Published in final edited form as:

Biochem Biophys Res Commun. 2010 January 15; 391(3): 1415–1420. doi:10.1016/j.bbrc.2009.12.079.

Residue Glu83 Plays a Major Role in Negatively Regulating α -Synuclein Amyloid Formation

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Abstract

α -Synuclein (α -Syn) amyloid filaments are the major ultrastructural component of pathological inclusions that define several neurodegenerative disorders, including Parkinson disease and other disorders that are collectively termed synucleinopathies. Since the aggregation of α -syn is associated with the etiology of these diseases, defining the molecular elements that influence this process may have important therapeutic implications. The deletions of major portions of the hydrophobic region of α -syn ($\Delta 74$ -79 and $\Delta 71$ -82) impair the ability to form amyloid. However, mutating residue E83 to an A restored the ability of these proteins to form amyloid. Additionally supporting an inhibitory role of residue E83 on amyloid formation, mutating this residue to an A enhanced amyloid formation in the presence of small molecule inhibitors, such as dopamine and EGCG. Our data, therefore, suggest that the presence and placement of the highly charged E83 residue plays a significant inhibitory role in α -syn amyloid formation and these findings provide important insights in the planning of therapeutic agents that may be capable of preventing α -syn amyloid formation.

Keywords

α -synuclein; amyloid; Parkinson disease

INTRODUCTION

α -Syn is a small 140-amino acid long protein that is highly soluble, “natively unfolded,” and heat-stable [1]. It is predominantly expressed in central nervous system neurons, where it is localized in close proximity to vesicles within presynaptic terminals [2–4]. The α -syn protein is characterized by an amino-terminal region containing several imperfect KTKEGV repeats, a hydrophobic center domain and a highly negatively charged carboxy-terminus region [4] (see Fig. 1). Although the function of α -syn is still poorly understood, several studies suggest that it is involved in modulating synaptic transmission, the density of synaptic vesicles, neuronal plasticity, vesicle recycling and synaptic integrity [3–7].

α -Syn can polymerize into amyloidogenic fibrils that coalesce to form large intracellular inclusions that are present in a spectrum of neurodegenerative diseases, collectively termed

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synucleinopathies [4;8]. These inclusions, such as Lewy bodies and Lewy neurites, can be found in surviving neurons post-mortem and are one of the defining characteristics of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [4;8–10]. In addition, α -syn amyloidogenic inclusions can occur in oligodendrocytes, typically termed glial cytoplasmic inclusions, a hallmark of multiple system atrophy [11;12]. Disease causing mutations in the α -syn gene (*SNCA*) provide the initial and is the most direct evidence for a pathogenic role of α -syn [4;8;13]. Genetic studies have identified three autosomal dominant missense mutations (A30P, E46K and A53T) in α -syn that are causative of PD and/or DLB [13–15]. In addition, short chromosomal duplications or trisomies containing the *SNCA* gene, plus relatively short flanking regions on chromosome 4, can result in PD and DLB [16;17].

Although the amino- and carboxy-terminal regions of α -syn [18–25] can modulate the rate of polymerization, the middle hydrophobic regions plays a more critical role in amyloid formation [26–28]. For example, the deletion of several hydrophobic stretches in this region can completely prevent the polymerization into amyloid [26–30]. In addition, some modeling and biochemical studies have suggested “a possible role of E83 as a gatekeeper residue to reduce the aggregation propensity” [27;31]. To investigate the inhibitory properties of the E83 residues on the polymerization of α -syn into amyloid fibrils, this residue was mutated to an A in α -syn proteins that are impaired in amyloid formation due to the deletions of hydrophobic residues. In addition, the effect of the E83A mutation in promoting amyloid in the presence of small molecular inhibitors was studied. These studies support the role of the E83 residue in modulating α -syn polymerization.

MATERIALS AND METHODS

Expression and Purification of Recombinant α -Syn and Mutants Thereof

The bacterial expression vector pRK172 cloned with full-length wild-type (WT) or E83A human α -syn cDNA or full-length human α -syn cDNA with deletion of the nucleotide sequence coding for residues 71-82 or 74-79 were previously described [21;27]. The Δ 71-82 and Δ 74-79 α -syn pRK172 constructs with mutations at residue E83A were created with oligonucleotides corresponding to the amino acid substitutions by QuickChange site-directed mutagenesis. All mutations were confirmed by DNA sequencing. All α -syn recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified as previously described [21;27].

Filament Assembly and Centrifugal Sedimentation

α -Syn proteins (345 μ M) were assembled into filaments by incubation at 37°C in 100 mM sodium acetate, pH 7.4 with continuous shaking. For some experiments, incubation was conducted in the presence of equimolar concentration of dopamine, baicalein, or (–)-epigallocatechin gallate (EGCG; Sigma-Aldrich). A fraction of each sample was set aside for K114 and Thioflavin T (ThT) fluorometry and electron microscopic (EM) analysis. The remainder of each sample was centrifuged at 100,000 $\times g$ for 20 min. SDS-sample buffer (10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM DTT, 1% SDS, 10% sucrose) was added to pellets and supernatants, which were heated to 100°C for 15 min. Equal volumes of α -syn proteins in the supernatants and pellets were separated by SDS-PAGE and were quantified by densitometry of Coomassie Blue R-250 stained gels.

K114 and ThT Fluorometry

α -Syn fibrils are amyloidogenic [32] and their formation can be quantified using the fluorescent amyloid binding dye K114 or ThT [32;33]. K114 is derived from the structure of Congo Red and demonstrates a tremendous increase in fluorescence in solution assays upon binding to amyloidogenic fibrils [33]. This assay was conducted, as previously described [33], by incubating a fraction of each sample with the K114 (50 μ M) in 100 mM glycine, pH 8.6 and

measuring fluorescence ($\lambda_{\text{ex}}=380$ nm, $\lambda_{\text{em}}=550$ nm, cutoff = 530 nm) with a SpectraMax Gemini fluorometer and SoftMax Pro 4.0 software. ThT fluorometry was performed using ThT (20 μM) diluted into 100 mM glycine, pH 8.0 using the follow parameters: $\lambda_{\text{ex}}=450$ nm, $\lambda_{\text{em}}=482$ nm, cutoff = 475 nm.

Negative Staining EM

Assembled α -syn filaments were absorbed onto 300 mesh carbon coated copper grids, stained with 1% uranyl acetate and visualized with a JEOL 1010 transmission electron microscope (Peabody, MA). Images were captured with a Hamamatsu digital camera (Bridgewater, MA) using AMT software (Danvers, MA). For diameter determination, the width of 100 to 120 filaments was measured using Image-Pro Plus software (Media Cybernetics, Del Mar, CA).

RESULTS

Mutation of E83 to A promotes amyloid formation despite short deletion in the hydrophobic middle region of α -syn

The deletion of residues 74-79 ($\Delta 74-79$) or 71-82 ($\Delta 71-82$) in α -syn impairs the ability of α -syn to polymerize into amyloid fibrils [26–28]. Instead of polymerizing, these proteins form spherical oligomers. However, α -syn with the deletion of residues 73-83 is still capable of polymerizing into amyloid fibrils [27;28]. To investigate the role of E83 on α -syn amyloid formation, this residue was mutated to an A in the α -syn protein concurrent with the deletion of residues 74-79 ($\Delta 74-79/\text{E83A}$) or 71-82 ($\Delta 71-82/\text{E83A}$). The polymerization and amyloid formation of these proteins were analyzed using 4 readouts: 1) quantitative sedimentation analysis, 2) ThT fluorometry, 3) K114 fluorometry, and 4) EM analysis. Sedimentation analyses showed that proteins containing the E83A mutation were capable of forming large polymerized complexes, while $\Delta 74-79$ and $\Delta 71-82$ proteins with the normal E83 residue could not (Fig. 2A). ThT fluorometry and K114 fluorometry demonstrated that these proteins with the E83A mutation also acquired the ability to polymerize into structures with amyloidogenic properties (Fig. 2B,C).

EM analysis verified that the E83A mutation promoted the formation of amyloid fibrils (Fig. 3A–C). $\Delta 74-79/\text{E83A}$ α -syn formed fibrils (width = 11.5 nm $\pm$ 2.8) with a negatively stained pattern and width similar to WT α -syn (width = 10.7 $\pm$ 2.0 nm). $\Delta 71-82/\text{E83A}$ α -syn also formed amyloid fibrils; however, the width was slight reduced (8.7 nm $\pm$ 1.9), and the fibrils were observed to have spiny projections. These fibrils showed significant alterations from the oligomers observed with $\Delta 71-82$ and $\Delta 74-79$ α -syn in the absence of the E83A mutation [27].

Despite the finding that the E83A mutation altered the amyloidogenic properties of $\Delta 74-79$ and $\Delta 71-82$ α -syn, the propensities to polymerize were still less than that of WT α -syn. In addition, relative differences in ThT, K114 and sedimentation assays were observed between WT α -syn, $\Delta 74-79/\text{E83A}$ α -syn and $\Delta 71-82/\text{E83A}$ α -syn. $\Delta 74-79/\text{E83A}$ exhibited diminished ThT fluorescence when compared to relative sedimentation and K114 analyses. However, $\Delta 71-82/\text{E83A}$ exhibited augmented K114 fluorescence when compared to relative sedimentation and ThT.

The effect of the E83A mutation in the presence of small molecular inhibitors

It was previously shown that the E83A mutation can promote the propensity of α -syn to polymerize [21], and the data herein indicate that the E83A mutation can promote fibril formation of α -syn deletion-mutants that would otherwise not be assembly competent. Taken together, these data support the notion that the E83 residue can significantly negatively

modulate α -syn fibril formation. Therefore, the ability of the E83A to drive amyloid formation in the presence of small molecular inhibitors was studied.

Dopamine [34–36], baicalein [37–39], and EGCG [38;40] are three of the best studied molecular inhibitors of α -syn amyloid formation. Our data confirmed that all three compounds were potent inhibitors of α -syn amyloid formation (Fig. 4). Each inhibitor was then tested on α -syn with the E83A mutation. Protein with the E83A mutation was capable of amyloid formation even in the presence of dopamine and EGCG, as shown using quantitative sedimentation analyses and K114 fluorometry (Fig. 4A,C). However, ThT fluorescence was relatively blunted in the presence of the E83A mutation (Fig. 4B), preventing adequate assessment with this measure in the presence of inhibitors.

By EM analysis, clusters of fibrils were observed with the E83A mutation in the presence of dopamine (Fig 3E). However, the E83A mutation in the presence of EGCG formed short fibrils with abnormal positive/negative staining (Fig. 3F). Although the E83A mutant in the presence of baicalein exhibited a modest increase in sedimentation, K114, and ThT measures, no filamentous polymers were observed by EM analysis.

DISCUSSION

The polymerization of soluble monomeric α -syn into mature amyloid fibrils can be influenced by amino acid segments and specific residues in the amino- and carboxy-terminals regions [18–25]. However, the central hydrophobic region in α -syn is necessary for polymerization, and alterations in the region has a more profound role on fibrillization [26;27;29;41]. For example, the deletion of short stretches of hydrophobic residues, such as 71-82 and 74-79, in this region impairs the ability of α -syn to polymerize into mature amyloid, and instead these proteins preferentially form oligomers [26;27;30]. In addition, single point mutations in many different residues, especially those creating a P or adding charged residues, have been shown to inhibit amyloid formation [26;27;42].

In striking contrast to the 71-82 and 74-79 deletions, Δ 73-83 α -syn was shown to be able to polymerize into mature amyloid, albeit at a slower rate than WT α -syn [26–28]. These findings are apparently counterintuitive since both of these deletions remove a similar region and they both include residues 74-79. However, it is important to note that the deletion of residues 73-83 results in a new hydrophobic stretch from the adjacent upstream and downstream sequences (⁶⁷GGAVVT⁷²⁻⁸⁴GAGSIAAATGF⁹⁴).

One possible explanation is the notion from modeling studies suggesting that “E83 plays a possible role as a gatekeeper residue to reduce the aggregation propensity” [31]. This notion is supported by the increased rate of α -syn polymerization in the presence of the E83A mutation [21]. To test this hypothesis, residue E83 in both Δ 74-79 α -syn and Δ 71-82 α -syn were mutated to an A. The E83A mutation dramatically rescued the ability of both proteins to form amyloid fibrils, even if the propensity was less that of WT α -syn. This rescue effect was previously noted for Δ 76-77 α -syn [27], but these data further underscore the importance of the E83 residue, given the ability of the E83A mutation to rescue polymerization despite the extended deletions of hydrophobic residues in this study.

Of additional note was the relative disparity between sedimentation, K114, and ThT measures when comparing WT, E83A, Δ 74-79/E83A and Δ 71-82/E83A α -syn. Polymerized E83A and Δ 74-79/E83A α -syn demonstrated blunted ThT fluorescence compared to polymerized WT α -syn, while Δ 71-82/E83A α -syn exhibited comparative ThT, but elevated K114 fluorescence. These differences could be due to direct inhibition of ThT binding and/or conformational changes within α -syn polymers that differentially affect ThT and K114 binding. K114 is a soluble styrylbenzene molecule that is structurally derived from Congo Red [33]. The

interaction of the fluorescent dyes Congo Red and ThT with amyloid is one of the characteristic defining properties for these protein structures [43]. The interaction of these compounds with amyloid fibrils induce spectral shifts and increased excitation that does not occur in the presence of other structural forms of the protein, such as monomer or amorphous aggregates [33;44–46]. However, the ThT and Congo Red amyloid binding sites are believed to be mutually exclusive [47]. For example, the binding of styrylbenzene-based compounds to amyloid fibrils can be competed with the dye Chrysamine G, but not with ThT [48]. Despite the widespread use of amyloid binding dyes to detect pathological lesions and diagnose diseases, the understanding of the molecular structure of amyloid and the interactions of amyloid with binding dyes remain incomplete. Several models have been proposed, and it is believed to be dependent on the presence of a protein complex with cross- β structure [33;43;47–51].

We further investigated the inhibitory role of E83 on α -syn amyloid formation in the presence of small molecular inhibitors. Three well-characterized inhibitors, dopamine, baicalein, and EGCG, each promote α -syn to form spherical oligomers that share similar properties with the oligomers formed by $\Delta 74$ -79 and $\Delta 71$ -82 α -syn [27;34;35;37;40]. The E83A mutant partially overcame the inhibition of polymerization due to dopamine and EGCG, consistent with a study of dopamine/ α -syn interaction [36]. We also found that dopamine, EGCG, and baicalein effectively inhibited amyloid formation of α -syn with the deletion of residues 73-83 (data not shown). Therefore, the ability of the E83A mutant to overcome the inhibition by these molecular inhibitors is more likely due to the increase propensity of E83A to directly drive fibril formation than direct interference of these molecules binding with the E83 residue. In addition, these molecules have been shown to inhibit fibril formation predominantly due to interaction with the flexible carboxy-terminal region [34;40;52]. However, despite the mutation of the E83 residue, baicalein continued to effectively inhibit α -syn fibril formation. This finding suggests that baicalein is the most potent of the inhibitors examined.

The effect of the E83 residue as a negative regulator of amyloid formation is likely due to the size of the residue and its negative charge as well as its focal inhibition of β -pleated sheet formation [21;53;54]. The inhibitory role is likely magnified in the presence of major deletions to the hydrophobic region of α -syn that drive fibril formation or other inhibitors that decrease the propensity of α -syn to form amyloid. This role is further supported by the ability of α -syn 1-74, where most of the hydrophobic middle region is removed, to still form abundant amyloid fibrils [30]. However, the propensity of α -syn to form amyloid is a complex process that also depends on residue context, conformational preferences, solubility, charge state, hydrophobicity and the ability to pack into a fibrillar structure [27;55–58].

The E83 residue in α -syn, situated in the center of the hydrophobic domain, appears to attenuate an already dynamic process of α -syn amyloid formation. In the absence of this residue the abilities of effective inhibitors of amyloid formation are diminished. This notion is further underscored by the ability of E83A mutations to allow fibril formation when large portions of the hydrophobic domain are deleted. The ability to impede amyloid formation may therefore be influenced by this “gate-keeper” residue. These findings indicate that defining the specific critical roles of amino acid residues in driving amyloid formation provide important insights into the mechanisms of polymer assembly and may enable the generation of therapeutic agents capable of preventing the assembly of amyloid.

Acknowledgments

This work was funded by grants from the National Institute on Aging (AG09215) and the National Institute of Neurological Disorders and Stroke (NS053488). E.A.W. was supported by a training grant (T32 AG00255) from the National Institute on Aging.

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Figure 1. Schematic of human α -syn protein

The diagram shows the 6 imperfect "KTKEGV" repeats (black boxes), the three point mutations causal of disease (A30P, E46K, and A53T) and an expanded view of the amino acid sequence in the middle hydrophobic region. Residue stretches from 74-79 and 71-82 are underlined, and residue E83 is enlarged.

Figure 2. Studies of the effects of mutating E83 to on A in α -syn proteins with $\Delta 74-79$ and $\Delta 71-82$ deletions

(A) Quantitative sedimentation analysis, (B) ThT fluorimetry, and (C) K114 fluorimetry of the formation of large polymers and amyloid for wild-type (WT), $\Delta 74-79$, $\Delta 74-79/E83A$, $\Delta 71-82$, and $\Delta 71-82/E83A$ α -syn after 0, 2, 4 and 9 days of incubation under assembly conditions, as described in “Material and Methods”. All proteins were incubated at 345 μ M. (C) Data represent average $\pm$ SD. n = 6 for all data sets.

Figure 3. EM analysis of fibril formation

Negative staining EM analysis of the polymerization of (A) WT α -syn after 2 days of incubation, (B) $\Delta 74-79/E83A$ α -syn after 9 days of incubation, (C) $\Delta 71-82/E83A$ α -syn after 9 days of incubation, (D) E83A after 2 days of incubation, (E) E83A α -syn with equimolar concentration of dopamine after 2 days of incubation, and (F) E83A α -syn with equimolar concentration of EGCG after 2 days of incubation. All proteins were incubated at 345 μ M. Bar scale = 100 nm.

Figure 4. Effects of the E83A mutation on overcoming the inhibition of α -syn fibril formation due to small molecules

WT or E83A α -syn was incubated alone or in the presence of equimolar concentrations of dopamine (DA), baicalein (Baic), or EGCG for 2 days. The formation of large polymers and amyloid formation were assessed by (A) quantitative sedimentation, (B) ThT fluorimetry, and (C) K114 fluorimetry. All proteins were incubated at 345 μ M. Data represent average $\pm$ SD. $n = 6$ for all data sets.