Expression, Purification and Characterization of Recombinant Human α, β, and γ Synuclein Proteins Associated with Neurodegeneration

Synopsis Submitted by

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Introduction:

Neurodegenerative diseases are characterized by a number of brain disorders with different symptoms such as dementia, disordered movements etc. and often it occurs in later stages of life. Aggregation and deposition of misfolded proteins in brain cells is the main cause of these disorders (**Taylor et al., 2002**). The proteins involved in neurodegenerative disorders are characterized by the presence of intracellular or extracellular protein deposits in the form of proteinaceous fibrils or amyloids (**Caughey and Lansbury, 2003**). These deposits have a characteristic of cross-β structure and are defined by their distinct morphological properties (**Sunde and Blake, 1997; Sipe and Cohen, 2000)**.

Human synucleins are natively unfolded proteins and are characterized by low overall hydrophobicity and large net charge. The hydrodynamic radii of these proteins are closer to random coils than for globular proteins. However, these proteins may be significantly folded in their normal cellular milieu after binding to specific ligands (**Uversky, 2002, Davidson et al., 1998; Eliezer et al., 2001).** The human synucleins family (α, β and γ) are comprised of small proteins (~14k Da) and expressed at high levels in the neurons. These proteins are heat stable and relatively soluble (**George et al., 1995; Jakes et al., 1994**) and characterized by absence of cysteines and tryptophans.

α-synuclein (SNCA/ α-syn):

The α-gene has been specifically implicated in the Parkinson's (PD) and Alzheimer's (**Maroteaux et al., 1988; Jakes et al., 1994**) and has been found in the Lewy body inclusions in neurons **(Spillantini et al., 1998**). *In vitro* studies have revealed that α syn is more fibrillogenic than β and γ syn (**Biere et al., 2000**). Furthermore, α and β syn interact with each other and β syn inhibits α syn aggregation due to its chaperonic activity (**Uversky et al., 2002; Park and Lansbury, 2003**). α syn protein might also have a role in synaptic development, function and plasticity (**Clayton and George, 1998; Cabin et al., 2002**). α syn binds to lipid membranes (**Davidson et al., 1998**) and also interacts with other proteins such as synphilin (**Kawamata et al., 2001**) and tubulin (**Payton et al., 2001**).

β-synuclein (SNCB/ β-syn):

Human β syn is a 134 amino acid protein. β-syn is deficient in the non-amyloidogenic component (NAC) domain that is believed to be responsible for the aggregating properties of α-syn (**Jensen et al., 1995**). Therefore β-syn is considered to be a non-amyloidogenic homolog of α-syn **(El-Agnaf and Irvine, 2000**). It is postulated that β syn could act as a physiological inhibitor of α syn aggregation (**Hashimoto et al., 2001**).

γ- synuclein (SNCG/ γ- syn):

γ syn is believed to be involved in neurodegenerative diseases and in non-neural disease like breast cancer. γ syn is principally expressed in the brain, particularly in the substantia nigra. γ syn is primarily found in the peripheral nervous system (in primary sensory neurons, sympathetic neurons, motor neurons and retina) (**George, 1995**). It is also detected in the brain, ovarian tumors, and in the olfactory epithelium. γ syn is the least conserved of the synuclein proteins in comparison with the α syn and β syn (**Lavedan, 1998**).

Human synuclein proteins share high sequence similarities amongst themselves and are intrinsically disordered proteins (IDPs) in nature, having partially folded structure. The function of the human synucleins and their involvement in various synucleiopathies are not clear till date. While most of the studies are available for α-syn, few studies have been carried out on β-syn and γ-syn. In the present work, we have studied pH, concentration and temperature dependent conformational changes and aggregation behavior of the three human synucleins. We have also carried out studies on the effect of structure stabilizing cosolvent trehalose, known to stabilize the native state of the proteins, on the structure and aggregation of synucleins. In addition, the effect of Ca(II) and dopamine (DA), known to play a significant role in the signaling process, on the stability, aggregation, binding and cytotoxicity effect of synucleins has also been investigated. Various morphological changes in amyloids fibers in the above conditions have also been analyzed and the results correlated with the cytotoxicity effect on human neuroblastoma cells (SHSY5Y) of the species formed under different conditions.

Methodology:

Protein expression and purification

Expression and purification of the human recombinant α-Syn**,** β-syn, γ-Syn and familial mutants (A30P, E46K and A53T), H50A of human α-Syn from *E. coli* was carried out according to **Volles and Lansbury Jr., 2007** and the identity confirmed using Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) while the mutations were identified using DNA sequencing to know the exact position of the mutation.

Gel filtration chromatography

Gel filtration chromatography of the human recombinant α-Syn**,** β-syn and γ-Syn from *E. coli* was carried out using an AKTA Basic 10 FPLC instrument and a Hi Prep 16/60 Sephracryl S-200 HR column from GE Healthcare at 25ºC.

Spectroscopic measurements of human synucleins

Intrinsic absorbance of tyrosine and temperature-induced conformational changes of human synucleins was recorded using UV-visible spectrometric measurements on a Cary Varian UV-visible spectrometer attached with a peltier temperature controller. Fluorescence emission spectra in different conditions were recorded on a Cary Varian Eclipse fluorimeter attached with a peltier temperature attachment. Tyrosine was used as a probe for fluorescence studies. Acrylamide quenching studies of intrinsic tyrosine fluorescence were carried out by adding different concentrations of the quencher into a cuvette containing the protein solution and the additives at a particular concentration and data were analyzed using the Stern-Volmer equation. 8-anilino-1-naphthalene-sulphonic acid (ANS) binding spectra were recorded from 400-600 nm with excitation at 350 nm at 25˚C. The final ratio of ANS to protein concentration was equal to 5. Thioflavin T (ThT) fluorescence assay for fibril formation were carried out using Thioflavin T assay at a protein concentration of 1.0 mg/ml at 37°C, pH 7.5 and shaking at 200 rpm for several days. ThT fluorescence was excited at 445 nm and the emission intensity was recorded at 480 nm.

Circular Dichroism (CD) measurements:

Far-UV CD (190-260nm) and near UV-CD (250-350nm) spectra were recorded using Jasco J-815 model spectropolarimeter equipped with a peltier based temperature controller.

Thermal stability of the human synucleins using Differential Scanning Calorimetry (DSC):

DSC experiments were carried out using a VP-DSC instrument from Micorcal LLC., Northampton, USA. Protein samples (70 μM) are prepared in 10 mM phosphate buffer at pH 7.4 and dialyzed overnight against respective buffer.

Isothermal Titration Calorimetry (ITC) to calculate the binding affinity of Ca(II) with human synucleins:

The binding energetics of human synucleins and familial mutants A30P, E46K and A53T of α-syn with Ca(II) were studied using a isothermal titration calorimeter (VP-ITC) from Microcal LLC., Northampton, MA. Integrated heat data were fitted using the MicroCal Origin software.

Morphological analysis of aggregates using transmission electron microscopy (TEM):

Morphological states of aggregates have been observed using JEM-2100, JEOL Transmission electron microscope (TEM) by negative staining method.

MTT cytotoxicity assay using human neuroblastoma cells SHSY5Y:

Cell toxicity assay for various protein species formed during the aggregation pathway viz. oligomers, multimers, protofibrils and fibrils of human synucleins in presence of Ca(II), dopamine and trehalose were carried out using human neuroblastoma cells SH-SY5Y in a time dependent manner. Cell viability was determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) toxicity assay at 570 nm using Spectra Max 384 plus microplate reader from Molecular devices.

Results and Discussion:

Results and discussion section has been divided into the following chapters:

<u>Chapter 1:</u> Characterization of human synucleins (*α,* β and γ) on the basis of their conformational and fibrillation properties

Followed by expression of the recombinant human synucleins (α, β and γ), purity was confirmed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and SDS-PAGE. Native gel electrophoresis data indicate that β-syn is more structured than α-Syn while γ-syn is less structured than both α- and β-syn based on their mobility. Gel filtration chromatography of human synucleins denatured by 8M urea or 6M GdmCl resulted in the denatured protein eluting out first in the elution volume as compared to the native human synucleins suggesting increased hydrodynamic radius of the denatured protein compared with the native protein. Human synucleins have been structurally characterized using UV absorption and fluorescence spectrophotometry and CD spectroscopy. Differential scanning calorimetry (DSC) was employed to monitor thermal transitions and their reversibility. While β- and γ-syn were observed to be reversible after heating up to 100 ºC, α-syn was irreversible and aggregated at high temperatures. Extensive studies on conformational changes as well as aggregation propensity of human synucleins as a function of pH, temperature, concentration of protein and denaturants have been carried out. Low pH, high temperatures and high protein concentration promoted fibril formation. ANS binding studies at different pH values showed pH-induced transformation from the natively unfolded state to a partially folded compact conformation in α- and γ-syn. α syn was also observed to have the highest propensity for fibrillation. Morphological characterization of the aggregates was done by using TEM. On the basis of sequence analysis using ClustalW, human synucleins shared more than 70% sequence homology. Out of these three proteins, α- and β-syn share high homology with each other in comparison to γ syn. C-terminal of γ syn is less conserved due to which its properties are slightly different from α- and β-syn. However, β syn does not form amyloids under physiological conditions due to the absence of amyloid

forming residues in its sequence. Thus, by using bioinformatics sequence analysis tools together with spectroscopy and other biophysical techniques, we have been able look into the sequence-structure relationship in human synucleins.

Chapter 2: Effect of Trehalose, a naturally occurring osmolytes on the conformational and fibrillation properties of human synucleins

Human synucleins are disordered proteins at physiological pH and it is expected that structure can be induced by using structure stabilizing cosolvent additives or osmolytes. Protein stabilization by cosolvent additives has been extensively studied in our laboratory (**Kaushik and Bhat, 1999, 2003**). Most of the osmolyte driven stabilization studies have been carried out for globular proteins. We used trehalose-a strong structure stabilizing agent, produced in organisms under stress conditions, in order to study its effect on the conformational and aggregation properties of human synucleins. Based on intrinsic tyrosine fluorescence, ANS binding, acrylamide quenching, far-UV and Near-UV CD data, it was observed that trehalose induces conformational changes from natively disordered state to partially folded state of human synucleins. Contrast to expectations, trehalose enhanced the fibril forming propensity of human synucleins and in familial mutants (A30P, E46K & A53T) of human α syn in a concentration dependent manner as monitored by ThT assay. Fibril morphology in the presence of trehalose was characterized using TEM. Aggregation species (oligomers, protofibrils and mature fibril) formed in presence of trehalose was toxic to human neuroblastoma cells. It is interesting to note that in case of globular proteins, trehalose is known to inhibit aggregation (**Liu et al., 2005; Vilasi et al., 2008; Kaushik and Bhat, 1999, 2003**) while in the case of human synucleins trehalose promoted fibrillation. Trehalose is known to be preferentially excluded from the proteins surface and favors the compaction of the protein molecules in case of globular protein (**Xie and Timasheff, 1997**). Therefore, presence of trehalose would favor more compact forms of human synucleins than the intrinsically disordered state and these folded intermediate states of synucleins may form a nucleus for further association to lead to fibril formation. The formation of a compact, partially folded state of human synucleins, in presence of trehalose would, thus, result in the shifting of the equilibrium from intrinsically disordered state-intermediate partially folded state- ordered fibrillar state.

Chapter 3: Calcium and dopamine-induced conformational and morphological changes in human synucleins and their effect on the propensity of fibrillation

Ca(II) is known to play an important role in the propagation of the action potentials in neurons and release of neurotransmitters (**Berridge, 1998a).** Ca(II) interacts with C-

terminus of human α-syn and is involved in the signaling process (**Nielsen et al., 2001)**. Also oxidative stress is linked to an increase in the Ca(II) influx and intracellular Ca(II) levels in the brain cells that disrupt normal cellular processes (**Cuschieri et al., 2005**). It has recently been hypothesized that high cytoplasmic Ca(II), elevated cytosolic DA and α-syn expression induced selective death of dopaminergic neurons from substantia nigra (SN) region of mid brain (**Mosharov et al., 2009**). Considering the importance of Ca(II), we studied its effect on the conformational changes, binding, aggregation propensity along with morphological changes in the amyloids fibrils of human synucleins. Various fluorescence techniques like intrinsic tyrosine fluorescence, ANS binding was used to investigate the interaction of Ca(II) which confirmed that Ca(II) binds to human α and β syn. Far-UV CD showed little changes in the secondary structures. ITC measurements showed that Ca(II) binds to the α, β syn and the familial mutants of α-syn. These binding interactions are weak and endothermic in nature. While the binding constant for the interaction of Ca(II) with α and β syn were 2.2×10^{-3} M^{-1}, 0.9×10^{-3} M^{-1} respectively. There was no appreciable binding between γ- syn and Ca(II). Fibrillation propensity of human α, γ syn and the familial mutants of α syn except β syn are enhanced in the presence of Ca(II).

We also studied the effect of dopamine (DA) alone and with Ca(II) on the fibrillation kinetics of human α syn. DA alone inhibits the propensity of fibrillation and induces the formation of oligomers. However, the presence of DA with Ca(II) shifts the fibril formation equilibrium from oligomers to protofibril state which is more toxic in nature. Cytotoxicity was determined at different time periods of incubation of the proteins on the aggregation pathway in the presence of Ca(II), DA and DA with Ca(II) using human neuroblastoma cells (SHSY5Y). It has been observed that Ca(II) and DA-induced aggregation is more toxic to the cells.

In the neuronal cytoplasm Ca(II), DA and human α syn are present together. The homeostasis amongst these is very critical to the survival of the dopaminergic neurons in the substantia nigra of the mid brain. As Ca(II) is a positively charged molecule and interacts with the negatively charged C-terminal of the human α syn. The binding of Ca(II) leads to subtle conformational changes in the human α syn resulting in toxic fibrillar species.

In summary, the results obtained in the present study are useful to understand the sequence and structure relationship of the three human synucleins. Trehalose induced fibril formation in human synucleins suggested that the mechanism of action of structure stabilizing osmolytes like trehalose is different for intrinsically disordered proteins compared with well folded globular proteins. Human α syn Ca(II) and DA homeostasis is very critical

for survival of neurons in the midbrain and interference with any of these three factors may be very useful in survival of neurons. These studies altogether may be helpful in the design of strategies to treat this old age motor dysfunction disease. The studies further show that even though Ca(II) and DA bind weakly to α-syn, they can regulate critically the nature of aggregate formation tendency of the protein in terms of forming fibrils, protofibrils or oligomers which could play a role in the onset of Parkinson's disease.

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