



COMMUNICATION

Conserved C-Terminal Charge Exerts a Profound Influence on the Aggregation Rate of α -Synuclein

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α -Synuclein (α -syn) is the major component of filamentous Lewy bodies found in the brains of patients diagnosed with Parkinson's disease (PD). Recent studies demonstrate that, in addition to the wild-type sequence, α -syn is found in several modified forms, including truncated and phosphorylated species. Although the mechanism by which the neuronal loss in PD occurs is unknown, aggregation and fibril formation of α -syn are considered to be key pathological features. In this study, we analyze the rates of fibril formation and the monomer–fibril equilibrium for eight disease-associated truncated and phosphorylated α -syn variants. Comparison of the relative rates of aggregation reveals a strong monotonic relationship between the C-terminal charge of α -syn and the lag time prior to the observation of fibril formation, with truncated species exhibiting the fastest aggregation rates. Moreover, we find that a decrease in C-terminal charge shifts the equilibrium to favor the fibrillar species. An analysis of these findings in the context of linear growth theories suggests that the loss of the charge-mediated stabilization of the soluble state is responsible for the enhanced aggregation rate and increased extent of fibril fraction. Therefore, C-terminal charge is kinetically and thermodynamically protective against α -syn polymerization and may provide a target for the treatment of PD.

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Parkinson's disease (PD) is a neurodegenerative disorder associated with the loss of dopaminergic neurons in the substantia nigra of the brain. A primary pathological feature of PD is the presence of Lewy bodies (LBs), which are cellular inclusions composed largely of aggregates of the protein α -synuclein (α -syn)¹ in the form of amyloid fibrils.² In addition to wild-type α -syn, LBs also contain

α -syn variants, including phosphorylated, ubiquitinated, acylated, and truncated forms of the protein.^{3,4} It has been shown, for example, that 15% of α -syn found in LBs is C-terminally truncated and 90% is phosphorylated.^{5,6} Although these variants exhibit significant differences in their tendencies to aggregate and disrupt cellular processes, the kinases and proteases responsible for phosphorylation and truncation, respectively, have not been clearly identified.⁷

The 140-amino-acid α -syn protein (Table 1) is natively unfolded in solution, but its N-terminus (residues 1–100) adopts an α -helical conformation upon interaction with membranes.^{8–10} The central

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Abbreviations used: PD, Parkinson's disease; LB, Lewy body; α -syn, α -synuclein.

region (residues 30–95), including the hydrophobic NAC sequence,¹¹ is involved in the formation of cross β -sheet structure in LB amyloid fibrils.¹² The net charge of the full-length α -syn is -9 , arising largely from the high density of acidic amino acids in the disordered C-terminal region (residues 96–140; Table 1). Previous work suggests that this highly charged C-terminal domain exerts an influence on the aggregation kinetics of α -syn.^{13,14} With regard to phosphorylation, which also alters polypeptide charge, there is considerable interest in the variant modified at Ser129, an abundant species in PD lesions.¹⁵ In this study, we report experiments involving an array of the natural α -syn variants found in LBs, including the dominant truncated and phosphorylated species. We find qualitatively, in accord with previous studies,^{13,14} that C-terminal charge and phosphorylation influence the kinetics of aggregation. By combining quantitative data from all species, however, we uncover a remarkable monotonic relationship between the α -syn charge and the lag time (τ_{lag}) associated with polymerization (Fig. 1).

The following α -syn species were used in the study: full-length wild-type α -syn (WT); α -syns with C-terminal truncation at residues 135 (D135), 133 (Y133), 122 (N122), and 119 (D119); Ser129-phosphorylated full-length α -syn (P-syn); and Ser129-phosphorylated truncates (P-Y133 and P-D135) (Table 1). The kinetics of fibril formation were monitored by thioflavin T fluorescence (Supplemental Information). All the experiments were conducted at pH 7.4 in 20 mM phosphate and 100 mM NaCl; lag times prior to the observation of fibril growth were extracted from an average of four trials. By comparing lag times of different modifications, we observe two main features. First, the phosphorylated α -syns, including both full-length and truncated species, form fibrils more slowly than their non-phosphorylated counterparts; these data support a previous finding that phosphorylation of α -syn at Ser129 inhibits fibril formation.¹⁶ Second, the truncated species form fibrils significantly faster than the full-length wild-type α -syn, with shorter sequences resulting in shorter lag times.

In order to explore a more global relationship among these factors, we evaluated lag times as a function of charge to compare the relative rates among all modified α -syns (Fig. 1a). The C-terminal charge on residues 91–140 for each modified α -syn (Table 1) was calculated using full-length wild-type α -syn (charge = -12) as a reference state. Larger truncations remove more of the acidic amino acids, resulting in a lower negative charge. Phosphorylation, on the other hand, increases the negative charge on the protein, as two of the three oxygens on the phosphate group are deprotonated at pH 7.4, thus contributing an additional charge of -2 to the C-terminus.

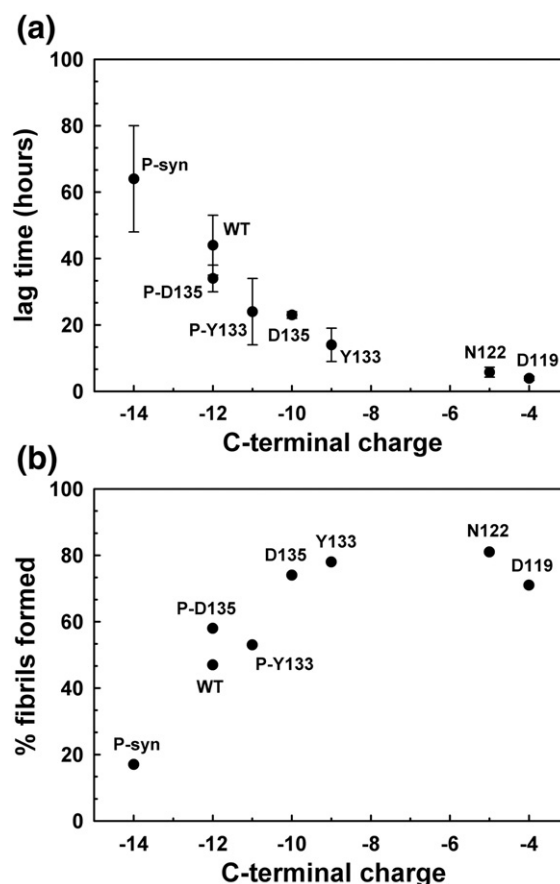


Fig. 1. Dependence of (a) lag time and (b) extent of aggregation on the C-terminal negative charge (residues 91–140; 1) of α -syn variants. Experimental details are described in the text and in Supplemental Information.

Figure 1a shows that the observed lag times exhibit a monotonic dependence on α -syn C-terminal charge, with more negative species aggregating more slowly.¹⁷ The lag times range over more than an order of magnitude, clearly emphasizing the importance of the C-terminal charge of α -syn in regulating the rate of aggregation. Phosphorylation modifies charge, without affecting polypeptide length, demonstrating that length alone cannot account for the relationship seen in Fig. 1a.

In addition to examining the lag times prior to fibril formation, we also quantified the fraction of fibrillar material at the end of the aggregation reaction (Supplemental Information). For each α -syn variant, fibrillar and non-fibrillar species were separated by centrifugation into insoluble and soluble fractions, respectively. The α -syn concentration in the soluble fraction was determined by UV absorbance at 280 nm. In addition, each sample was evaluated by dynamic light scattering, which revealed a dominant species ($>90\%$) with a radius of gyration consistent with that of a monomeric α -syn (2.1–2.9 nm). Subtraction of the monomer

Rat	K	K	D	Q	M	G	K	G	E	E	G	Y	P	Q	E	G	I	L	E	D	M	P	V	D	P	S	S	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Mouse	K	K	D	Q	M	G	K	G	E	E	G	Y	P	Q	E	G	I	L	E	D	M	P	V	D	P	G	S	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Human	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Chimpanzee	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Sumatran orangutan	K	K	D	Q	L	G	K	N	E	E	G	A	T	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Red guenon	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	Q	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Crab-eating macaque	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	Q	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Bonobo	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Rhesus macaque	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	Q	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Lowland gorilla	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Pig	K	K	D	Q	L	G	K	N	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Cow	K	K	D	H	M	G	K	G	E	E	G	A	S	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Black-handed spider monkey	K	K	D	H	S	G	K	S	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Common woolly monkey	K	K	D	H	S	G	K	S	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A
Red-chested mustached tamarin	R	K	D	H	L	G	K	S	E	E	G	A	P	Q	E	G	I	L	E	D	M	P	V	D	P	D	N	E	A	Y	E	M	P	S	E	E	G	Y	Q	D	Y	E	P	E	A

Fig. 2. Alignment of the α -syn C-terminal region (residues 96–140) showing conservation of the negatively charged residues (red columns). Data were obtained from UniProtKB.

concentration from total peptide concentration at the beginning of a trial gave the percentage of fibril. As a final check, UV absorbance measurements performed directly on resuspended fibrils agreed with the more rigorous assay based on monomer to within 10%.¹⁸ Our results (shown in Fig. 1b) demonstrate that the equilibrium between dissociated and aggregated proteins is strongly influenced by C-terminal charge in a systematic manner, with more negative species possessing an increased tendency to remain in their soluble states.

The physiological significance of negative charge in the C-terminal region was further investigated with a sequence alignment of α -syn orthologs from 15 different mammals. The results show an almost perfect conservation of acidic residues and a complete conservation of Ser129 in the C-terminal region (Fig. 2) and suggest that α -syms have evolved to adopt a state that is relatively resistant to aggregation and that reduction of this protective feature in α -syn truncates could play a role in PD pathology.

Posttranslational modifications are implicated in many proteins associated with neurodegenerative disorders. For example, N-terminal truncations of the amyloid β peptide found in Alzheimer's plaques enhance aggregation and neurotoxicity;^{19,20} the hydrophilic N-terminal domain of amyloid β flanks the β -core region and is thought to promote fibril formation by aiding protofilament packing into the fibril structure.¹⁹ Along with our studies, it appears that domains adjacent to an amyloidogenic core can remotely regulate aggregation and, thus, might play a key role in modulating the development of neurodegenerative disorders.

Amyloid formation is characterized by several microscopic rate constants describing fibril elongation (k_+), fibril depolymerization (k_{off}), and the dominant

nucleation mechanism (k_N), which can take the form of primary nucleation,²¹ monomer-dependent secondary nucleation,²² or fragmentation.²³ All of these processes can contribute to the timescale of the observed lag phase. For instance, it is clear that an increase in the monomer addition rate k_+ reduces the time taken for a threshold amount of monomer to be incorporated into aggregates. In order to determine which microscopic process is primarily affected by the change in C-terminal charge, we considered quantitatively the effect on the lag time and the fibril fraction (χ) after the reaction has come to completion following the lag phase, when we vary each of the rate constants individually, while holding the other rate constants fixed. Based on a previous analysis,²⁴

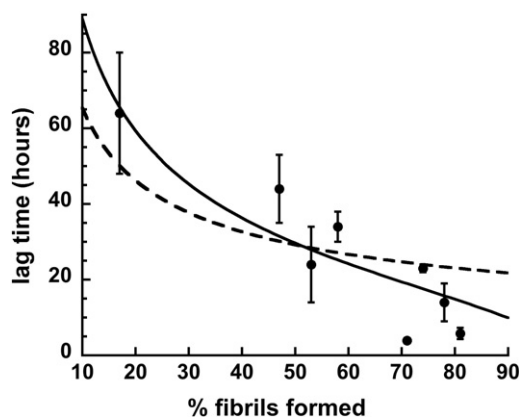


Fig. 3. Relationship between lag time and degree of aggregation at the end of a kinetic trial for truncated and phosphorylated α -syn variants. Best fits to the filament assembly model are shown for variation in the fibril dissociation rate (broken line) and in the monomer addition rate (continuous line). Full details and additional mechanisms are provided in Supplemental Information.

Table 1. α -Syn sequences

WT ^{a,b}	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEP
P-syn ^c	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQDYEP
D135	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQD
P-D135 ^c	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQD
Y133	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGY
P-Y133 ^c	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGY
N122	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGY
D119	α -syn(1-90)-ATGFVKKDQLGKNEEGAPQEGILEDMPVD

^aSequence details are shown for residues 91–140.^bAcidic residues are in red.^cPhosphorylated Ser is in blue.

we note first that, if the dominant nucleation rate k_N is increased while holding all of the other rates constant, the lag time would decrease, while the final fibril fraction would remain approximately constant (see Supplemental Information), which is inconsistent with the significant changes in fibril fraction observed in our data (Fig. 3). However, an increase in either the rate of monomer addition to fibrils (elongation) (k_+) or the rate of monomer dissociation from fibrils (depolymerization) (k_{off}) would result in the observed trend (Fig. 3) of a concurrent increase in fibril fraction χ and decrease in lag time τ_{lag} , but with different functional forms (see Supplemental Information):

$$\tau_{lag} \propto \sqrt{\frac{1}{\chi}} \quad k_{off} \text{ varied; } k_N, k_+ \text{ constant} \quad (1)$$

$$\tau_{lag} \propto \sqrt{\frac{1-\chi}{\chi}} \quad k_+ \text{ varied; } k_N, k_{off} \text{ constant} \quad (2)$$

We systematically compared the predictions of Eqs. (1) and (2) to the experimental data (Fig. 3). While both Eqs. (1) and (2) give a decrease in lag time with an increase in fibril fraction, the case where k_{off} is affected results in a dependence that is too weak to capture the trend in our data. In contrast, variation of the fibril elongation rate k_+ is able to capture quantitatively the significant decrease in lag time with fibril fraction observed in our measurements. From a qualitative perspective, the different dependencies of Eqs. (1) and (2) originate from the fact that both the depolymerization rate k_{off} and the elongation rate k_+ affect the fibril fraction with an equal weighting, whereas the net fibril growth, as reflected in reduced τ_{lag} , is controlled primarily through the elongation rate.

Previous studies suggest that both electrostatic and hydrophobic long-range interactions, between the acidic C-terminal domain of α -syn and the remainder of the protein, tend to inhibit aggregation.²⁵ Spin-label NMR experiments using paramagnetic relaxation enhancement to extract long-range distances identify contacts between the hydrophobic NAC region and

the acidic C-terminus.^{26,27} As proposed in these studies, hydrophobic segments in the C-terminal tail may interact with the NAC region, while the high density of negative charges serves to maintain solubility. In this scenario, both charge conservation and separation between the charges, maintained by specific hydrophobic segments, are important and explain the near-perfect sequence conservation of this partially ordered domain (Fig. 2). Within the hydrophobic segments are several conserved proline residues that slow aggregation, as demonstrated by recent Pro \rightarrow Ala mutagenesis studies.²⁸ However, the most significant decrease in lag time observed for these mutants relative to wild type is approximately 2-fold and significantly less than the 10-fold reduction in lag time observed for the truncated species here. By examining detailed kinetics and thermodynamic properties from a range of disease-associated truncated and phosphorylated species, our studies provide quantitative support for a protective role of the α -syn C-terminus and demonstrate unequivocally the contribution of electrostatics in this inhibitory mechanism. The α -syn C-terminus plays an essential role in monomer stabilization as a result of thermodynamic and kinetic factors, and loss of this protective mechanism through truncation may be a critical first step in the development of PD lesions.

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Supplementary Data

Supplementary data to this article can be found online at [doi:10.1016/j.jmb.2011.05.046](https://doi.org/10.1016/j.jmb.2011.05.046)

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