Effects of Interaction Between Tyrosine and Methionine on Methionine Oxidation and Fibrillation of Alpha Synuclein

Introduction

Alpha-synuclein is a protein naturally found in the central nervous system. Its functions are still not well understood, though there have been several proposals in recent literatures. It is suggested that alpha-synuclein may play role in regulating synaptic vesicles at synapse terminals, remodeling membranes, interacting with mitochondria, control synaptic plasticity, etc. (1) Alpha-synuclein consists of 140 amino acids, of which a long-range intra-molecular interaction between the ending of C-terminus (residue 120 -140, very acidic, negatively charged) and the central region (residue 30 – 100, slightly positive) is frequently observed. It was suggested that this interaction is due to the difference in charges between the two regions, and acts as an auto-inhibitory factor toward the aggregation of the protein. (2)

Alpha-synuclein is a major component in Lewy bodies, formation of which is recognized as the hallmarks of Parkinson's Disease. Pathological aggregates of the protein are usually found in the form of beta-sheet rich amyloid fibrils (1). Recent findings suggest that methionine oxidation in alpha-synuclein controls the aggregation levels of the protein (3) (4). Additionally, imbalance between the oxidized and non-oxidized form of the protein in vitro can also influence the fibrillation kinetics of the protein (3). The core of the fibrillary filaments of alpha-synuclein is composed largely of the central region (residue 31 to 109) of the protein. Additionally, the ability of alpha-synuclein to form beta-sheet rich aggregates is thought to be promoted by its hydrophobic non-amyloid component (NAC) region (residue 61 to 95) (5).

Recently, it has been proposed that oxidative stress is an important risk factor leading to the aggregation of alpha-synuclein and degeneration of dopaminergic neuron in Parkinson's Disease (6) (7). Apha-synuclein found in Lewy bodies purified from Parkinson's patient is abundant in oxidative post-translational modification – nitrated tyrosine, oxidized methionine, etc (8). Available reactive oxygen species (ROS) in physiological environment to react directly or indirectly with the amino acid residues in the protein includes superoxide, hydrogen peroxide, and hydroxyl radicals, etc. (6) Due to the under-presence of aromatic and sulfur-containing residues (such as tryptophan and cysteine) in alpha-synuclein, methionine is the most reactive residue toward oxidation (3).

Methionine is reversibly oxidized to methionine sulfoxide by addition of an oxygen to sulfur atom, and further oxidized irreversibly to methionine sulfone. In alpha-synuclein, there are 4 methionine residues (2 at N-terminus- Met1 and Met5, and 2 at C-terminus- Met116 and Met127). While oxidation of Met1 and Met5 can be repaired by methionine sulfoxide reductases (Msrs), oxidation of Met116 and Met127 is not (8). Between Met116 and Met127, the latter is shown to be the more preferred target for oxidation (9).

Particularly in alpha-synuclein, methionine oxidation is found to significantly inhibit the aggregation of the protein. Incubation of methionine-oxidized alpha-synuclein at physiological pH shows little evidence of aggregation, measured by ThT assay. Besides, it was observed that the magnitude of this inhibition effect is proportional to the number of methionine residues that are oxidized (3). Also, methionine-oxidized alpha-synuclein can interact with its non-oxidized form and create soluble hetero-oligomer. Upon addition of methionine-oxidized alpha-synuclein to its non-oxidized form at ratio 2:1, lag-time till aggregation greatly increases; and upon further addition of methionine-oxidized form (at ratio 4:1), aggregation of the protein is completely inhibited (3). Hence, the kinetics of the interaction between methionine and oxidative species remain an important aspect when studying fibrillation of alpha-synuclein.

In addition to the influence of reactive oxygen species available in biological environment, methionine is also subject to interaction with nearby amino acids. Lately, interaction between methionine and aromatic moieties (S-aromatic motif) has been shown to be very common among intra-protein interaction (10). Since alpha-synuclein is lack in tryptophan residues, most of the interaction of this type is between the divalent sulfur atom of methionine and the aromatic ring from tyrosine. Through modeling the DMS-Benzene interaction – analogous to sulfur-aromatic motif in methionine and tyrosine interaction, it is suggested that this interaction is formed due to dispersion effects, which provide additional stabilization for the binding between two moieties (10). Beside stabilizing protein structure, especially in secondary and tertiary structure, it also stabilizes interaction between protein complexes, hence contributing to the function(s) of many proteins. Additionally, this S-aromatic motif interaction is also proposed to reduce the propensity of methionine to be oxidized by solvent (11). In the sequence of alpha-synuclein, there are four tyrosine residues – Tyr39, Tyr125, Tyr133, and Tyr 136- which are accessible to interact by the four present methionine residues.

By mutating tyrosine into alanine, it is possible to confirm if the interaction between tyrosine and methionine influence the oxidation of methionine, and hence, the fibrillation of alpha-synuclein. Here, we consider the tyrosine residues in proximity to Met127, the more targeted methionine between two methionines in C-terminus. The three tyrosines sequentially close to Met127 includes Tyr125, Tyr133, and Tyr 136. In addition, due to the intra-protein long range interaction in alpha-synuclein, Tyr39 is also considered to be interacted with Met127.

Methods

Four single mutants, including Y39A, Y125A, Y133A, and Y136A, as well as one quadruple mutant containing all former mutations Y39A/Y125A/Y133A/ Y136A were created by site-directed mutagenesis. Mutant proteins were overexpressed in bacteria. Collected proteins were purified by Ion Exchange Chromatography and Size Exclusion Chromatography, as well as confirmed afterward by electrophoresis with SDS-PAGE.

Thioflavin-T (ThT) assay was conducted to study fibrillation of alpha-synuclein mutants as well as oxidized wildtype and non-oxidized wildtype as control. ThT molecule has high

binding affinity to amyloid moieties. As the thiazole nitrogen from ThT molecule binds with hydroxyl groups from the amyloid, the free rotation about the shared carbon-carbon bond of the benzylamine and benzathiole ring is eliminate (12). Hence, an increase in fluorescence intensity is detected, compared to sample where no binding occurs.

Results

Alpha-synuclein DNA mutants were confirmed to contain the desired sequence. Confirmed sequence of the quadruple mutant are shown below with mutation points printed in bold.

ATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCTGCTGAG
AAAACCAAACAGGGTGTGGCAGAAGCAGCAGGAAAGACAAAAGAGGGTGTTCTC**GCG**GTAGGCTC
CAAAACCAAGGAGGGAGTGGTGCATGGTGTGGCAACAGTGGCTGAGAAGACCAAAGAGCAAGTG
ACAAATGTTGGAGGAGCAGTGGTGACGGGTGTGACAGCAGTAGCCCAGAAGACAGTGGAGGGAG
CAGGGAGCATTGCAGCAGCCACTGGCTTTGTCAAAAAGGACCAGTTGGGCAAGAATGAAGAAGGA
GCCCCACAGGAAGGAATTCTGGAAGATATGCCTGTGGATCCTGACAATGAGGCT**GCG**GAAATGCCT
TCTGAGGAAGGG**GCG**CAAGAC**GCG**GAACCTGAAGCC

Overexpressed protein mutants after purification with Ion Exchange Chromatography and Size Exclusion Chromatography (SEC) showed high purity and concentration, confirmed by SDS- PAGE gel. Alpha-synuclein sizes at 140kDa, and the bands seen on SDS gels below marked the desired molecular weight. After SEC, impurity in protein was significantly reduced, shown by decreasingly faint bands above the major of the proteins.

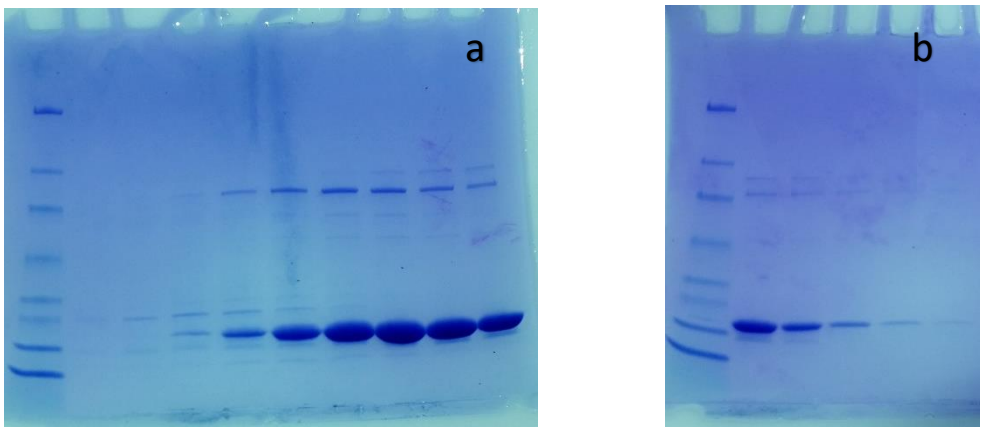

Figure 1: (a) SDS-PAGE gel after Ion Exchange Chromatography and (b) SDS-PAGE gel after Size Exclusion Chromatography confirm the purity of the overexpressed protein.

Discussions

As mentioned previously, methionine oxidation has an inhibiting effect on the aggregation kinetics of alpha-synuclein. Additionally, interaction between methionine and tyrosine (S-aromatic motif) has also been shown to reduce the tendency of the involved methionine toward oxidation, and contribute to protein structure stabilization.

Due to the common presence of the S-aromatic motif among many protein structures, it is expected that interaction between Met127 and four tyrosine residues (Tyr39, Tyr125,

Tyr133, and Tyr 136) also occurs in alpha-synuclein and influence its aggregation. In mutant protein, since, tyrosine has been mutated into alanine- which eliminates the presence of the aromatic ring originating from tyrosine structure, the protein mutants may not experience this S-aromatic motif interaction at Met127. Hence, compared to the wildtype, mutants are more likely to involve in methionine oxidation and decreased protein aggregation.

Statistically, among the mutants, since there has been no suggested evidence as of which tyrosine residues Met127 would preferably interact with, all presented single mutants should have same probability of methionine oxidation. Also, compared to wildtype, each mutant would have only slightly higher probability of oxidation, because they eliminate one of the four possible methionine-tyrosine interactions. Meanwhile, the quadruple mutant will have the highest probability of oxidation because it aborts all four possible S-aromatic motif interactions between Met127 and proximal tyrosine residues. Therefore, in the case where all other three methionines (Met1, Met5, and Met116) are oxidized, and S-aromatic motif occur with same probability among Met127 and four tyrosine residues, the protein mutants would more likely to undergo methionine oxidation than the wildtype; hence, they would have lower degree of fibrillation and lower intensity of fluorescence in ThT assay. In contrast, when consider the possible different probability of interaction between Met127 and the four tyrosine residues, the results predicted above may not hold. Under such circumstance, this fibrillation study (upon eliminating influence from other methionine residues) can conceivably predict the preference of Met127 for one/more of the four present tyrosines, and consequently, provide further understanding regarding the structure of the protein as well as the effects of the structure on intra-molecular interactions among amino acids.

Nevertheless, in alpha-synuclein fibrillation study, beside Met127, it is also essential to consider the effects of methionine oxidation at the other three methionine positions to the fibrillation of the protein Due to the complexity of long-range intra-molecular interactions present in secondary and tertiary protein structure (2), as well as the consequent interactions between the newly-proximal amino acids, and other solvent factors (solvent composition, residue accessibility, etc.) (13), each methionine may interact with nearby tyrosine residues and/or participate in oxidation with different propensity and to different extent. Therefore, they would contribute differently to the aggregation of alpha-synuclein. Further study on the interaction between methionine and tyrosine at other methionine residues may contribute to a more thorough and comprehensive view on the influence of this motif to the aggregation of alpha-synuclein. When accompanied by research on post-translational modifications consequential to these interactions, the study can provide insight on the mechanism behind the fibrillation of the protein as well as the progress of Parkinson's Disease.

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