

ACCELERATED COMMUNICATION

N-terminal acetylation of α -synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer

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Abstract: The conformational properties of soluble α -synuclein, the primary protein found in patients with Parkinson's disease, are thought to play a key role in the structural transition to amyloid fibrils. In this work, we report that recombinant 100% N-terminal acetylated α -synuclein purified under mild physiological conditions presents as a primarily monomeric protein, and that the N-terminal acetyl group affects the transient secondary structure and fibril assembly rates of the protein. Residue-specific NMR chemical shift analysis indicates substantial increase in transient helical propensity in the first 9 N-terminal residues, as well as smaller long-range changes in residues 28–31, 43–46, and 50–66: regions in which the three familial mutations currently known to be causative of early onset disease are found. In addition, we show that the N-terminal acetylated protein forms fibrils that are morphologically similar to those formed from nonacetylated α -synuclein, but that their growth rates are slower. Our results highlight that N-terminal acetylation does not form significant numbers of dimers, tetramers, or higher molecular

Abbreviations: α syn, alpha synuclein; Ac- α syn, acetylated α syn; BOG, β -octyl glucoside; EM, fluorescence electron microscopy; ESI-IMS-MS, ion mobility spectrometry combined with ESI-MS; ESI-MS, noncovalent electrospray ionization mass spectrometry; IDP, intrinsically disordered protein; NatB, N-acetyltransferase B; SEC, analytical size-exclusion chromatography; ThT, Thioflavin T.

Additional Supporting Information may be found in the online version of this article.

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weight species, but does alter the conformational distributions of monomeric α -synuclein species in regions known to be important in metal binding, in association with membranes, and in regions known to affect fibril formation rates.

Keywords: α -synuclein; N-terminal acetylated α -synuclein; NMR; mass spectrometry; aggregation; Parkinson's disease; fluorescence; amyloid fibril

Introduction

Alpha synuclein (α syn) is a small primarily neuronal protein that is known to make a structural transition to amyloid fibrils in several neurodegenerative diseases. It is a major component of Lewy Bodies in patients with Parkinson's, a disease resulting from a loss of dopaminergic neurons.^{1,2} While a large body of evidence over many years has supported the characterization of α syn as an intrinsically disordered monomer, a recent study by Bartels *et al.* in which α syn was isolated from red blood cells, as well as neuronal and non-neuronal cell lines, reported that in its physiological form, α syn exists as a helical tetramer that is resistant to amyloid formation, and has a mass corresponding to the sole modification of the monomer by an acetyl group.³ Shortly thereafter, a GST recombinant α syn protein purified from the micellar reagent β -octyl glucoside (BOG), similarly showed the existence of a dynamic α syn tetramer.⁴ In response to these papers, Faivet *et al.* and an assemblage of groups went on to demonstrate that α syn isolated from rodent and human nervous system tissues and erythrocytes presents as an intrinsically disordered monomer. In this work, Fauvet *et al.* was the first to address the role of the acetyl group, referred to in the Bartel *et al.* paper, and showed the acetylated and nonacetylated proteins migrate similarly on nondenaturing gels.⁵ A follow-up report by Trexler *et al.* has indicated that recombinant acetylated α syn (Ac- α syn) is monomeric under physiological conditions, but that it may display a greater preference for helical structure and higher-order oligomerization states when purified in the presence of BOG.⁶

It has been demonstrated that soluble and insoluble fractions of brain tissues from patients suffering from Parkinson's and from dementia with Lewy bodies universally contain N-terminal Ac- α syn.^{7,8} While an uncommon modification to prokaryotic proteins, the N-termini of eukaryotic proteins are often processed at the initiating amino acid with the addition of an acetyl group by *N*-acetyltransferase complexes.⁹ The role of N-terminal acetylation, however, is poorly understood, but has been suggested to affect the thermal stability of proteins.^{10,11} Because N-terminal Ac- α syn is now believed to be the physiologically relevant species in the brain, it is critically important to characterize the conformational properties and fibrillation kinetics of this protein to under-

stand how acetylation impacts on the mechanism of fibril formation and disease.

Here we present the first direct experimental evidence that N-terminal acetylation affects the secondary structure propensities and kinetics of fibril assembly of Ac- α syn, relative to the nonacetylated protein. Using NMR, noncovalent electrospray ionization mass spectrometry (ESI-MS), ion mobility spectrometry combined with ESI-MS (ESI-IMS-MS), Thioflavin T (ThT) fluorescence, and electron microscopy (EM), we demonstrate that the 100% N-terminal acetylated recombinant α syn protein purified under mild physiological conditions presents primarily as a disordered monomer. Our results highlight that N-terminal acetylation impacts on secondary structure propensity in important functional regions including the N-terminal and His-50 metal binding regions¹² and the regions of the three familial mutations A30P, E46K, and A53T.^{13–15} The removal of the positive charge at the N-terminus arising from acetylation thus has short and long-range conformational effects that impact on the distribution of states sampled by the intrinsically disordered α syn monomer and on the rate of fibril assembly.

Results and Discussion

To determine the oligomeric status and the conformational properties of Ac- α syn and to compare this with α syn, Ac- α syn was generated from an *E. coli* coexpression system containing the yeast N-terminal acetyltransferase B(NatB)^{6,16} and purified using mild physiological purification conditions (herein described as "mild" purification) that avoid steps involving the application of heat or salting out (herein described as "harsh" conditions) (see Supporting Information for detailed protocols of "mild" and "harsh" purification). Taking into account recent suggestions that harsh purification steps such as boiling can destroy tetramer formation^{3,4} or that purification under nonphysiological conditions⁶ can promote higher-order oligomerization states of α syn, a mild physiological purification protocol that applies only homogenization and liquid chromatography was adopted for Ac- α syn in this work. ESI-MS confirms that purified Ac- α syn coexpressed with this eukaryotic modification system exists as 100% acetylated protein (observed mass 14502.5 Da, expected mass 14502.1 Da) [Fig. 1(A,B)].

Investigation of the existence of higher-order oligomeric states in the Ac- α syn was performed using

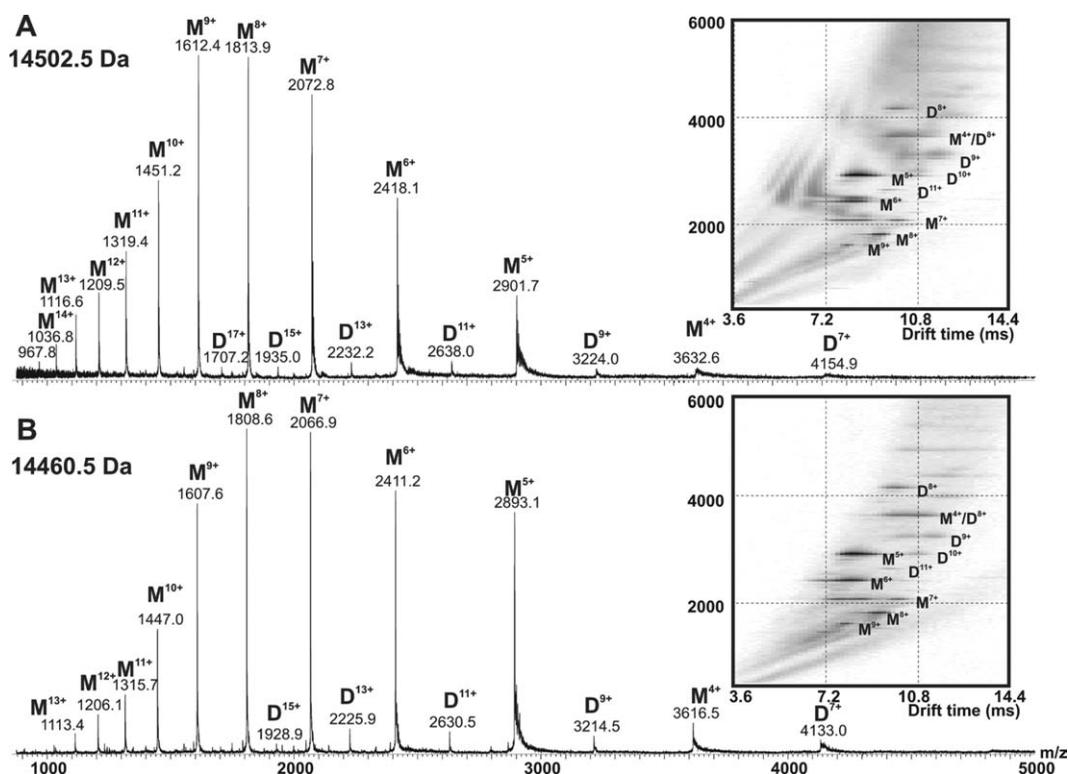


Figure 1. Native ESI-IMS-MS analysis of (A) Ac- α syn and (B) α syn, showing both samples to be predominantly monomeric. Ac- α syn or α syn (35 μ M) were dissolved or buffer exchanged into 165 mM ammonium acetate, pH 7.4. Inset shows the driftscope plot of each sample acquired under conditions optimized for the detection of large noncovalent species (cone voltage 170 V). ESI mass spectra of α syn purified under “harsh” and “mild” conditions indicate that the purification procedure does not affect the results obtained (not shown).

ESI-MS and ESI-IMS-MS (Fig. 1), along with solution methods including analytical size-exclusion chromatography (SEC) and migration on a native gel (Supporting Information Fig. S1). The latter two methods show that both proteins elute at the same volume as a single peak using SEC and migrate at the same position on the native gel. These data show that the acetylated protein exhibits indistinguishable hydrodynamic dimensions from the nonacetylated protein purified under mild conditions. Furthermore, comparison by native gel, SEC, ^1H - ^{15}N HSQC and ESI-MS (Supporting Information Fig. S2) of the nonacetylated α syn purified by harsh or mild purification protocols indicate that they are essentially indistinguishable, consistent with other comparisons made by Fauvet et al.⁵

ESI-IMS-MS experiments add further information about the oligomerization status of Ac- α syn, as the population distributions can be obtained quantitatively from these experiments by the ability of ESI-IMS-MS to separate peaks of identical m/z and to quantify their population and conformational properties.¹⁷ Comparison of the ESI-IMS-MS spectra of Ac- α syn and α syn indicate that acetylation does not perturb the oligomerization status of α syn, as both α syn and Ac- α syn appear predominantly monomeric (\sim 90–95%), with the presence of a small popu-

lation of dimeric species (\sim 5–10%), where the former appears in both more compact and extended forms at physiological pH [Fig. 1(A,B)]. These data are consistent with previous analyses using ESI-MS,^{18–20} and interchain NMR PRE experiments that have shown weak dimer N- to C-terminal interchain interactions in α syn under physiological conditions.²¹ Because of the suggestion in the recent literature that Ac- α syn purified under micellar BOG conditions can shift the monomer populations toward oligomeric species,⁶ the possibility of higher-order transient oligomeric species purified under physiological conditions was further probed using a higher cone voltage (170 V) in ESI-IMS-MS, which favors detection of large noncovalent aggregates. These experiments revealed no difference between the oligomeric distribution of acetylated and nonacetylated samples and no evidence for the population of higher-order species, consistent with results obtained by Trexler et al. when purification was performed under physiological conditions.⁶ While the biophysical techniques inform about the hydrodynamic radii, ESI-IMS-MS provides definite evidence that both proteins are predominantly monomeric in aqueous solution at pH 7.4.

Residue-specific analysis of Ac- α syn by NMR was next pursued to enable the secondary structure

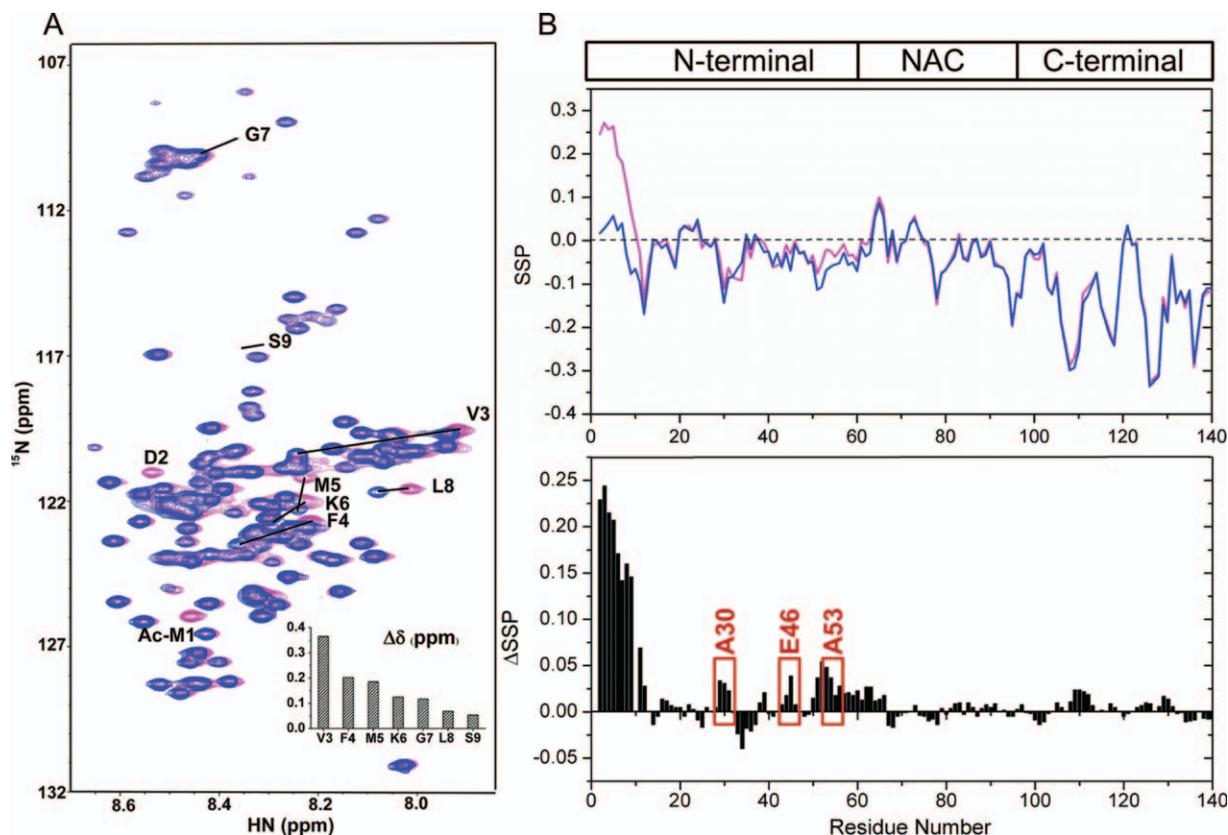


Figure 2. A. ^1H - ^{15}N HSQC spectra of Ac- α syn (magenta) versus α syn (blue) at 15°C . The changes in the first nine residues of Ac- α syn are indicated in the spectrum. The $\Delta\delta$ (ppm) for the inset is calculated by the expression $((\Delta H)^2 + (0.159 \times \Delta^{15}\text{N})^2)^{1/2}$ (inset). B. Top: Schematic of α syn divided into N-terminal, NAC and C-terminal regions. Middle: SSP analysis of Ac- α syn (magenta) and α syn (blue) using $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ chemical shifts as input and a five residue sliding window with Zhang et al. random coil references.²³ ^{13}C assignments for Ac- α syn were obtained from a $350 \mu\text{M}$ doubly labeled protein in PBS buffer at 15°C and ^{13}C assignments for α syn were used from a previous report.²⁴ Bottom: Differences of SSP ($\Delta\text{SSP} = \text{SSP}(\text{Ac-}\alpha\text{syn}) - \text{SSP}(\alpha\text{syn})$) between Ac- α syn and α syn with boxes shown at positions of familial mutations. ^1H - ^{15}N HSQC comparison of α syn purified under mild and harsh conditions indicate that they are very similar (Supporting Information Fig. S2).

propensities of Ac- α syn and α syn monomers to be compared (Fig. 2). Backbone assignments by triple resonance experiments confirmed that Ac- α syn is acetylated on the N-terminal residue Met-1 (Supporting Information Fig. S3). An overlay of the ^1H - ^{15}N spectrum of Ac- α syn and α syn at pH 7.4, shows that the two proteins share a high degree of similarity, except at the first nine N-terminal residues [Fig. 2(A)]. Both Ac- α syn and α syn display narrow chemical shift dispersion, characteristic of a predominantly unfolded protein, consistent with analyses using far UV CD (Supporting Information Fig. S4). Acetylation of the N-terminus results in the appearance of the Met-1 and Asp-2 resonances in the Ac- α syn ^1H - ^{15}N HSQC spectrum possibly because of changes in hydrogen exchange rates arising from the modification at Met-1. Additionally, acetylation results in up-field shifting of residues observable in both spectra in the region of the first nine N-terminal residues, [inset Fig. 2(A)] further supporting the site of acetylation as Met-1 and demonstrating the extent to which N-terminal acetylation alters the conformational properties of the polypeptide chain.

The secondary structure propensity of Ac- α syn was next examined by analyzing NMR chemical shift perturbations and was compared with that of α syn [Fig. 2(B)]. A number of methods have been developed to determine the secondary structural propensities of unfolded proteins.^{25–27} Here we use secondary structure propensity scores (SSP)²⁶ which represent ensemble-averaged values over a distribution of states to obtain the secondary structure propensities of both Ac- α syn and α syn. Paralleling the chemical shift deviations observed in ^1H - ^{15}N HSQC spectra [Fig. 2(A)], increased SSP values up to 0.3 are observed for the first 12 residues in the N-terminal region of Ac- α syn. These values are significantly larger than those observed previously for α syn and mutants of this protein^{24,28–30} and represent a significant stabilization of transient helix and hence a redistribution of the structural ensemble sampled by the monomeric protein within the N-terminal region [Fig. 2(B)]. Longer-range perturbations, although small, are observed, in other regions of the N-terminus in the regions between residues 28–31, 43–46, 50–66 and are marked by a decrease in β -sheet

propensity in Ac- α syn [Fig. 2(B)]. By contrast, the NAC and C-terminal regions [Fig. 2(B)] remain relatively unperturbed by acetylation. The change of secondary structure propensities arising from acetylation may relate to important structural and functional properties of the protein. Specifically, changes are observed at Met-1, Asp-2, and His-50, the high affinity copper binding regions,^{12,31–33} and at the three familial mutants A30P, E46K, and A53T associated with Parkinson's disease that affect the rate of fibril formation.^{34,35} Together, the results reveal that N-terminal acetylation of α syn does not by itself cause the intrinsically disordered protein (IDP) to self-assemble into tetrameric or other higher oligomeric forms, however, marked short range and subtle long-range effects of N-terminal acetylation are observed on the disordered monomer.

While N-terminal acetylation has been shown to increase helicity in peptides,³⁶ the data presented here represent the first investigation of acetylation at the N-terminus of a full-length IDP. The increased helicity in Ac- α syn can be rationalized by stabilization of the helix macrodipole,³⁷ where removal of the α -amino positive charge upon acetylation is favorable to the overall dipole moment of the helix that this IDP transiently samples. The acetyl group is also known to form a highly favorable helix N-cap, in which the acetyl-carbonyl group interacts favorably with unsatisfied hydrogen bond donors in the N-terminal turn of the helix.^{38,39}

The fibrillation properties of Ac- α syn and α syn were also examined using ThT fluorescence to provide macroscopic information about the role of N-terminal acetylation in modifying the efficiency of fibril nucleation and elongation. The normalized ThT fluorescence of Ac- α syn and α syn at pH 7.4 exhibit typical sigmoidal curves for fibril assembly (Supporting Information Fig. S5). The lag times of the samples were highly variable, thereby ruling out analysis of the role of N-terminal acetylation in fibril nucleation. The elongation rates are slower in the acetylated versus nonacetylated α syn proteins for all experimental trails. For both the lag times and elongation rates we note that the aggregation kinetics are sensitive to the purification methods [Fig. 3(A)]. The change in fibrillation rate may result from stabilization of the N-terminal region of the protein by acetylation, or changes in secondary structure propensities at residues 50–66, which have been shown previously to have significant effects upon the kinetics of fibril formation,^{24,28,40} or both. Physicochemical changes resulting from acetylation may affect electrostatic and hydrophobic interactions, resulting in alterations in transient long-range contacts between the highly charged C-terminal region and the helix stabilized N-terminal region. This redistribution of states may have effects upon fibril formation.

This work presents the first NMR structural characterization of N-terminal Ac- α syn and illus-

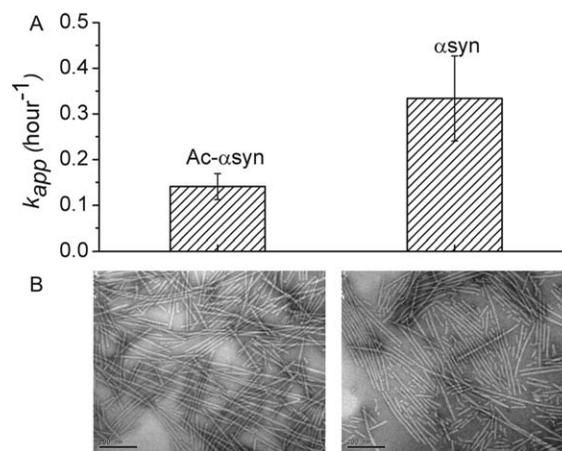


Figure 3. A. The apparent rate of fibril elongation of Ac- α syn and α syn performed at 37°C with 600 rpm shaking at a protein concentration of $\sim 150 \mu\text{M}$ measured using ThT fluorescence. Both proteins formed fibrils within 230 h. B. Negatively stained electron micrographs of the end products of fibril formation of Ac- α syn (left) and α syn (right). The scale bar is 200 nm.

trates the effect of N-terminal acetylation on the fibrillation rates of the protein. We demonstrate conclusively using ESI-IMS-MS that the equilibrium states that are sampled in the Ac- α syn are primarily monomer and a small population of dimer. NMR data support the view that both Ac- α syn and α syn exist at pH 7.4 as intrinsically disordered monomers, and further shows that N-terminal acetylation results in significant stabilization of transient helical propensity in the first 9 residues of the protein along with longer range changes in secondary structure between residues 50–66 and around the three familial mutants A30P, E46K, and A53T. These regions represent important functional regions associated with metal ion binding and with familial mutants that affect aggregation rates. We show that N-terminal acetylation not only changes the distribution of the intrinsically disordered monomeric conformers within the structural ensemble of α syn, but that this cotranslational modification disfavors fibril formation, presumably caused by the conformational redistribution of the monomeric protein.

Materials and Methods

Protein expression and purification

Expression of acetylated protein was achieved by coexpressing the α syn plasmid and the NatB plasmid in *E. coli* using a procedure similar to that described by Trexler et al.⁶ The NatB plasmid was kindly gifted by Dr. Daniel Mulvihill. Acetylated α syn was purified only under “mild” physiological conditions and nonacetylated α syn protein was purified under both “mild” physiological and “harsh” conditions for comparison (see Supporting Information

for detailed descriptions of Ac- α syn expression and “harsh” and “mild” purification protocols).

Biophysical characterization

NMR assignments, ThT fluorescence and TEM experiments were performed as described previously.²⁴ Native gel, SEC and CD procedures are described along with Supporting Information Figures S1 and S2, respectively. ESI-IMS-MS experiments were performed using similar procedures to Smith *et al.*¹⁷ α syn proteins (35 μ M) were dissolved or buffer exchanged into 165 mM ammonium acetate, pH 7.4 and the sampling cone voltage was varied from native conditions (30 V) to conditions that allow for the detection of large noncovalent species (170 V).

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