α-Synuclein Phosphorylation and Nitration in Parkinson's Disease

Stephanie Valtierra*
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Summary

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting six million people worldwide. PD results from the specific loss of substantia nigra dopaminergic neurons. Aggregation of one protein, α-synuclein, is characteristic of PD. This aggregation is thought to be a critical step in the etiology of the disease. While the molecular mechanism of α-synuclein aggregation remains unknown, nitrative stress and phosphorylation have been implicated in α-synuclein modification and aggregation. In fact, nitration of α-synuclein tyrosine residues 39, 125, 133 or 136, may be an early event in aggregates, Lewy bodies, seen in PD. Furthermore, nitrative stress leads to the induction of α-synuclein aggregation at a higher rate than seen in other PD mutants. This aggregation may result from a stabilization of pre-assembled α-synuclein filaments, which, upon nitration, may withstand denaturing conditions and enhance formation of SDS-insoluble, heat-stable high mass aggregates. Phosphorylation of a-synuclein also appears to play a critical role in the formation of aggregates. Extensive studies indicate that α-synuclein found in PD patient brains is extensively phosphorylated. Phosphorylation of ser-129 may enhance formation of aggregates reminiscent of Lewy bodies *in vitro* and *in vivo*. Nitration and phosphorylation of α-synuclein may be key to the mechanisms underlying the formation of Lewy bodies in PD.

Introduction

Parkinson's disease (PD) is a common, fatal neurodegenerative disease that affects millions worldwide. This disease, characterized by postural instability, resting, and bradykinesia affects 1 in 50 individuals over the age of 60 (Olanow and Tatton, 1999; Periquet *et al*, 2007). There are two forms of PD: sporadic and familial. The sporadic form of PD is the most common, constituting ~90-95 of PD cases. The remaining 5-10% of PD cases are familial (Dauer *et al.,*2003). Although there is an increasing understanding of PD and its causes, there is still much to be investigated. Our knowledge is still very limited, and because of that, there is unfortunately still no cure for this tragic affliction.

Pathology

Both familial and sporadic forms of PD are linked to the death of midbrain dopaminergic substantia nigra neurons, which accumulate as misfolded and aggregated protein α-synuclein (Lee *et al.,*2004). As these cells die, there is diminished release of the neurotransmitter dopamine, which regulates the activity of parts of the brain that control movement initiation and coordination (Parkinson's disease, 2007). This diminished amount of dopamine renders patients with a decreased ability to control and initiate movement, resulting in the clinical manifestations of PD.

Upon autopsy, neurons of the substantia nigra are found to contain large filamentous aggregates called Lewy bodies. The major component of Lewy bodies is a protein called α-synuclein (Periquet *et al.,*2007).

Characteristics of α-Synuclein

This natively unfolded protein of 140 amino acids has three major regions. The first major region of this protein is an N-terminal amphipathic region consisting of amino acids 1-61, while the central region consists of amino acids 61-95 (Periquet *et al.,*2007). Deletion of this region prevents α-synuclein aggregation *in vivo* and *in vitro,* suggesting that this region is essential for aggregation of the protein (Periquet *et al.,*2007). Finally, the third region, a highly acidic C-terminal region consists of amino acids 95-140. This C-terminal region may have an inhibitory role in the aggregation of α-synuclein, as C-terminally truncated forms of α-synuclein are found to aggregate into filaments more readily than full-length wild-type α-synuclein (Periquet *et al.,*2007). About 15% of Lewy bodies contain C-terminally truncated α-synuclein (Gaisson *et al.,*2000).

This highly conserved protein of unknown function is found throughout the central nervous system and is abundant in neurons, especially in pre-synaptic terminals (Gaisson *et al.,*2000). As previously stated, the aggregation and misfolding of this protein contributes to the formation of Lewy bodies, which are hallmarks of the disease.

Recent evidence has shed light on several post-translational events that can influence protein folding and function. This review will focus on the nitration and phosphorylation of α-synuclein. An emphasis will be made on the affects of the previously mentioned post-translational modifications on α-synuclein and the link of these modifications on pathology of the disease.

Phosphorylation of α-synuclein

Much research has gone into understanding the mechanism of Lewy Body formation and PD pathogenesis. While it is known that phosphorylation plays a key role in the functional properties of several proteins, there is limited knowledge on phosphorylation on PD-associated α-synuclein (Okoshi *et al.,*2000)

Initially, specific post-translational modifications that underlie the aggregation of α-synuclein were not known. More recently, however, our knowledge has increased in regards to these post-translational modifications due to several studies that have been conducted using a variety of models.

*This author wrote the paper based on original scholarship conducted in BIO 493, taught by Dr. Shubhik K. DebBurman.

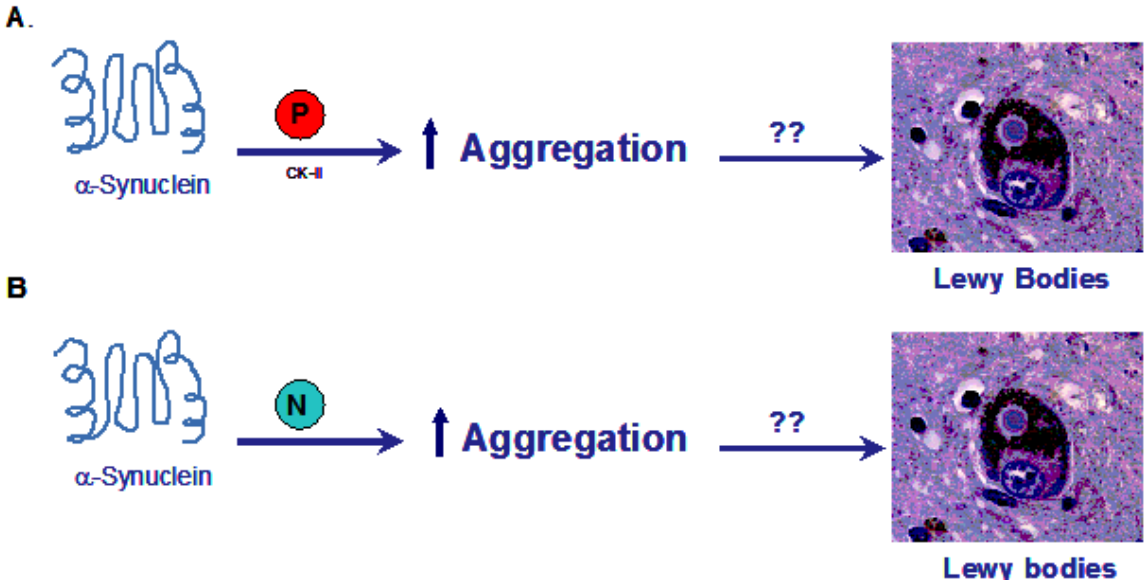

Figure 1. A) Effects of α-synuclein phosphorylation : α-synuclein is a natively soluble, unfolded protein characterized by the lack of rigid, well-defined three-dimensional structure. Pre-synaptic α-synuclein aggregates to form Lewy bodies, the filamentous agrregates that characterize PD. Phosphorylated α-synuclein have been found in Lewy bodies in PD patient brains. α-synuclein is phosphorylated by casein kinase II at residue 125, and is phosphorylated at residues 87 or 129. These post-translational modifications results in increased aggregation of α-synuclein.
B) Effects of α-synuclein nitration: Nitrated α-synuclein have been found in Lewy bodies in PD patient brains. a-synuclein is nitrated at residues 39, 125, 133, or 136. This post-translational modifications has been found to result in increased aggregation of α-synuclein and may result in the presence of agrregates reminiscent of Lewy bodies.

Phosphorylation of Serine and Tyrosine

α-synuclein is phosphorylated *in vitro* at several residues, including serines-87, and 129. These residues are phosphorylated by casein kinase 1 (CK-1) and casein kinase 2 (CK-2), but not by two other kinases, PKA or PKC (Okochi *et al.,*2000).

Tyrosine residues are phosphorylated as well. A comparison of α-synuclein family members reveals that all four tyrosine residues of α-synuclein are conserved in all orthologs. This conservation suggests that these tyrosine residues may be of functional importance. *In vitro* analysis of α-synuclein demonstrates that tyrosine phosphorylation occurs primarily on tyrosine 125 by Src protein-tyrosine kinase family members (Ellis *et al.,* 2001).

Using purified α-synuclein from insoluble fraction of Lewy bodies, Fujiwara et al. established that Ser-129 of α-synuclein is selectively and extensively phosphorylated in PD aggregates. Furthermore, phosphorylation of α-synuclein promotes the formation of fibrils *in vitro,* establishing the importance of phosphorylation in the pathogenesis of Parkinson's disease (Figure 1A.) (Fujiwara *et al.,*2002). Recent studies of Lewy bodies confirm Fujiwara's findings that Ser-129 is extensively phosphorylated. This modification was found to be the dominant pathological modification of α-synuclein in Lewy bodies (Anderson *et al.,*2006)

α-Synuclein or Synphilin-1?

While many studies focus on α-synuclein phosphorylation and its consequences, another study points to another protein's phosphorylation as playing a critical role in aggregate formation.

α-Synuclein, along with the protein synphilin-1, are found as components of Lewy bodies. The interaction between these two proteins is found to be phosphorylation dependent, as inhibition of CK-2, a kinase previously established to phosphorylate α-synuclein, results in an inhibition of binding of the two proteins. Furthermore, the inhibition of α-synuclein and synphilin-1 binding results in a significant decrease in the percentage of cells that contain cytoplasmic aggregates (Lee *et al.,*2004). Interestingly, mutations in α-synuclein that result in no α-synuclein phosphorylation have no effect on α-synuclein/synphilin-1 interaction or aggregate formation. These results suggest that the interaction of these two proteins is phosphorylation dependent and that phosphorylation of synphilin-1, not α-synuclein is critical for the formation of cytoplasmic aggregates (Lee *et al.,* 2004).

While one study implicates synphilin-1 phosphorylation as key to aggregate formation, α-synuclein, the major component of Lewy bodies, is still of major interest and many studies still focus on this protein in an effort to understand the pathogenesis of PD. Using neuroblastoma cells, Smith and colleagues examined the effect of α-synuclein phosphorylation on aggregate formation. Co-expression of mutant S129A of α-synuclein, which abolishes phosphorylation and synphilin-1, results in few or no aggregates forming. Furthermore, co-immunoprecipitation assays reveal that there is a decreased interaction between α-synuclein and synphilin-1. Together, these findings suggest that α-synuclein phosphorylation is important for α-synuclein/synphilin interaction, as well as aggregate formation (Smith *et al.,* 2005).

Previous attempts at uncovering mechanisms for aggregate formation have been done in different

cells *in vivo* and *in vitro*. One study, however, uses a *Drosophila* model in order to examine the role of α-synuclein phosphorylation, specifically the phosphorylation of serine 129, on neurotoxicity and aggregate formation. When serine 129 is altered to a negatively charged residue, aspirate, mimicking phosphorylation, significant α-synuclein toxicity is observed (Chen *et al.,* 2005). Other studies, however, show that phosphorylation and nitrosylation-deficient mutants are toxic to yeast (Herrera *et al.,* 2005).

In vivo, α-synuclein is phosphorylated by G-protein receptor kinase (Gprk2). Moreover, blocking the phosphorylation of α-synuclein results in a progressive accumulation of cytoplasmic aggregates resembling Lewy bodies (Chen *et al.,* 2005).

α-synuclein Nitration

Nitrative stress caused by oxidative injury has been implicated in the pathogenic mechanism of PD (Souza *et al.,* 2000; Takahashi et al, 2002). Previous studies revealed the presence of nitrated proteins in several neurodegenerative disorders, and available evidence support the notion that α-synuclein is a target for nitration (Souza *et al.,* 2000; Gaisson *et al.,*2000).

Nitration's Link to Formation of Aggregates

While it is known that α-synuclein is nitrated in PD aggregates, the effects of this nitration remained to be elucidated (Duda *et al.,*2000). Initial studies focused on attempting to determine the effects of α-synuclein nitration of the protein and its' aggregation. Exposure of human recombinant α-synuclein to nitrative and oxidative agents results in the formation of α-synuclein aggregates (Souza *et al.,* 2000; Paxinou *et al.,* 2001). These aggregates are in the form of dimers, trimers and oligomers, suggesting cross-linking between nitrated tyrosine residues (Souza et al. 2000; Takahashi *et al.,* 2002.)

To examine how concentration of nitrating agent affects the formation of aggregates, Souza and colleagues expose the cells to an increasing proportion of nitrating agent (Souza *et al.,* 2000). Increasing the ratio of nitrating agent, such as peroxynitrtie/CO_2, to protein not only results an increased nitration of dimers and trimers, but also in an augmentation in insoluble oligomers. Further examination of the protein reveals that it is nitrated at tyrosine 39, as well as tyrosine 125, 133, and 136. Tyrosine-125, however, appears to have a critical role in α-synuclein dimerization, as a lack of this residue significantly decreases dimerization compared to α-synuclein lacking tyrosine 39, 133 or 136 (Souza *et al.,*2000).

Effects of Nitrative Stress

Previous studies have established that nitrative and oxidative stress lead to the formation of aggregates in α-synuclein transfected cells. It has been hypothesized that tyrosine cross-linking may be the cause of this event. To test this hypothesis, Norris *et al.* mutated tyrosine residues and examined fibril formation in these mutant proteins. Results showed that one or more tyrosine residues are required for cross-linking resulting from the exposure to nitrating agents. Tyrosine residues are not required, however, for cross-linking resulting from the exposure to oxidative agents. Furthermore, the formation of aggregates after nitrative and oxidative stress in cells requires tyrosine residues (Norris *et al.,* 2003).

Norris et al. propose a model to explain the role of nitration and oxidation in the formation of aggregates of α-synuclein. It is proposed that soluble-α-synuclein can be formed as a result of both nitration and oxidation. Nitration-induced oligomers and nitrated monomers, however, are incapable of assembling fibrils, the intermediate to aggregates. α-synuclein monomers and oligomers that have been modified due to oxidation, however, are capable of forming fibrils, as they undergo a structural change. These monomers and oligomers go from a α-helix or random coil conformation to a β-pleated sheet conformation. Sheets are then capable of assembling into protofibrils and eventually fibrils. Both nitrative and oxidative modifications resulting in cross-links cans stabilize the filaments, which can then aggregate with other intracellular proteins to form aggregates.

Further examination of the modification of α-synuclein upon nitration indicates that nitration leads to α-synuclein's formation of a rapidly folded conformation of the protein with an increased secondary structure. This modification is a critical step in the formation of fibrils, as a non-modified protein does not have the ability to fibrillate.

Lipid Binding

Nitration has been found to modify α-synuclein's structure and aggregate formation. Recent studies also implicate nitration as the cause of diminished lipid binding to vesicles. This is important because the interaction of α-synuclein with lipid vesicles is believed to be critical in the regulation of neurotransmission at presynaptic terminals. Examination of the protein in the presence of lipid vesicles shows that purified monomer-nitrated α-synuclein did bind to lipid vesicles at a diminished rate.

Furthermore, association of α-synuclein with biological membranes protects the protein from oxidation and nitration, thereby decreasing the formation of molecules able to form aggregates.

Summary: Effects of α-synuclein modification

Several studies have focused on examining Lewy bodies. PD aggregates are phosphorylated at several resides; however, ser-129 is the major phosphorylation site of α-synuclein in Lewy bodies (Fujiwara *et al*., 2002). CK1, CK2 and Gprk2 and Src protein-tyrosine kinase family members phosphorylate the protein *in vitro* (Chen et al., 2005; Ellis et al., 2001; Okochi et al., 2000). The interaction between α-synuclein and another protein synphilin-1 is critical to aggregate formation and is phosphorylation dependent (Lee *et al*., 2005). The question of which protein's phosphorylation is crucial to this aggregation is still under debate (Lee *et al*., 2004; Smith *et al.,* 2005)

Nitrated α-synuclein has also been shown to be a major component of Lewy bodies. The protein is nitrated at tyrosine 39, as well as tyrosine 125, 133, and 136. Tyrosine-125 though, appears to have a critical role in α-synuclein dimerization, as a lack of this residue significantly decreases dimerization compared to α-synuclein lacking tyrosine 39, 133 or 136 (Souza *et al.,*2000).

α-synuclein has been found to be modified as a result of nitration and phosphorylation. Modifications include the change in conformation seen in the protein,

which allows it to aggregate more readily. The nitration and phosphorylation of α-synuclein result in the increased aggregation of the protein (Chen *et al.,* 2005; Norris *et al.,* 2003; Smith *et al.,* 2005; Fujiwara *et al.,* 2002).

The relation of aggrigation to toxicity has yet to be discovered. There are still conflicting studies concerning aggregate formation and its correlation to toxicity. While some studies suggest that phosphorylation of the protein increases aggregate formation and increases toxicity (Chen *et al,* 2000),

Future Research

As previously stated, the link between aggregation and toxicity in post-translation mutants is still under debate. Post-translational modification, such as phosphorylation and nitration, increase the rate of fibrillation and aggregate formation of α-synuclein. If this increased aggregation is linked to diminished toxicity, then perhaps these modifications serve a protective purpose.

Moreover, it is necessary to understand if there is a relationship between post-translational modifications and neurodegeneration, as this relationship is not yet fully understood. nitrosylation-deficient mutants exhibit significant toxicity (Herrera *et al.*, 2005).

Conclusion

Parkinson's disease is the second most common neurodegenerative disease in the world, affecting millions of people. Extensively phosphorylated and nitrated α-synuclein has been found in PD aggregates known as Lewy Bodies. Recent studies show that these post-translational modifications of α-synuclein increase the rate of fibril formation and increase the rate of aggregate formation. Examinations of the role of phosphorylation and nitration on toxicity have not come to conclusive results, therefore, future research is necessary to understand these post-translational modifications' role in neurodegeneration.

Research has shed light on the effects of phosphorylation and nitration of α-synuclein on this protein's aggregation and misfolding, providing important evidence of this protein's role in the pathogenesis of PD.

Acknowledgments

I would like to thank Dr. Shubhik DebBurman and Michael White in their help for writing this manuscript.

References

Anderson, J.P et al. 2006. Phosphorylation of Ser-129 is the dominant pathological Modification of α-synuclein in familial and sporadic lewy body disease. Journal of Biological Chemistry 281 (40): 29739-29752.

Arawaka S. 2006. The role of G-protein-coupled receptor kinase 5 in pathogenesis of sporadic Parkinson's disease. Journal of Neuroscience 26(36):9227-38.

Chen, Li & Feany M.B. 2005. A-synuclein Phosphorylation controls neurotoxocity and agrregate formation in a Drosophila model of Parkinson's Disease. Nature Neuroscience 8 (5): 657-663

Dauer W. & Przedborski S. 2003. Parkinson's Disease: Mechanisms and models. Neuron 39: 889-909.

Duda, J.E. et al. 2000. Widespread nitration of pathological agrregates in neurodegenerative synulceinopathies. American Journal of Pathology 157(5):1439-1445.

Ellis C.E, et al 2001. A-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases. The Journal of Biological Chemistry 276 (6): 3879-3884.

Fujiwara H et al. 2002. A-synuclein is phosphorylated in synocleinopathy lesions. Nature Cell Biology 4: 160-164.

Giasson B.I. et al 2000. Oxidative damage linked to neurodegeneration by selective α-synuclein nitration in synocleinopathy lesions. Science 290: 985-989.

Hasegawa M et al. 2002. Phosphorylated α-synuclein is ubitiquinated in α-synucleinopathy lesions. The Journal of Biological Chemistry 277: 49071-49706.

Herrera S. & Shrestha R. 2005. Newly discovered α-synuclein familial mutant E46K and key Phosphorylation and nitrosylation-deficient mutants are toxic to yeast. Eukaryon 1 : 95-101.

Hirai H. 2004. Phosphorylated α-synuclein in normal mouse brain. FEBS Letters. 572(1-3):227-32.

Hodora R. et al. 2004. Functional consequences of α-synuclein tyrosine nitration. Journal of Biological Chemistry 279: 47746-47753.

Kahle P.J. et al. 2002. Hyperphosphorylation and insolubility of α-synuclein in transgenic mouse oligodendrocytes. Embo Reports 3: 583-588.

Ishii A. et al. 2007. Casein kinase 2 is the major enzyme in brain that phosphorylates Ser129 of human α-synuclein: Implication for α-synucleinopathies. FEBS Letters. 581(24):4711-7.

Negro A. et al 2002. Multiple phosphorylation of α-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation. FASEB Journal. 16(2):210-2.

Norris E.H. et al 2003. Effects of oxidative and nitrative challenges on α-synuclein fibrillogenesis involve distinct mechanisms of protein modifications. Journal of Biological Chemistry 278: 27230-27240.

Okoshi M. et al. 2000. Constitutive phosphorylation of the Parkinson's Disease associated α-synuclein. Journal of Biological Chemistry 275: 390-397.

Paxinou E. et al. 2001. Induction of α-synuclein aggregation by intracellular nitrative insult. The Journal of Neuroscience 21: 8053-8061.

Payton T.E. et al. 2004. Structural determinants of PLD2 inhibition by α-synuclein. Journal of Molecular Biology. 337(4):1001-9.

Periquet M. et al. 2007. Aggregated α-synuclein mediates dopaminergic neurotoxicity in vivo. The Journal of Neuroscience 27: 3338-3346.

Reynolds M.R et al. 2007. Nitration in neurodegeneration: Deciphering the "Hows" "nYs" Biochemistry 46:7325-7336.

Saito Y. et al. 2003. Accumulation of α-synuclein in aging human brain. Journal of Neuropathology and Experimental Neurology. 62: 644-654.

Sasakawa H., 2007. Ultra-high field NMR studies of antibody binding and site-specific phosphorylation of α-synuclein. Biochemical and biophysical research communication. 363(3):795-799.

Smith W.W. et al 2005. A-synuclein phosphorylation enhances eosinophilic cytoplasmic agrregate formation in SH-SY5Y cells. The Journal of Neuroscience. 25: 5544-5552.

Souza J.M. et al 2000. Dityrosine cross-linking promotes formation of stable α-synuclein polymers. Journal of Biological Chemistry. 275: 18344-18349.

Takahashi M et al. 2003. Phosphorylation of α-synuclein characteristic of synucleinopathy lesions is recapitulated in α-synuclein transgenic Drosophila. Neuroscience Letters. 336: 155-158.

Takahashi M. et al. 2006. Identification of G-protein coupled receptor kinase 2 in paired helical filaments and neurofibrillary tangles. Journal of Neuropathology and Experimental Neurology. 65(12):1157-69.

Takasuya T. et al 2002. Tyrosine 125 of α-synuclein plays a critical role for dimerization following nitrative stress. Brain Research 938: 73-80.

Trostchansky A. 2006. Interaction with phospholipids modulates α-synuclein nitration and lipid-protein adduct formation. Biochemistry Journal. 393: 343-349.

Uversky V.N. 2005. Effects of nitration on the structure and aggregation of α-synuclein. Molecular Brain Research. 134: 84-102.

Uversky V.N. 2007. Neuropathology, biochemistry, and biophysics of α-synuclein aggregation. Journal of Neurochemistry 103: 177.

Wakamatsu M. et al. 2007. Accumulation of phosphorylated α-synuclein in dopaminergic neurons of transgenic mice that express human α-synuclein. Journal of Neuroscience Research. 85(8):1819-25.

Windisch M. et al 2007. Is α-Synuclein Pathology a Target for Treatment of Neurodegenerative Disorders? Current Alzheimer's Research. 4(4):446-57

Yamada M. et al. 2004. Overexpression of α-synuclein in rat substantia nigra results in loss of dopaminergic neurons, phosphorylation of α-synuclein and activation of caspase-9: resemblance to pathogenetic changes in Parkinson's disease. Journal of Neurochemistry. 91(2):451-61.