Intricacies of alpha-synuclein aggregation

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Chapter 1

Introduction[1]

1.1. Neuropathology of Parkinson's disease

Almost two centuries ago James Parkinson first described the manifestation of shaking palsy and the disease was named after him as Parkinson's disease (PD). Presently, PD takes second place on the list of the most common neurodegenerative disorders of the human brain [1] affecting approximately 6.3 million people around the world. Symptoms like tremor, rigidity, bradykinesia, speaking difficulties and postural instability are characteristic for PD that ultimately may lead to total disability followed by death. The disease develops not only in the central, but also in the peripheral (PNS) and the enteric nervous system [2]. When the disease is diagnosed, approximately 70% of the striatum dopamine has already been depleted and it advances in time [3]. In advanced PD most of the dopaminergic neurons are lost, suggesting significant cell death during the process [4]. Moreover, choline neurons present in the dorsal vagal nucleus degenerate too [5].

It took a full century from the time of Parkinson's discovery until Fritz Heinrich Lewy identified the abnormal inclusions in nerve cell bodies to be a hallmark of the disease. They were named Lewy bodies (LBs) after him. LBs and Lewy neurites (LNs) are not unique to PD, they are also found, for example, in the PNS of patients with pure autonomic failure [6]. Electron microscopy helped in finding out that LBs and LNs are largely composed of 200-600 nm long α-synuclein (α-syn) filaments [7]. α-Syn is a protein that is mostly present in the human brain, but also found in the heart and muscles. This thesis focuses on the properties of α-syn and on its propensity to form aggregates. We will briefly describe the properties of proteins in general and of α-syn in particular.

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1.2. Proteins and protein folding

Protein molecules are made of building blocks - amino acids - connected into linear chains through covalent peptide bonds [8]. Although only 20 amino acids are considered to be involved in building proteins, there are nearly 100,000 different proteins in the human body [9]. The majority of the proteins have to be folded properly into a unique 3-dimensional (3D) structure in order to function correctly [10]. Synthesized proteins usually adopt their unique structure through the folding process on ribosomes. It is well-known, however, that folding also takes place in the cytoplasm after their release from ribosomes, mitochondria or the endoplasmic reticulum [11]. The folding process itself is strongly influenced by the specific environmental conditions under which it takes place. It is also widely known that the basic principles of folding are common. Sometimes the folding process goes wrong and it needs to be supported by molecular chaperones and folding catalysts in order to prevent such behavior [11,12]. If the assistance of molecular chaperones and folding catalysts also fails, the cell then tends to degrade the misfolded protein, *e.g.*, *via* the ubiquitin-proteasome system [13].

1.3. Protein misfolding and disease

A large number of proteins, however, do not adopt a well-defined 3D structure, although they are functional and biologically active [14,15]. They are called intrinsically disordered or natively unfolded proteins (IDPs). Some IDPs are associated with neurodegenerative diseases, clinging together to form aggregates [9,16]. Others are very quick in reacting to changes in their surroundings and tend to involve themselves in numerous signaling, regulation and recognition processes, often modulating and controlling their binding partners [17]. IDPs biological activities are controlled *via* posttranslational modifications [15] and alternative splicing [18,19]. While IDPs can operate as hubs (one-to-many signaling), they also can bind to hubs [20–23] (many-to-one signaling). When interacting with different binding partners, IDPs can fold in a different fashion [24,25].

Protein misfolding and aggregation play a key role in numerous human diseases such are Parkinson’s, Alzheimer’s, Huntington’s disease, type II (late-onset) diabetes, Amyotrophic Lateral Sclerosis and prion disease [26] (Table 1.1). These diseases all correlate with the formation of inter- and intracellular inclusions consisting of insoluble amyloid fibrillar aggregates. The structure of amyloids is very similar, although they may be composed of proteins with very different original function. Amyloids are fibrillar aggregates with a length

of up to a few microns, which exhibit a characteristic cross-β structure which plays a key role in the interaction and binding of adjacent, individual proteins within the fibrils [27].

Table 1.1: Intrinsically disordered proteins associated with human diseases.

Protein/peptide	**Disease(s)**	**Polypeptide length (number of amino acid residues)**	**Protein/peptide structure**
α-synuclein	Parkinson's disease Synucleopathies Dementia with Lewy body Multiple system atrophy Lewy body variant of Alzheimer's disease	140	Intrinsically disordered
Amyloid-β peptide	Alzheimer's disease	37-43	Intrinsically disordered
Huntingtin fragments	Huntington's disease	Variable	Mostly intrinsically disordered
Amylin	Type II diabetes	37	Intrinsically disordered
TDP43	Amyotrophic lateral sclerosis	414	Intrinsically disordered
Prion protein	Prion disease Creutzfeld-Jacob disease Bovine spongiform encephalopathy	231	Intrinsically disordered and α-helical

1.4. Parkinson's disease and α-synuclein

In 1997 two breakthrough discoveries shed light onto the molecular basis of PD. The first one connected the rare, familiar form of PD to a missense mutation in the gene that codes for a small, relatively unknown protein, α-syn, consisting of 140 amino acid residues [28] (SNCA).

The second one brought to light that LBs and neurites of PD patients were immunoreactive for α-syn [7]. After the first identified missense mutation (Ala53Thr) appeared to be associated with dominantly inherited PD with Lewy pathology, two other missense mutations, Glu46Lys [29] and Ala30Pro [30], were found to cause PD or dementia with LBs. The abovementioned mutations are positioned in the α-syn region made of seven 11-amino-acid repeats with a common KTKEGV sequence [31]. Residues 1-60 (Figure 1.1) make up the positively charged amino-terminal domain of α-syn, which contains 5 repeats of this sequence [31]. The central domain of α-syn, *i.e.*, residues 61-95, is described as the "non-amyloid-beta-component" (NAC) of Alzheimer's disease plaques [32], and is now identified as the hydrophobic part that is responsible for α-syn aggregation involving β-sheet formation [33,34]. The carboxyl-terminus of α-syn is rich in acidic residues, especially glutamic acid [31]. It has been suggested that particularly the negatively charged amino acids 104, 105, 114, 115 at the carboxylic-terminus reduce the α-syn aggregation propensity [35].

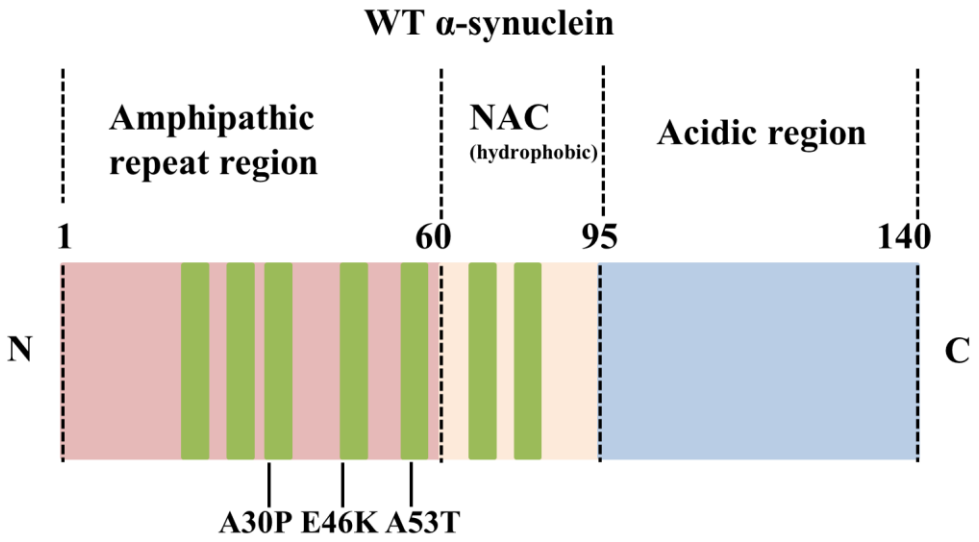

Figure 1.1: Primary structure of WT α-syn. The main domains: N (amphipathic), C-terminus (acidic) and NAC (hydrophobic) regions are indicated. The green marked regions are KTKEGV repeats.

1.5. The mechanism and molecular basis of α-synuclein aggregation

The aggregation of α-syn is thought to be a key aspect of the pathology of Parkinson's disease. The protein aggregation process is represented in a simplified way in Figure 1.2. Oligomers are intermediate species between the monomeric and the fibrillar from of the protein, and believed to be toxic, possibly by destructing the cell membranes [36–38]. The

oligomer formation is a rather obscure state of α-syn aggregation. It is still challenging to follow the dynamics of oligomer formation experimentally, due to their transient nature and their low concentration in the sample solution. It also remains unclear how the oligomers transform into fibrils and whether all oligomers have the same ability to do so. The oligomers that are involved in fibril formation are referred to as the on-pathway species (Figure 1.2).

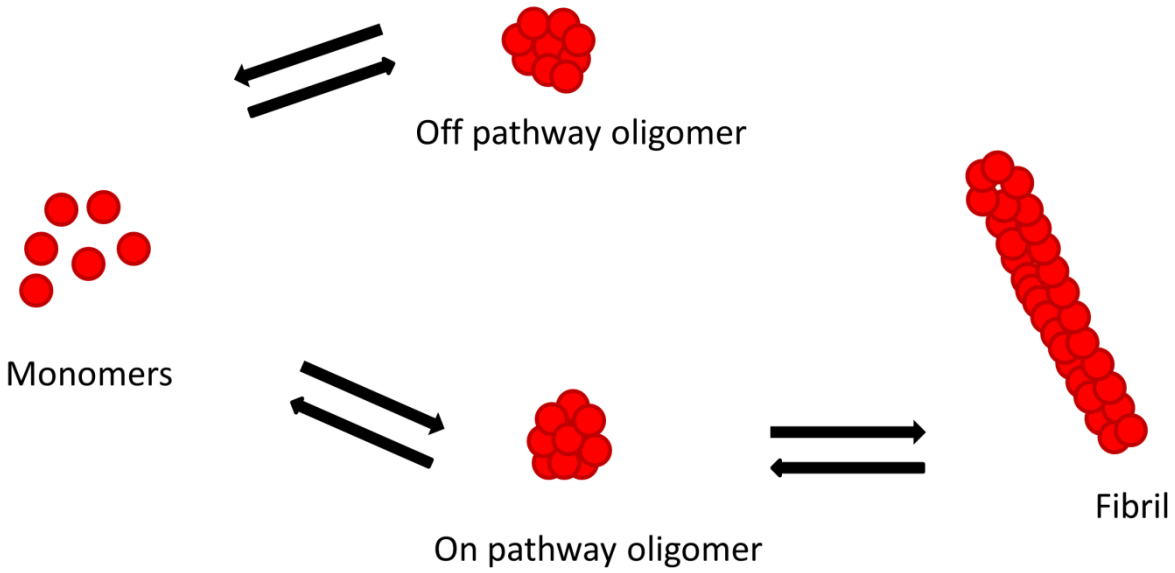

Figure 1.2: Simplified representation of the protein aggregation.

We can identify three stages in the process of α-syn aggregation, starting from a solution of α-syn monomers. The first stage is the nucleation phase with the (relatively slow) formation of small oligomers that eventually become precursors for fibril formation. Once these precursors are formed the aggregation process is dominated by fibril elongation and secondary nucleation. The latter mechanism involves, for example, fibril breaking, which is also the reason why the rate of fibrillar growth of α-syn in solution is enhanced by shaking the sample. Ultimately, equilibrium is achieved between monomer binding to and dissociation from the fibril ends.

Kinetic studies on fibril growth found a first-order dependence on monomer and fibril concentration [39]. Early α-syn aggregates formed through nucleation processes seem to elongate by addition of monomer to the existing fibrils ends [40,41]. There has been much speculation, and recently increasing evidence, that growth may also occur through oligomer addition [42], although it has been shown that the concentration of oligomers does not depend linearly on monomer concentration.

In bulk studies of α-syn elongation, a fluorescent assay based on Thioflavin T (ThT) is often used to detect the presence of aggregates. Recently, single fibril growth measurements of fluorescently labeled α-syn, using total internal reflection (TIRF) microscopy [43] and two-color direct stochastic optical reconstruction microscopy [41,44] (dSTORM), have provided deeper insight into α-syn elongation kinetics.

1.6. Methods to determine kinetics of α-synuclein aggregation

1.6.1. Thioflavin T fluorescent assay

A widely used method to establish and detect the formation of amyloid aggregates, including α-syn, uses the fluorogenic dye ThT. In general, when ThT binds to β-sheet secondary structures such as those in amyloid aggregates, its fluorescence intensity is enhanced and the emission spectrum is red shifted. α-Syn aggregation can be monitored by measuring the increase of ThT fluorescence intensity as a function of time. A typical aggregation curve shows a sigmoidal increase of ThT fluorescence intensity described by the initial, so-called lag phase, followed by the exponential-like growth phase and the plateau or saturation phase. A typical example of such an aggregation curve is shown in Figure 1.3. The lag phase is associated with the primary nucleation, and its duration varies strongly from sample to sample because of the highly stochastic character of the nucleation and initial elongation processes which occur more or less simultaneously [45]. The elongation rate is often determined as the maximal slope of the sigmoidal curve [45].

1.6.2. TIRF microscopy

Although a huge leap in elucidating the α-syn aggregation kinetics was made by bulk kinetic experiments, the information at the single-fibril level was missing until recently. To better understand α-syn aggregation at the molecular level, high resolution imaging methods proved to be essential. Ban et al. showed the use of ThT staining and TIRF in monitoring elongation of amyloid proteins such are amyloid beta [46], glucagon [47], amylin [48,49] and β_2-microglobulin [50]. Wördenhoff et al. [43] applied TIRF microscopy using ThT fluorescence to follow α-syn growth and reported its elongation rate to be 8.6×10^3 $M^{-1}s^{-1}$. The experimentally determined elongation rate of α-syn is lower than that of other amyloid proteins. The lower elongation rate of α-syn might be related to its size, as it is longer compared to other amyloid proteins and its incorporation into the fibril core may take more time [51].

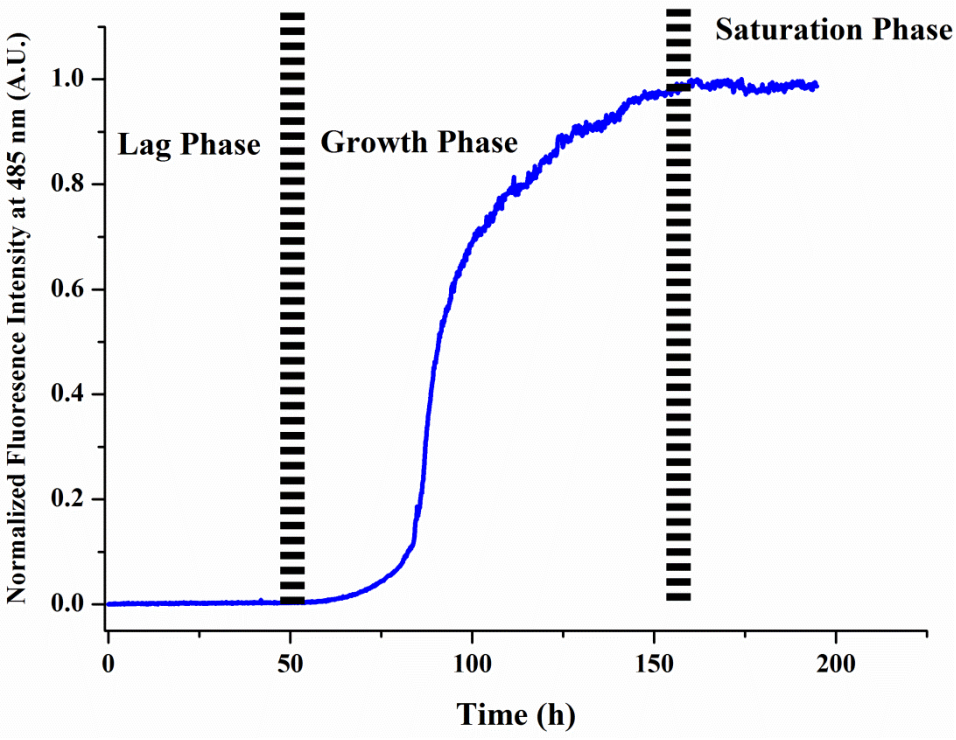

Figure 1.3: A typical aggregation curve monitored using ThT fluorescence intensity, showing lag, growth and saturation phases. The aggregation of 100 µM WT α-syn was performed in 6 mM sodium phosphate buffer at pH 7.4 with 0.1 mM EDTA, 1,000 rpm, and 37°C. Fluorescence spectra of ThT in solution were subtracted from each α-syn sample measurement.

1.7. The factors influencing α-synuclein aggregation

A large number of studies have reported extrinsic factors that markedly influence α-syn aggregation kinetics. Membranes [52], salts, pH, metal ions, temperature and shaking are just a few of them. Although intrinsic properties of the protein determine to a large extent the reaction rate, for example the net charge of α-syn, tuning the solution conditions, like pH or ionic strength [53], will modulate the effective charges of the protein and influence the screening of self-repulsion. Hoyer et al. [54] provided details on the differences in morphology of α-syn aggregates formed under different pH and NaCl concentrations by atomic force microscopy (AFM). Later on, the detailed study by Buell et al. [53] addressed similar solution conditions using bulk kinetic measurements and provided accurate rate constants for the process. An important question relates to the effect that membranes have on the α-syn aggregation process. Numerous recent studies have suggested their impact on the primary nucleation process [52] as well as the morphology of α-syn aggregates [55].

1.8. Thesis outline

In this thesis we describe the results of our study on α-syn aggregation *in vitro* with the help of several experimental approaches. As optical techniques were our main tool, the first step was the fluorescent labeling of α-syn.

Chapter 2 is dedicated to the influence that fluorescent dyes have on the morphology of α-syn fibrils. AFM images of wild type (WT) and the A140C α-syn mutant mature fibrils show a twisted, ribbon-like appearance with a pitch at 127 nm and an average height of 9.4 nm. The effect of fluorescent labels on the α-syn fibril morphology is remarkable, especially with increasing fractions of the labeled α-syn, affecting mostly the length of fibrils and their general architecture. The reason for significantly reduced fibril length with increasing fractions of labeled α-syn is most probably due to a change of the affinity of monomers to the fibril ends, while the short-range interactions between dyes attached to the C-terminus of the protein presumably plays an important role in the disappearance of the twisted morphology of the α-syn fibrils.

Chapter 3 focuses on the isolation of early aggregates in α-syn aggregation. We succeeded in detecting dimers and tetramers using fluorescence-based imaging of gels and confirmed the presence of small species with additional methods. The results of diffusion time measurements by fluorescence correlation spectroscopy in the electrophoresed gels confirm the difference in molecular weight between isolated species. The intensities of the bands in electrophoresed gels allow the assessment of the time profile of species accumulation and disappearance during the aggregation process.

Chapter 4 describes a systematic study on the relationship between α-syn aggregation kinetics, ionic strength and pH. We use bulk kinetic measurements in a plate reader to elucidate this important phenomenon. Since the initial elongation rate decreases significantly with increasing ionic strength (more than 30 mM NaCl), we assign an important role to electrostatic interactions in the α-syn aggregation process.

Real-time TIRF imaging was performed to monitor fibril elongation in order to determine the rate of monomer binding to fibril ends, and, subsequently, to establish the average fibril length in time. Surprisingly, our findings reported in **Chapter 5** show that it is not possible to describe the evolution of fibril growth according to the existing 3-step amyloid formation model. In fact, our results suggest that, in time, many α-syn fibrils tend to lose their ability for

binding additional monomers. For this reason, we introduced quenching rate constant (k_q) in the kinetic model to analyze our results.

Chapter 6 addresses the impact of the substrate surface on the morphology of α-syn aggregates. When studying the aggregation process in real-time by TIRF, significant differences appeared between the species formed on a charged surface and on a zwitterionic supported lipid bilayer. The morphology of aggregates formed on the charged surface displayed the greater heterogeneity compared to the ones formed on neutral surface, suggesting a significant effect of substrate surface properties on the morphology of α-syn aggregates.

In the closing **Chapter 7** we summarize the work done in this thesis with highlighting the most important findings, and present prospects for future work.

1.9. References

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