT and H50Q α-Syn that we have presented here shows only a small increase (<7%) in the polyproline II population, specific to the site of the mutation (Fig. 6D). By comparison, a similar study of the A53T variant observed a 10% increase in the population of β-structure around the site of the mutation (54). An analysis of perturbations to secondary structure populations measured systematically for a series of α-Syn variants (55) highlighted that the population of β-structure correlated strongly (r = 0.93) to the aggregation propensity (54). However, much weaker correlations were observed with the formation of α-helical (r = 0.24) or polyproline II (r = 0.19) structure. Given that only eight variants were analyzed (55), the specific values of these coefficients of correlation should be considered with caution, and further work will be needed to firmly establish the association between the changes in the α-helical or polyproline II populations and the changes in aggregation rate.

In this context, it is notable that both β and polyproline II secondary structures are suitable for the formation of intermolecular hydrogen bonds and could thus promote aggregation. These structured elements, if exposed, may be able to promote...
In conclusion, we have presented an analysis of the modulation of the aggregation process of the H50Q variant α-Syn by the WT form and of the features in their sequence and structure that are associated with their different aggregation behavior. Our results show that the changes in the ratio between the concentrations of the WT and H50Q α-Syn, in the secondary structure populations of the monomeric ensemble, and in the thermodynamic stability of the fibrils, are closely associated with an alteration of the aggregation behavior of this protein.

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more stable initial interactions than those established by coil regions, leading to increased aggregation potential (Fig. 7). Incorporating experimental measurements of β and polyproline II populations into calculations of aggregation propensity may in the future be used to improve the accuracy of these predictions. Such calculations are illustrated in Fig. 7, using secondary structure populations determined by NMR (Fig. 6D) together with a scoring function with a form similar to that used to correct for solvent exposure (Equation 4).

In addition to the perturbations to secondary structure in the monomeric ensembles of WT and H50Q α-Syn, we have also identified a significant increase in the thermodynamic stability of H50Q α-Syn fibrils compared with the WT species, ΔΔGel = −5.0 ± 1.0 kJ mol⁻¹ (Fig. 7). This finding might be consistent with some morphological differences revealed by EM (18) and our AFM analyses, but the difficulty in determining the structure of amyloid fibrils precludes a detailed structural interpretation of these differences. However, structural studies of amyloid fibrils are rapidly improving in resolution and scale (56), and several solid state NMR models of the α-Syn fibril core indicate that His50 is close to the edge of a β-strand or in an adjacent loop region (57–59). The increased stability of H50Q fibrils may therefore arise from a stabilization or extension of this β-strand within the fibril core, similar to recent observations of the core structure of Aβ3T α-Syn fibrils (60). Alternatively, however, the variant may favor the formation of a fibril polymorph with more extensive rearrangements in the core structure, as observed for fibrils of the E46K variant (60).

It is also possible that the increased thermodynamic stability of H50Q α-Syn fibrils could contribute toward its increased rate of aggregation. The macroscopic aggregation rate, kapp (Fig. 2), is dependent on the microscopic rate of fibril elongation (61), which in turn depends on the free energy of the transition state, as depicted in Fig. 7. If the H50Q variant stabilizes this state (for example, if residue 50 is partially structured in the transition state), then this will also contribute toward the increased rate of aggregation, alongside the perturbations to the monomeric ensemble discussed above. Thus, it is important in the analysis of aggregation kinetics to consider both the fibrillar “products” of the reaction, as well as the monomeric “reactants.”

Finally, and importantly, the increased stability of fibrils formed from the H50Q variant corresponds to an order of magnitude reduction in the critical concentration, from 4.1 ± 0.1 μM for WT to 0.6 ± 0.1 μM for the variant. It is worth noting that the concentration of α-Syn in synaptosomes is ~20 μM (62). It has been previously noted that the expression levels of proteins are finely tuned, together with their critical concentrations and aggregation rates, to avoid aggregation under normal physiological conditions (25–27). Indeed, many proteins, including α-Syn, are present in vivo at concentrations that exceed their critical levels and therefore exist in a metastable, or supersaturated, state in which only large kinetic barriers prevent their aggregation (25–27). In this context, the lowering of the critical concentration for the H50Q variant, particularly together with the acceleration of its aggregation, may tip this delicate balance toward aggregation and ultimately the onset of disease.
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