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The Aggregation and Fibrillation of α-Synuclein

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ABSTRACT

α-Synuclein is a small (14 kDa), abundant, intrinsically disordered presynaptic protein, whose aggregation is believed to be a critical step in Parkinson's disease (PD). The kinetics of α-synuclein fibrillation are consistent with a nucleation-dependent mechanism, in which the critical early stage of the structural transformation involves a partially folded intermediate. Although the basis for the toxic effects of aggregated α-synuclein are unknown, it has been proposed that transient oligomers are responsible, possibly by forming pores in membranes. In this Account, I discuss our investigations into the molecular basis for α-synuclein aggregation/fibrillation, including factors that either accelerate or inhibit fibrillation, effects of molecular crowding, oxidation, point mutations, and lipid membranes, as well as the variety of conformational and oligomeric states that α-synuclein can adopt. It is apparent that neuronal cells must have a very fine balance of factors that control the levels and potential aggregation of α-synuclein.

Introduction

Substantial evidence suggests that the aggregation of α-synuclein is a critical step in the etiology of Parkinson's disease (PD).[1] Parkinson's disease is predominantly a movement disorder resulting from degeneration of dopaminergic neurons in the substantia nigra. The cause of the disease is unknown, but considerable evidence suggests a multifactorial etiology involving genetic susceptibility and environmental factors.

The following observations are among the most compelling for the involvement of α-synuclein aggregation in PD: familial early onset PD is caused by overexpression of α-synuclein due to duplication or triplication of the α-synuclein gene locus;[2] point mutations that increase the aggregation propensity of α-synuclein lead to familial early onset Parkinson's disease;[3−5] fibrils of α-synuclein are observed in Lewy bodies, the cardinal hallmark of PD pathology.[6] Lewy bodies are spherical protein inclusions found in the cytoplasm of surviving nigral neurons consisting of a dense core surrounded by a halo of radiating fibrils of α-synuclein and containing a variety of other proteins. The fibrils observed in PD are structurally similar to those found in amyloid diseases and are linear rods of 5−10 nm diameter (Figure 1). The production of wild-type (WT) α-synuclein in transgenic mice or of WT or the familial variants A30P and A53T in transgenic flies leads to motor deficits and neuronal inclusions suggestive of PD. Currently, it is not known how the aggregation of α-synuclein triggers cell death, although it has been postulated that certain oligomeric forms may result in enhanced membrane permeability.[7]

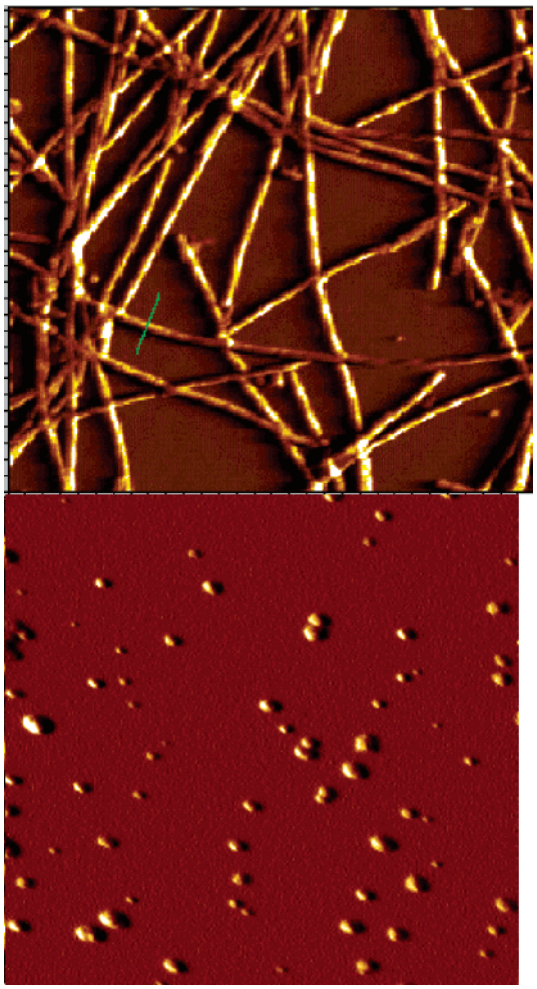

FIGURE 1. AFM images of α-synuclein fibrils (top) and oligomers (bottom). The fibrils show the characteristic twist of mature fibrils and are 10 nm high. The oligomers are from the late lag phase period and are mostly 5 or 7 nm high. The images are 3 μm square.

α-Synuclein is an abundant brain protein of 140 residues, lacking both cysteine and tryptophan residues. A variety of spectroscopic studies, including NMR, indicate that α-synuclein is an intrinsically disordered protein. This is believed to be due to the very high net (negative) charge at neutral pH and the low intrinsic hydrophobicity of the molecule, reflecting the amino acid composition of α-synuclein.[8] α-Synuclein is present in high concentration at presynaptic terminals and is found in both soluble and membrane-associated fractions of the brain. Several possible functions have been suggested, and it appears to be involved in vesicle release and trafficking. α-Synuclein deposits are implicated pathologically in several other neurodegenerative diseases including Alzheimer's disease.

Anthony L. Fink received his Ph.D. degree from Queen's University, Canada, in 1968 and did postdoctoral research at Northwestern University. In 1969, he joined the University of California, Santa Cruz, as an Assistant Professor and is currently Professor. His research focuses on protein misfolding and aggregation, the molecular basis of protein deposition diseases, such as Parkinson's, and the development of drugs to prevent protein deposition.

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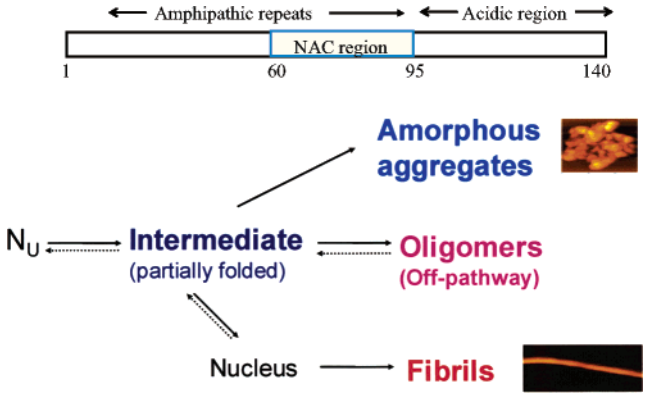

FIGURE 2. α-Synuclein structure (top) and competing kinetics pathways for the aggregation of α-synuclein (bottom). N_u represents the natively unfolded α-synuclein monomer. Partial folding leads to formation of an aggregation-prone intermediate that, depending on the conditions, can form soluble oligomers, insoluble amorphous aggregates, or insoluble fibrils.

The structure of α-synuclein can be divided into three regions: residues 1−60, which contain four 11-amino acid imperfect repeats (coding for amphipathic helices) with a conserved motif (KTKEGV); residues 61−95, which contain the highly amyloidogenic NAC region and two additional repeats; and the highly charged C-terminal region, residues 96−140 (Figure 2).

Extensive data support the hypothesis that "pathological" or abnormal aggregation[9] arises from a key partially folded intermediate precursor.[10] Such intermediates have sizable nonpolar patches (i.e., contiguous hydrophobic side chains) on their surface, which lead to hydrophobic interactions between molecules, resulting in specific intermolecular interactions and aggregation. These hydrophobic patches are absent in the fully unfolded state, as in monomeric α-synuclein. Factors that increase the concentration of such intermediates will favor aggregation.

Fibrillation of α-Synuclein

When monomeric α-synuclein is incubated at 37 °C, pH 7.4, it forms fibrils with a condition-dependent rate. Agitation, in particular, will greatly accelerate the process. Typically with 70 μM (1 mg/mL) α-synuclein, fibrillation will be completed within 3 days with agitation, whereas without agitation it will take months. The in vitro kinetics of α-synuclein fibril formation show an initial lag phase followed by an exponential growth phase and a final plateau, usually attributed to a nucleation-dependent polymerization (e.g., see Figure 5). As anticipated, increasing concentrations of α-synuclein lead to increased rate of fibrillation.[11]

α-Synuclein fibrils grown in vitro typically vary in length from about 500 nm to 3 μm. Based on atomic force microscopy (AFM) images three different heights of fibrillar species are observed: protofilaments, 3.8 ± 0.6 nm; protofibrils, 6.5 ± 0.6 nm; and fibrils, 9.8 ± 1.2 nm.[12] EM images show similar species, although of slightly larger diameters.[13] Figure 1 shows typical fibrils of α-synuclein.

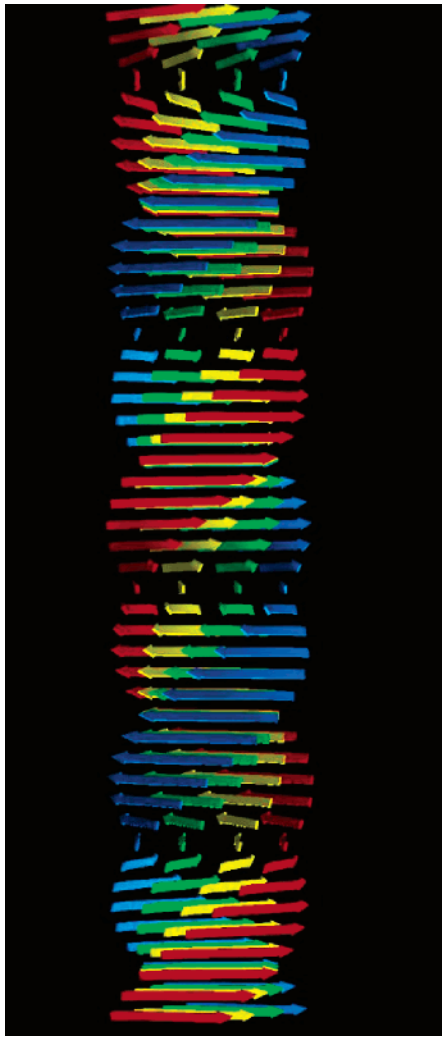

FIGURE 3. The cross-β structure of fibrils.

α-Synuclein Forms a Critical Amyloidogenic Partially Folded Intermediate

A partially folded intermediate has been shown to be critical for α-synuclein fibrillation.[14] In fact, substantial data supports the hypothesis that partially folded intermediates are critical species in the early stages of most if not all pathological aggregation and especially fibrillation reactions.[10,15] This is readily rationalized as follows: all fibrils, independent of the original structure of the given amyloidogenic protein, have a common cross-β structure (Figure 3), consisting of β-sheets in which the β-strands are perpendicular to the axis of the fibril. In order to attain this structure, folded proteins have to undergo significant conformational rearrangements to allow the necessary topological changes required. Evidence for a model for the fibrillation of α-synuclein in which the first step is the conformational transformation of the natively unfolded protein into an aggregation-competent partially folded intermediate initially came from observations correlating partial folding with fibrillation.[14] This suggests a simple kinetic model of fibrillation involving conversion of monomeric α-synuclein into the critical partially folded intermediate, which leads to formation of the amyloidogenic nucleus and fibrils. Consequently, factors that shift the

equilibrium from unfolded monomer in favor of the intermediate will facilitate fibril formation. Thus, an increase in protein concentration is predicted to increase the concentration of the intermediate, and accordingly the rate of fibrillation, as observed.

The kinetics of α-synuclein fibrillation are very sensitive to ionic strength.[16] This has been attributed to the presence of anions and cations inducing partial folding of α-synuclein, forming the critical amyloidogenic intermediate, which leads to significant acceleration of the rate of fibrillation.[11,17]

Multiple Pathways of α-Synuclein Aggregation

Analysis by electron microscopy and AFM has revealed that different final products may arise from the aggregation of α-synuclein depending on the experimental conditions: fibrils, soluble oligomers, and insoluble "amorphous" aggregates. The presence of these three apparently distinct aggregation products reflects the underlying energetics and thermodynamic stabilities of different aggregated forms of α-synuclein. The particular product formed under a given set of conditions will be determined by the kinetic partitioning among the different pathways leading to these products, Figure 2.

Three point mutations of α-synuclein are associated with early onset familial PD, A53T, A30P, and E46K, two of these fibrillate more rapidly than wild-type in vitro. A variety of investigations have demonstrated that these variants of α-synuclein self-associate more rapidly than wild-type.[18−21] A number of non-naturally occurring mutants of α-synuclein have been synthesized and their properties examined. From these, it is clear that very small changes in side-chain structure can have significant effects on the kinetics of fibrillation. For example, replacing methionine with leucine residues in a series of mutants led to slower kinetics of fibrillation in all cases.[22] Thus, the introduction of the more hydrophobic leucine residues increased the propensity for self-association to off-pathway oligomers.

Investigations in a variety of alcohol solvents demonstrated that, depending on their concentration, α-synuclein could adopt several different stable conformations, including soluble β-sheet and α-helix rich structures, suggesting that the energy landscape of α-synuclein stability is complex.[16]

Transient Oligomers

As seen in Figure 4, when α-synuclein is incubated at pH 7.4, 37 °C, with agitation, the kinetics of fibril formation are typical of a nucleated polymerization. It is now clear that transient soluble oligomeric species are present during the lag phase, Figure 4.[7,23−26] A key question is whether these transient oligomers are on the direct pathway to fibrils or are off-pathway but in equilibrium with monomer that can add to fibrils or can add directly to growing fibrils.

The oligomers present during the lag phase have been studied by a variety of different biophysical techniques. For example, dynamic light scattering (DLS) shows the buildup of oligomers of α-synuclein starting soon after

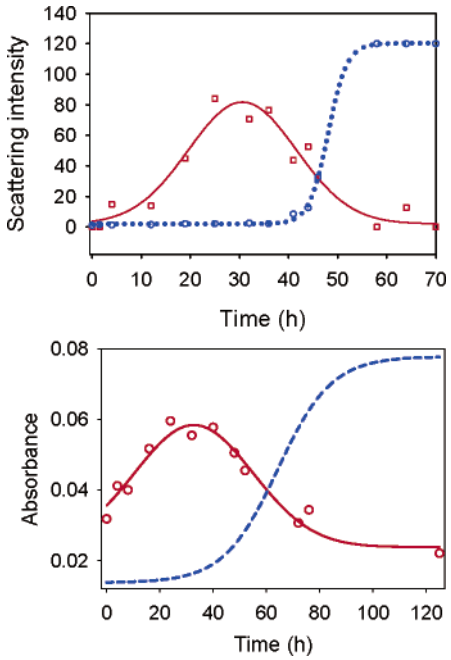

FIGURE 4. Formation of transient oligomers during the lag phase of α-synuclein fibrillation: (A) the presence of the transient oligomer was monitored by dynamic light scattering; (B) the oligomer was monitored by changes in the amount of β-structure (1630 cm^{-1}) in the soluble fraction, using FTIR. In both panels, the rate of formation of fibrils, monitored by thioflavin T fluorescence, is shown in blue. The time scales are different since different protein concentrations and experimental conditions were used.

incubation (Figure 4). These typically reach a maximum concentration of 15−25% toward the end of the lag period and then decline in concentration as fibril growth accelerates. This raises the critical question of whether the oligomers directly transform into fibrils or dissociate into monomeric species, which then add to the growing fibril tips. Similar kinetics of oligomerization are observed using FTIR to monitor changes in secondary structure of the soluble material (Figure 4).[24] The major structural changes observed occur around 1630 cm^{-1}, reflecting changes in β-structure. The amount of β-structure initially increases and then decreases, suggesting build-up and decay of a soluble transient intermediate.

The aggregation of α-synuclein has been investigated with fluorescence resonance energy transfer (FRET) using the donor/acceptor pair of tyrosine and tryptophan to minimize perturbations of the α-synuclein structure. For example, Y125W/Y133F/Y136F α-synuclein has a single tyrosine donor at position 39 and a single tryptophan acceptor at position 125. Energy transfer in the supernatant fractions showed that the changes in FRET correspond predominantly to the formation and decay of the oligomeric intermediates.[24] By the middle of the lag phase, there is a jump in the energy transfer efficiency as oligomers with closer proximity for the two fluorophores become significantly populated.

Replacing one of the four tyrosine residues in α-synuclein with a tryptophan provides a strong intrinsic fluorescence signal in α-synuclein: Y39W α-synuclein was studied by a variety of biophysical methods, including

tryptophan fluorescence, to monitor the kinetics of aggregation.[29] These probes revealed the significant population of two classes of oligomeric intermediates, one formed during the lag period of fibrillation and the other present at the completion of fibrillation. As expected for a natively unfolded protein, Trp39 was highly solvent-exposed in the monomer and is solvent-exposed in the two oligomeric intermediates; however, it is partially, but not fully, buried in the fibrils.[29]

The oligomers have also been characterized by small-angle X-ray scattering (SAXS) and AFM. SAXS measurements on aliquots removed from the incubation solution at $t = 0$ showed only monomer, with $R_g = 4.2$ nm, whereas already by 2 h, oligomeric species with $R_g = 7.2$ nm were observed, consistent with DLS analysis. However, these early formed oligomers are not stable because they are not readily detected by size exclusion chromatography (SEC) high-performance liquid chromatography (HPLC), indicating their dissociation rates are relatively fast. AFM images of aliquots removed from the incubation mixture reveal two populations of oligomers during the lag phase, the first formed have a height of 2−3 nm, and those formed toward the end of the lag have a height of 5−7 nm (Figure 1). Based on FTIR analysis, the latter oligomers have substantial β-sheet structure and similar size (AFM) to the mature fibril. The fact that these oligomers disappear at the same rate that fibrils appear suggests that fibrils could be assembled directly from them via longitudinal association of the oligomers, rather than by binding of monomeric species to the growing end of fibrils.

Stabilized Oligomers

Several modifications of α-synuclein lead to the formation of stable oligomers. These include oxidation of the four methionine residues to methionine sulfoxide,[22,30,31] specific nitration of the tyrosine residues,[32,33] interaction with polyphenols such as baicalein and rifampicin,[34,35] and covalent modification by 4-hydroxynonenal.[36] Many of these oligomers are extremely stable with very slow rates of dissociation. For example, incubation of α-synuclein with the flavonoid baicalein leads to formation of oligomers that show a C_m of 3.3 M guanidinium hydrochloride (Gdn·HCl) for dissociation to monomer, but even at 6 M Gdn·HCl, significant oligomer remains. Typically these oligomers are formed more rapidly than fibrils, and as a consequence, no significant fibrillation occurs from these modified forms of α-synuclein. Biophysical characterization of these oligomers suggests that they have significant secondary and tertiary structure and are substantially more compact than monomeric α-synuclein. The stability of these oligomers indicates that the underlying structure of their subunits is different than that in the transient oligomers. Consequently it is not clear whether they would necessarily be neurotoxic.

Interactions of α-Synuclein with Membranes

Although α-synuclein is found in the cytosolic fractions, membrane-bound α-synuclein has been suggested to play an important role in the function(s) of α-synuclein. This is one of the most contentious areas concerning α-synuclein; recent studies suggest that there may be major differences between the situation in vivo and in vitro. The presence of membranes has been reported to both accelerate and inhibit fibrillation, and this probably reflects the varying conditions used in the experiments.[37]

In vitro, α-synuclein preferentially interacts with membranes containing acidic phospholipids.[38,39] The nature of the interaction of α-synuclein with vesicles is highly dependent on the phospholipid composition, the ratio of α-synuclein to phospholipid, and the size of the vesicles. The strongest interactions are between α-synuclein and vesicles composed of phosphatidic acid (PA)/phosphotidylcholine (PC) and phosphatidylglycerol (PG)/PC and involve formation of helical structure in α-synuclein. A strong correlation is observed between the induction of α-helix in α-synuclein and the inhibition of fibril formation.[37]

α-Synuclein−membrane interactions have been found to affect both protein and membrane properties, and both electrostatic and hydrophobic interactions are important in the association of the protein with the bilayer.[40] The ability of α-synuclein to disrupt membranes correlated with the binding affinity of α-synuclein for the particular membrane composition and with the induced helical conformation of α-synuclein. Protofibrillar or fibrillar α-synuclein caused a much more rapid destruction of the membrane than soluble monomeric α-synuclein,[40] indicating that protofibrils (oligomers) or fibrils are likely to be significantly neurotoxic.[25,40−42]

Effects of Oxidation on α-Synuclein Fibrillation

Oxidative stress has been implicated in the pathogenesis of Parkinson's disease.[43] This is another area of controversy: it is not clear whether oxidative stress and mitochondrial dysfunction are causative factors or a result of α-synuclein aggregation or other pathogenic aspects of the disease. Methionine and cysteine are the most readily oxidized amino acids. The absence of cysteine means that α-synuclein is most easily oxidized to methionine sulfoxide. Rather surprisingly, the fibrillation of α-synuclein at neutral pH was inhibited by methionine oxidation.[30] This is due to the formation of a relatively stable oligomer. Interestingly, the addition of small molar excesses of methionine-oxidized α-synuclein to α-synuclein led to inhibition of fibrillation. A 4-fold molar excess of the oxidized protein was sufficient to completely inhibit α-synuclein fibril formation.[30] This suggests that the oligomers of the methionine-oxidized α-synuclein can incorporate unmodified α-synuclein and still remain sufficiently stable so as to prevent fibrillation. The degree of inhibition of fibrillation by methionine-oxidized α-synuclein is proportional to the number of oxidized methionines.[22] Interestingly, the presence of certain metals (Pb^{2+}, Zn^{2+}, Al^{3+}, and Ti^{3+}) overcame the inhibition.[44]

Effects of Molecular Crowding on α-Synuclein Fibrillation

A common property of the interior of all cells is the high concentration of macromolecules present. The typical cell

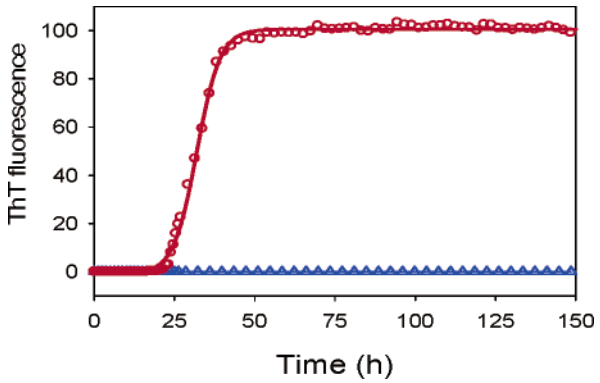

FIGURE 5. Macromolecular crowding dramatically increases the rate of α-synuclein fibrillation. α-Synuclein in the presence of 100 mg/mL PEG-3350 (red) and in the absence of the crowding agent (blue). The experiments were performed at 37 °C, pH 7.4, 100 mM NaCl, with minimal agitation.

contains ~25% protein by volume, along with substantial amounts of RNA and other biopolymers. The excluded volume of these macromolecules results in very little room for additional macromolecules. This has major thermodynamic and kinetic consequences on the properties of macromolecules present in the cell.[45]

Macromolecular crowding leads to a dramatic acceleration in the rate of α-synuclein aggregation and fibrillation, as shown in Figure 5. Various types of polymers, from neutral poly(ethylene glycol)s and polysaccharides (Ficolls, dextrans) to inert proteins, accelerate α-synuclein fibrillation.[46−49] The acceleration of fibrillation increases with increasing length of polymer, as well as increasing polymer concentration. Pesticides and metals, which are linked to increased risk of Parkinson's disease by epidemiological studies, accelerate α-synuclein fibrillation under conditions of macromolecular crowding.[49,50] The very dramatic increases in rate of α-synuclein fibrillation in the presence of macromolecular crowding suggests that in the cell there must be mechanisms to prevent α-synuclein aggregation from occurring.

Factors Affecting the Kinetics of α-Synuclein Fibrillation

A variety of endogenous and exogenous factors can significantly accelerate the rate of α-synuclein fibrillation; conversely, many factors can inhibit the rate of fibrillation. Among factors that accelerate α-synuclein fibrillation are certain pesticides, metals, polycations, glycosaminoglycans (GAGs), lipids, membranes, and macromolecular crowding. Acceleration typically arises from conditions that increase the concentration of the critical amyloidogenic intermediate. Inhibition typically arises from situations in which either the monomer or non-fibrillogenic oligomers are stabilized.

Providing a potential explanation of the increased risk of PD associated with exposure to pesticides, in vitro studies have shown that several commonly used pesticides induce a conformational change in α-synuclein and significantly accelerate the rate of formation of α-synuclein fibrils.[51−53] Similarly, the increased risk of PD associated with exposure to certain metals may in part be explained by the observation that di- and trivalent metal ions cause significant accelerations in the rate of α-synuclein fibril formation in vitro.[44,53−55] The most effective cations in vitro include aluminum(III), copper(II), iron(III), and lead(II) and show a strong correlation with increasing ion charge density.[11,44] The mechanism for this presumably involves the metal ions binding to negatively charged carboxylates, thus masking the electrostatic repulsion and facilitating collapse to the partially folded conformation. There also appear to be specific binding sites for some metals, for example, copper.

In vitro studies show that certain glycosaminoglycans (GAGs) (e.g., heparin) and other highly sulfated polymers significantly stimulate the formation of α-synuclein fibrils.[56,57] α-Synuclein also shows strong affinity for polycationic species, such as polyamines (e.g., spermine, histones), which accelerate the fibrillation of α-synuclein.[58,59] The magnitude of the accelerating effect depends on the nature of the polymer, its length, and its concentration and suggests a potential critical role of electrostatic interactions in which neutralization of the net negative charge on the protein leads to collapse of the intrinsically disordered α-synuclein monomer to the partially folded amyloidogenic intermediate and thence fibrillation.[58]

Osmolytes both accelerate and inhibit α-synuclein fibrillation, depending on their concentration. For example, concentrations of trimethylamine *N*-oxide (TMAO) in the vicinity of 1 M induced partial folding of α-synuclein and significantly increased the rate of fibrillation. At higher concentrations (≥3 M) of TMAO, α-synuclein became tightly folded, with substantial helical character, and formed oligomers. Fibrillation was inhibited at these high concentrations of TMAO, presumably due to the stability of the oligomers.[60]

Inhibitors of Fibrillation

Some of the α-synuclein in Lewy bodies has been covalently modified by tyrosine nitration.[61,62] Whether this occurs before or after fibril formation and whether nitration of the monomer accelerates or retards fibrillation are controversial.[63] The effect of tyrosine nitration on the propensity of α-synuclein to fibrillate in vitro was examined; fibril formation was completely inhibited by nitration, due to the formation of stable soluble oligomers.[32,64] In addition, the presence of substoichiometric concentrations of nitrated α-synuclein prevented fibrillation of nonmodified α-synuclein.[32] It should be noted that in vivo nitration of tyrosine occurs via reaction with oxidative derivatives of NO, and under these conditions additional products of α-synuclein oxidation could arise, for example, dityrosine.

Two homologous proteins, β- and γ-synucleins, are also abundant in the brain. γ-Synuclein forms fibrils much more slowly than α-synuclein, and β-synuclein does not form fibrils under typical in vitro or in vivo conditions.[65,66] Complete inhibition of α-synuclein fibrillation was observed at 4:1 molar excess of β- and γ-synucleins.[65] The

lack of fibrils formed by β-synuclein is most readily explained by the absence of a stretch of hydrophobic residues from the middle region of the protein and formation of stable oligomers. It is thus likely that β-synuclein may help negatively regulate formation of α-synuclein fibrils.

One of the interesting aspects of Parkinson's disease is that the most sensitive neurons to degeneration are the dopaminergic neurons in the substantia nigra. Since dopamine is very sensitive to oxidation, this has led to the suggestion that the oxidation of dopamine may contribute to the disease. It has been shown that dopamine and related catecholamines can inhibit the formation of α-synuclein fibrils in vitro.[67−69] The mechanism for this inhibition is not totally clear at this time, but it is likely that covalent modification by quinones, arising from the oxidation of the catecholamines, is involved. Interestingly, catechols can also dissolve existing α-synuclein fibrils formed in vitro,[70] and intraneuronal α-synuclein deposits formed in a mouse model were dissolved by incubation of tissue slices with levodopa.[70]

We have shown that a variety of flavonoids and polyphenols can inhibit the fibril formation of α-synuclein and in some cases also disaggregate existing fibrils.[34,35,70] These compounds bind to monomeric α-synuclein and lead to formation of stable α-synuclein oligomers.

Implications for Parkinson's disease

Although many details remain to be discovered as to the cause of Parkinson's disease, it is clear that the aggregation of α-synuclein is a critical factor in the etiology of this debilitating disorder. This Account has focused on the underlying biophysical properties of α-synuclein and how they relate to its aggregation and fibrillation. Such studies provide essential knowledge regarding the basic molecular mechanisms that must occur in vivo. For example, the cellular concentration of α-synuclein, the presence of various endogenous and exogenous factors, macromolecular crowding, and covalent modification due to oxidative stress all have significant effects on the aggregation and fibrillation of α-synuclein and are likely to play important roles in the disease state. Similarly, the inhibitory effects of certain polyphenols such as baicalein and rifampicin could lead to the development of effective therapies to prevent progression of Parkinson's disease.

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