# STUDIES ON DNA HELICITY, STABILITY AND α-SYNUCLEIN-DNA INTERACTIONS IN RELEVANCE TO PARKINSON'S DISEASE

***A Thesis submitted to the***

**UNIVERSITY OF MYSORE**

*for the award of the degree of*

**Doctor of Philosophy**
***in***
**BIOCHEMISTRY**

***by***

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**June 2005**

*Dedicated to*

*My parents*

*Poppa & Ayee*

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# DECLARATION

I hereby declare that the thesis entitled **"STUDIES ON DNA HELICITY, STABILITY AND α-SYNUCLEIN-DNA INTERACTIONS IN RELEVANCE TO PARKINSON'S DISEASE"** submitted to the **University of Mysore** for the award of degree of **Doctor of Philosophy** in **Biochemistry**, is the result of research work carried out by me under the guidance of **Dr. K.S. Jagannatha Rao**, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore - 570020, India, during the period 2001 – 2005. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date: **(MURALIDHAR L. HEGDE)**
Place: Mysore

# CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON DNA HELICITY, STABILITY AND α-SYNUCLEIN-DNA INTERACTIONS IN RELEVANCE TO PARKINSON'S DISEASE"** submitted by **Mr. Muralidhar L. Hegde,** for the award of **Doctor of Philosophy** in **Biochemistry** to the **University of Mysore** is the result of research work carried out by him in the Department of Biochemistry and Nutrition, under my` guidance during the period 2001 – 2005.

Date:
Place: Mysore

**(K.S. JAGANNATHA RAO)**
Guide
Dept. of Biochemistry and Nutrition

## TO WHOM SO EVER IT MAY CONCERN

**This is to certify that Mr. Muralidhar L. Hegde did doctoral degree work under my guidance during 2001 to 2005 and his Ph.D thesis entitled "STUDIES ON DNA HELICITY, STABILITY AND α-SYNUCLEIN-DNA INTERACTIONS IN RELEVANCE TO PARKINSON'S DISEASE" is submitted for the award of Doctor of Philosophy in Biochemistry to the University of Mysore on 27th June, 2005.**

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**Place: Mysore**

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# CONTENTS

## Abbreviations

| | |
|---|---|
| AD | Alzheimer's Disease |
| ANS | 1-aninilinonaphthalein -8-sulphonic acid |
| ATA | aurintricarboxylic acid |
| Aβ | amyloid β peptide |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| CD | circular dichroism spectroscopy |
| CSF | cerebrospinal fluid |
| DEPC | diethylpyrocarbonate |
| DL | detection limit |
| DLB | Dementia with Lewy bodies |
| DNAse I | deoxyribonuclease I |
| DSB | double strand breaks |
| dscDNA | double stranded circular DNA |
| E Coli | Escherichia coli |
| EDTA | ethylene diamine tetra acetic acid |
| EtBr | ethidium bromide |
| GPC | gel permeation chromatography |
| HEPES | N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid) |
| ICP-AES | Inductively Coupled Plasma Atomic Emission Spectroscopy |
| kDa | kilo daltons |
| LBs | Lewy body |
| LNs | Lewy Neurites |
| MSA | Multiple System Atrophy |
| NaCl | sodium chloride |
| NBT | nitroblue tetrazolium |
| NMR | nuclear magnetic resonance |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PD | Parkinson's Disease |
| PMI | post-mortem interval |
| RNAse A | ribonuclease A |
| scDNA | supercoiled DNA |
| SDS | sodium dodecyl sulphate |
| SN | substantia nigra |
| SNpc | substantia nigra pars compacta |
| SPs | senile plaques |
| SSB | single strand breaks |
| sscDNA | single stranded circular DNA |
| TEM | Transmission Electron Microscopy |
| Thio T | Thioflavin T, a fluorescent dye |
| TMAO | trimethylamine N-oxide |
| Tris | tris(hydroxymethyl) aminomethane |
| TUNEL | terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labelling |
| UPDRS | Unified Parkinson's Disease Rating Scale |

# *Synopsis*

***Synopsis of the thesis submitted for the award of Ph.D. degree (Biochemistry) to the University of Mysore, Mysore, India.***

***Title of thesis:***

**STUDIES ON DNA HELICITY, STABILITY AND α-SYNUCLEIN-DNA INTERACTIONS IN RELEVANCE TO PARKINSON'S DISEASE**

***Candidate:*** **Muralidhar L. Hegde**

Parkinson's disease (PD) is a progressive, neurodegenerative disorder that is characterized by the severe motor symptoms, including uncontrollable *tremor*, postural imbalance, slowness of movement and rigidity. The main pathological *hallmark* of PD is a pronounced loss of dopamine-producing neurons in the *substantia nigra* (SN), which results in a drastic decrease in dopamine in the striatum, to which these neurons project.

The etiology of PD has not been fully understood. Unproven hypotheses have included environmental toxins including metals, pesticides etc and genetic factors. It is proposed that a cross-talk of environmental and genetic factors may be playing a role in causing PD. A variety of mechanisms that are believed to cause accelerated cell death have also been suggested, including oxidative stress, excitotoxicity and mitochondrial dysfunction. Pathologically, PD is characterized by the loss of the pigmented dopaminergic neurons from the substantia nigra pars compacta, the presence of extracellular melanin (a dark pigment), released from degenerating neurons, reactive gliosis (increase in numbers of glial or support cells), and pink-staining cellular inclusions known as *Lewy Bodies.*

The exact causes and molecular pathogenesis of PD is largely undefined. However, accumulating evidence suggest the involvement of protein conformational deficit (α-synuclein toxicity), metal toxicity, oxidative stress, DNA instability etc. We focused on the DNA topology and stability and α-synuclein-DNA interactions in the present investigation.

The objectives of the present investigation were:

1. To study the helicity and stability (topology) of genomic DNA isolated from selected regions of Parkinson disease affected human post-mortem brain (*Chapter* 2)
2. To study the mechanism of DNA binding and nicking property α-synuclein (*Chapter* 3)
3. To study the conformation/aggregation of α-synuclein in presence of DNA and understanding the mechanism using the chaperon property of naturally occurring osmolytes (*Chapter* 4)
4. To map trace elemental homeostasis in the serum samples Parkinson's patients (*Chapter* 5).

The research work carried towards achieving these objectives makes the subject matter of the thesis. The thesis is divided in to five chapters.

### *Chapter 1.* General Introduction

This chapter begins with a general account of PD, followed by an overview of current literature on symptoms, causes, disease diagnosis, treatments available and the molecular mechanism of PD pathogenesis. The role of α-synuclein in PD has been discussed in detail. The chapter also highlights the aims and scope of the present study. We also provided a hypothesis on the complexity of α-synuclein toxicity in PD.

***Chapter 2.*** **Studies on Genomic DNA topology and stability in brain regions of Parkinson's disease**

Recently few studies showed that oxidative stress, DNA damage, chromatin condensation, and altered expression of genes are also associated with neurodegeneration in PD like Alzheimer's disease (AD). One of the consequences of redox imbalance is apoptosis and/or necrosis (programmed vs passive cell death) which are associated with neurodegeneration in PD. Studies have also shown that the levels of the nucleoside, 8-hydroxy -2'-deoxyguanosine (8-OHdG), a product of free radical attack on DNA were generally increased and differentially distributed in PD brains with highest levels in caudate, putamen, SN and cerebral cortex. Further, our laboratory evidenced that the genomic DNA undergoes a helicity change in Alzheimer's disease (AD) from B-form to left handed Z-DNA. These observations suggest that topological changes in the genetic material may be involved in the pathogenesis of PD also. So far no studies have been reported on the DNA conformation in PD affected brain cells.

**In this perspective**, we studied the stability, integrity and topology of DNA isolated from five clinically and neuropathologically confirmed PD cases and six age-matched controls. Genomic DNA was isolated from eight regions in the human brain, namely frontal, temporal and occipital cortex, hippocampus, caudate/ putamen, thalamus, cerebellum and midbrain collected at post-mortem from cases of PD and controls and were analyzed for single and double strand breaks in DNA, and their conformations and topology. The results showed that DNA from midbrain in PD accumulated significant number of strand breaks than age-matched controls. Caudate nucleus/ putamen, thalamus and hippocampus also showed more DNA fragmentation compared to control brains. Circular dichroism studies showed that DNA conformation was altered with imprecise base

stacking in midbrain, caudate nucleus/ putamen, thalamus and hippocampus in PD. However, DNA from frontal, temporal and occipital cortex, cerebellum was not affected significantly in PD group as compared to controls. This study provides a new data on stability, damage and conformations of DNA in different regions in brains of PD patients.

### ***Chapter* 3. α-synuclein nicks DNA: An evidence for a new toxic role**

The pathological hallmark of PD is the formation of insoluble protein aggregates known as *Lewy bodies*. The major constituent of these fibrillar structures is **α-synuclein**, a 140 amino acid protein with a basic amino terminal and an acidic carboxy terminal. There is little information available about the neurobiology of α-synuclein under normal and neurodegenerative conditions. In amyloid plaques of PD and other neurodegenerative disorders, α-synuclein has cross beta conformation while it adopts α-helical conformation in presence of phospholipids and lipid membranes. The formation of α-helix is suggested as a protective mechanism against formation of beta sheet and aggregated structures. The major efforts of drug development are focused on the prevention or delaying of the protein aggregation and formation of plaques and recently emphasis has been given to stabilizing the *non-toxic form of the protein*. Recent observations showed that α-synuclein is localized in the chromatin region of nuclei in the brain. Moreover, the presence of the majority of the lysine residues in the N-terminal region of α-synuclein suggests a possible DNA binding role for α-synuclein.

**We demonstrated in the present study**, two new and novel properties of α-synuclein. First, we showed that α-synuclein binds to DNA and alters the conformation of DNA. Second, α-synuclein was shown to have DNA nicking activity and it behaves like a nuclease

enzyme. These are new evidences in literature on α-synuclein binding to DNA. It was also observed that the nicking activity involves the formation of only single strand breaks. However, during long term incubations with α-synuclein, double strand breaks were formed chopping the DNA into small pieces indicating that α-synuclein preferentially nicks only single stranded DNA. Further, the ability of known nuclease inhibitors, Aurintricarboxylic acid (ATA) and Diethyl pyrocarbonate (DEPC) to abolish DNA nicking activity of α-synuclein reveals that histidine residue at 50$^{th}$ position in α-synuclein sequence plays a crucial role in the nicking activity. We further showed that conformational change or oligomerisation of α-synuclein would enhance the nicking activity. This indicates that the oligomers of α-synuclein are more toxic in terms of DNA nicking than monomers and aggregates. This work was awarded **International Alzheimer's Fellowship** and was presented in the 9$^{th}$ International Meeting on Alzheimer's disease and Related Disorders held in Philadelphia, USA in July, 2004.

We discussed the potential implications of the above in vitro findings to neurodegenerative changes associated with PD. We proposed that the DNA binding property of α-synuclein characterized in the present study may have a significant effect on nuclear-translocated α-synuclein functioning. In particular, α-synuclein may interact with histone-free DNA segments and induce nicking.

### ***Chapter 4.*** **DNA induces folding in α-synuclein: Understanding the mechanism using chaperon property of osmolytes**

Structurally, purified α-synuclein is a natively unfolded protein. This lack of folding has been shown to correlate with the specific combinations of low overall hydrophobicity and large net charge. *In vitro* α-synuclein readily assembles into fibrils, with morphologies and staining characteristics similar to those of fibrils extracted from PD

affected brain. It has been assumed that α-synuclein may exist in two structurally different isoforms *in vivo*: a helix-rich, membrane-bound form and a disordered, cytosolic form, with the membrane-bound α-synuclein generating nuclei that seed the aggregation of the more abundant cytosolic form.

**In this chapter, we tried to** understand the ability of different DNA to induce conformational changes in α-synuclein. We have used the effect of chaperonic properties of five osmolytes viz. glycerol, betaine, taurine, TMAO, sarcosine for their ability to induce folding in α-synuclein, as a model system to understand the DNA induced conformational changes in α-synuclein. We provided a comprehensive picture of DNA binding effect on α-synuclein fibrillation using different DNAs such as double and single stranded DNA, AT and GC sequence specific DNA, of different sizes, genomic DNA etc. We showed that only those DNA which induce a partial folding in α-synuclein (GC* rich DNA) promote its aggregation, while, sscDNA forms α-helix conformation in α-synuclein and also inhibit aggregation to a considerable extent. It was also observed that among the osmolytes used in the present study, glycerol, TMAO, betaine and taurine induced partial folding in α-synuclein and enhanced the fibrillation kinetics.

The ability of DNA and osmolytes in inducing conformational transition in α-synuclein, indicates that two factors are critical in modulating α-synuclein folding: (i) Electrostatic interaction as in the case of DNA, and (ii) Hydrophobic interactions as in the case of osmolytes. We feel that the property of sscDNA in inducing α-helical conformation in α-synuclein and inhibiting the fibrillation may be of significance in engineering **DNA-chip based therapeutic approaches** to PD and other amyloid disorders. Further, from the fact that other amyloidogenic peptides like Aβ, tau and prions also have DNA binding property, it appears that the DNA binding is a unifying property of

amyloidogenic peptides implicated in various neurodegenerative disorders.

***Chapter 5.*** **Serum trace elemental levels and the complexity of inter-elemental relationships in patients with PD**

Metals are well established to be risk factors for PD, though the significance of metals in the aetiopathogenesis of PD is still unknown. Several studies have shown decreased copper, increased zinc and iron in the *substantia nigra* and increased copper concentrations in the cerebrospinal fluid of PD affected patients. Though much work has been done on metal homeostasis in PD brain, limited data is available on trace elemental levels and their inter-relationships in serum samples of Parkinson's patients. Moreover, most of the available information is limited to few selected elements and there is no study, which examines inter-elemental relationships with regard to severity of PD. The aim of this study was to assess the serum levels of 12 elements (Na, K, Fe, Al, Cu, Zn, Ca, Mg, Mn, Si, P and S) in patients with early and severe PD compared with a control population and also to understand the possible relevance of inter-elemental relationships to the progression of PD.

**The results showed** a definite pattern of variation among certain elements in early and severe PD compared to control. In both early and severe PD serum, Al and S ($p<0.05$) were decreased compared to control. Fe ($p<0.01$) and Zn ($p<0.05$) were significantly low in severe PD, while K, Mg, Cu ($p<0.01$) and P ($p<0.05$) were high in early and severe PD compared to control. The data revealed a clear imbalance in the elemental interrelationship in both early and severe PD serum compared to control as shown by the direct and inverse correlations. These results suggested that a definite disturbance in the elemental homeostasis occurs during the progression of PD. There has been a controversy regarding metal levels in PD serum and also possible role of metals as risk factors for PD. It is not clear whether alteration in metal

homeostasis is a cause or consequence of disease pathology. So far, there is no detailed or comprehensive database on metal homeostasis and inter-relationships. The available reports only indicated the changes in levels of one or two individual elements, but fall short to correlate the element-to-element inter-relationship pattern with the disease progression. In this perspective, the present study provided a comprehensive database on concentrations of as many as 12 elements (majority of essential and few important toxic elements) in PD serum in comparison with control groups.

**We further advocated** from the above results that irrespective of metals being primary risk factors or consequences of disease mechanism, a moderate change in a single metal ion will upset the whole elemental homeostasis pool resulting in the significant imbalance in elemental levels in the body system (serum, CSF and brain). The effect of increase or decrease of a single metal is not restricted to the presenting metal alone, it will affect the total elemental and charge distribution pattern in the system.

The thesis ends with a comprehensive S*ummary and Conclusions* of the present study.

**In a nut shell the study provides the following significant contributions**

1. The study provided a new data on DNA stability, integrity and topology of genomic DNA isolated from eight different brain regions from PD affected human brain samples (*Chapter* 2).

2. We evidenced two new and novel properties of α-synuclein: i) DNA binding and ii) DNA nicking. We showed that α-synuclein behaves as an endonuclease. These have been very intriguing observations

which exposed a new toxic role of α-synuclein. The observations lead to a new debate on whether direct α-synuclein induced DNA damage has any role to play in DNA fragmentation observed in PD brain and hence open new avenues of research in this direction (*Chapter* 3).

3. We showed that single stranded circular DNA induces a stable, aggregation resistant, α-helical conformation in natively random-coil α-synuclein. This observation is very important in investigating the possibility of using DNA-chip based therapy for PD. We also showed that GC* rich DNA forms a partially folded intermediate conformation in α-synuclein and this conformation has high aggregation propensity. Using chaperonic properties of α-synuclein as model system we showed that increase in hydrophobicity and decrease in the net charge are critical in α-synuclein conformational transition and fibrillation (*Chapter* 4).

4. We generated a comprehensive database on the levels of 12 elements (both essential and toxic) in serum samples of PD affected patients and showed that there is a definite disturbance in inter-element homeostasis pattern in PD serum (*Chapter* 5).

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Research Supervisor

# CHAPTER 1

# *General Introduction*

## 1.1 Parkinson's Disease

Parkinson's disease (PD) is a common progressive neurological disorder that results from degeneration of nerve cells (neurons) in a region of the brain called '*substantia nigra*' (SN) (Fig. 1.1 & 1.2) that controls balance and coordinates muscle movement. This degeneration creates a shortage of the brain-signaling chemical (*neurotransmitter*) known as dopamine, which causes impaired movement. Dopamine is a neurotransmitter that carries information from neuron to neuron and eventually out to the muscles. When these dopaminergic neurons start to die, the lines of communication between the brain and the body become progressively weaker. Eventually, the brain is no longer able to direct or control muscle movement in a normal manner.

PD was first formally described in "An Essay on the Shaking Palsy," published in 1817 by a London physician named James Parkinson [Parkinson, 1817]. The symptoms and potential therapies for PD were mentioned in the Ayurveda, the system of medicine practiced in India as early as 5000 BC, and in the first Chinese medical text, Nei Jing, which appeared 2500 years ago.

In the United States, about a million people are believed to suffer from PD, and about 50,000 new cases are reported every year [Rajput and Birdi, 1997; Chung et al, 2003]. Because the symptoms typically appear later in life, these figures are expected to grow as the average age of the population increases over the next several decades. The disorder is most frequent among people in their 70s and 80s, and appears to be slightly more common in men than in women. PD is found all over the world. The rates vary from country to country, but it is not clear whether this reflects true ethnic or geographic differences or simply variations in data collection.

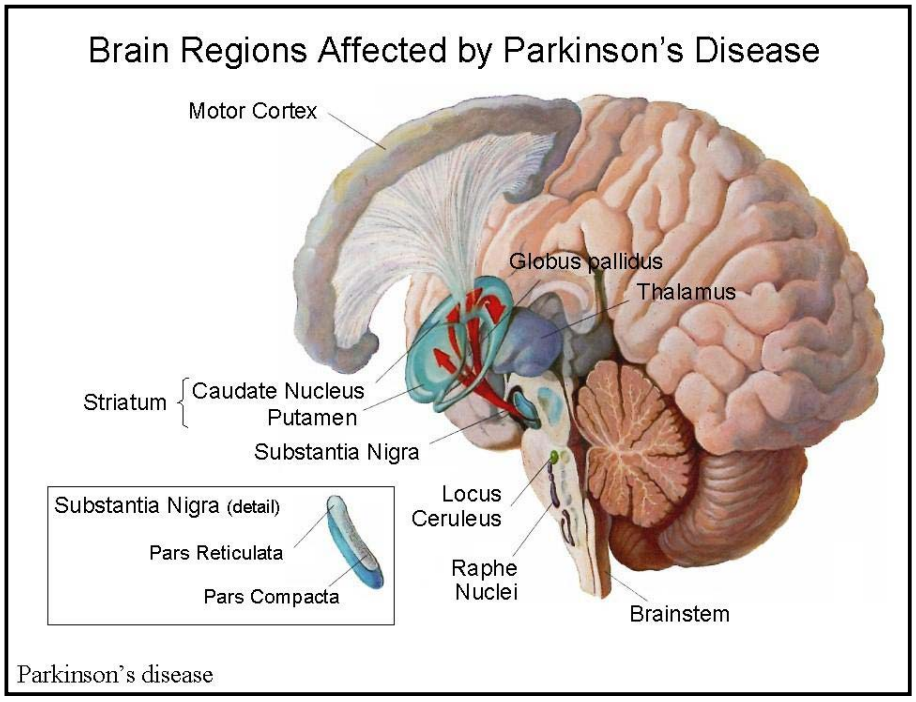



**Figure 1.1**
**Substantia nigra is located in the lower mid brain region is affected in Parkinson's disease** (picture**.** Courtesy, International Parkinson's disease research Foundation website, 2003)

## 1.2 Symptoms

Usually, the first symptom of PD is resting *tremor* (trembling or shaking) of a limb. The tremor often begins on one side of the body, frequently in one hand. Other common symptoms include slow movement (*bradykinesia*), an inability to move (*akinesia*), rigid limbs, a shuffling gait, and a stooped posture. People with PD often show reduced facial expression and speak in a soft voice. Occasionally, the disease also causes depression, personality changes, dementia, sleep disturbances, speech impairments, or sexual difficulties. The symptoms first appear, on average, at about age 60, and the severity tends to worsen over time.

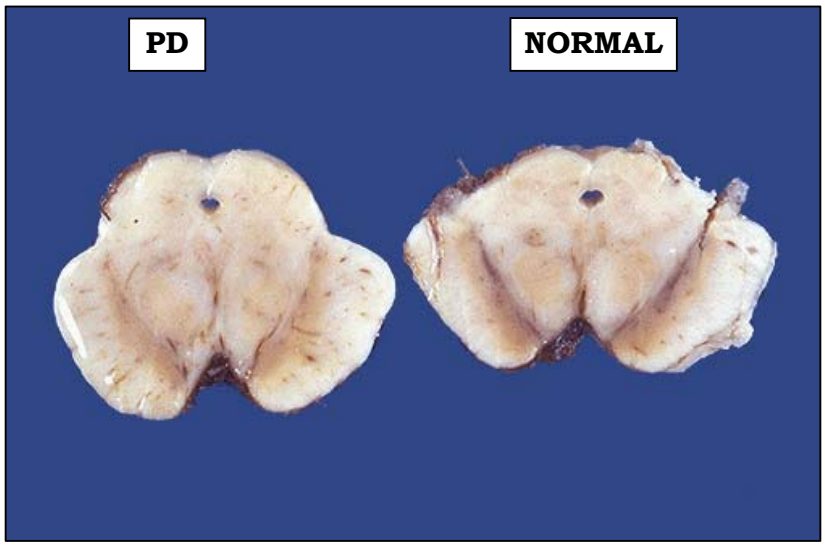



**Figure 1.2**
**Substantia nigra of PD affected and Normal brain**.
The loss of pigmentation and neuronal loss in the substantia nigra of the midbrain at the left in a patient with PD is contrasted with a normal midbrain at the right. (picture**.** Courtesy, International Parkinson's disease research Foundation website, 2003)

The symptoms vary from patient to patient and not every one is affected by all of them. In some people, the disease progresses quickly; in others it does not.

The four ***primary symptoms*** of PD often appear gradually but increase in severity with time. They are:

*Tremor* or trembling in hands, arms, legs, jaw, and face
*Rigidity* or stiffness of the limbs and trunk
*Slowness* of motor movements
*Postural instability* or impaired balance and coordination

**Tremor:** In the early stages of the disease, about 70% of people experience a slight tremor in the hand or foot on one side of the body, or less commonly in the jaw or face. It appears as a 'beating' or oscillating movement and is regular (4-6 beats per second). Because tremor usually appears when the muscles are relaxed, it is called "*resting tremor*". This means that the affected body part trembles when it is at rest and not doing work and often subsides with action. The tremor often spreads to the other side of the body as the disease progresses, but remains most apparent on the original side of occurrence.

**Rigidity:** Rigidity or increased muscle tone means stiffness or inflexibility of the muscles. Normally muscles contract when they move, and then relax when they are at rest. In rigidity, the muscle tone of an affected limb is stiff. Rigidity can result in a decreased range of motion. For example, a patient may not swing his or her arms when walking. Rigidity can also cause pain and cramps at the muscle site.

**Bradykinesia:** Bradykinesia is a slowing of voluntary movement. In addition to slow movements, a person with bradykinesia may also have incompleteness of movement, difficulty in initiating movements, and arrests of ongoing movement. Patients may begin to walk with short, shuffling steps (*festination*), which, combined with other symptoms such as loss of balance, increases the incidence of falls. They may also experience difficulty in making turns or abrupt movements. They may go through periods of "*freezing*," which is when the patient is stuck and finds it difficult to stop or start walking. Bradykinesia and rigidity can occur in the facial muscles, causing a "*mask-like*" expression with little or no movement of the face, often affecting speaking and swallowing.

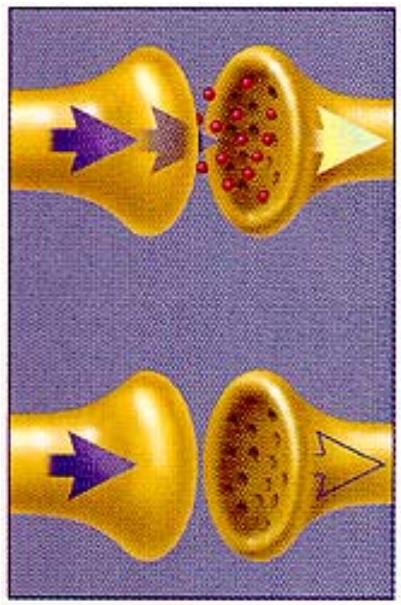



**Figure 1.3**
**Synapse in normal and PD neurons**
In normal individuals, a signal is sent down neurons from an area of the brain called the SN, and is received in the striatum, where the messages cross the gap between neurons as the chemical messenger dopamine. In people affected with PD, cells in the SN produce much less dopamine, so this pathway is blocked.

These above symptoms such as tremor, rigidity, and slowness of movement develop because of depleted dopamine levels in SN neurons. In normal individuals, a signal is sent down neurons from an area of the brain called the SN, and is received in the striatum, where the messages crosses the gap between neurons as the chemical messenger dopamine. In people affected with PD, cells in the SN produce much less dopamine, so this pathway is blocked, and leads to the appearance of above symptoms (Fig. 1. 3).

There are many ***secondary symptoms*** of PD. These include stooped posture, a tendency to lean forward or backward, and

speech problems, such as softness of voice or slurred speech caused by lack of muscle control. Non-motor symptoms also have impact on the life of a person with PD.

The following is a list of secondary symptoms of PD:

- Speech changes
- Loss of facial expression
- Micrographia (small, cramped handwriting)
- Difficulty in swallowing
- Drooling
- Pain
- Dementia or confusion
- Sleep disturbances
- Constipation
- Skin problems
- Depression
- Fear or anxiety
- Memory difficulties and slow thinking
- Sexual dysfunction
- Urinary problems
- Fatigue and aching
- Loss of energy

At present, there are no methods to predict or prevent PD. Symptoms usually appear after 80 percent or more of the dopamine-producing neurons in the SN have died, which significantly narrows the window of opportunity for preventive or protective treatments. Medications can relieve symptoms for a period of time but won't slow or stop the progression of the disease. The course of the disease

varies widely. Some people have mild symptoms for many years, while others have severe symptoms and a quicker progression.

## 1.3 Diagnosis of PD

There is no biochemical test that can clearly identify the disease. To this day, PD remains a clinical diagnosis that is based on the patient's history and the signs that are present on neurologic examination. One of the most powerful – and simple – tests used in the examination is a measure known as the *Unified Parkinson's Disease Rating Scale* (UPDRS) [Fahn and Elton, 1987].

An increasingly important area of diagnostic testing is neuroimaging. Over the past 15 years, imaging techniques have provided neurologists with a window through which they can directly measure the nerve cells that are affected in PD. While these techniques are not routinely available, they have contributed greatly to our understanding of how PD affects patients at different stages of life.

Sometimes people with suspected PD are given anti-Parkinson's drugs to see if they respond. Other tests, such as brain scans, can help doctors decide if a patient has true PD or some other disorder that resembles it. Microscopic brain structures called *Lewy bodies*, which can be seen only during an autopsy, are regarded as a hallmark of classical PD [Lewy, 1912], although they are occasionally found in other neurodegenerative disorders as well. Autopsies have uncovered Lewy bodies in a surprising number of older people without diagnosed PD- 8% of people over 50, almost 13% of people over 70, and almost 16% of those over 80. Some experts believe PD is something of an "*iceberg phenomenon*," lurking undetected in as

many as 20 people for each known PD patient. A few researchers contend that almost everyone would develop PD eventually if they lived long enough.

***Stages of PD***

The following scale, developed by Hoehn and Yahr [1967], is most widely used to describe PD:
Stage I: Signs of PD are unilateral (affecting one side of the body only).
Stage II: Signs of PD are bilateral. Balance is not impaired.
Stage III: Signs of PD are bilateral and balance is impaired.
Stage IV: PD is functionally disabling.
Stage V: Patient is confined to bed or wheelchair.

## 1.4 Treatments available

There is no cure for PD to date. Many patients are only mildly affected and need no treatment for several years after the initial diagnosis. When symptoms grow severe, doctors usually prescribe levodopa (L-dopa), which helps replace the brain's dopamine. Since most of the symptoms are due to the lack of dopamine in the brain, effective medications aim at temporarily replenishing or mimicking dopamine's actions. These drugs - levodopa and the dopamine agonists ropinirole, pramipexole, and pergolide - reduce muscle rigidity, improve speed and coordination of movement, and relieve tremor. In addition, many doctors recommend physical therapy or muscle-strengthening exercises to help people handle their daily activities. In patients who are very severely affected, various kinds of brain surgery have reportedly been effective in reducing symptoms. These surgical approaches include pallidotomy and implantation of an electrical stimulator to counteract the effect of the loss of

dopamine-producing cells in the substantia nigra. Another kind of brain surgery, in which healthy dopamine-producing tissue is transplanted into the brain, is also being tested [International Parkinson's disease research Foundation website, 2003].

Developing therapies to prevent PD, to suppress symptoms, to halt disease progression, and to repair damage are all fundamental goals in modern day research. Available drugs suppress symptoms early in PD, but progressively fail as more nerve cells die. The emergence of drug-induced dyskinesias and motor fluctuations often limits drug benefits. To achieve therapeutic goals, many separate studies are required, from the first steps in translating basic research advances, animal testing, preliminary safety studies in human patients, and finally large trials to evaluate the effectiveness of a therapy.

## Gene Therapy

Gene Therapy is defined by the Institute for Human Gene therapy as: "*a novel approach to treating diseases based on modifying the expression of a person's genes toward a therapeutic goal*; *based on correcting disease at the level of DNA molecules and thus compensating for the abnormal genes.*"

Theoretically, gene therapy may be performed in two ways:

***Somatic gene therapy,*** in which a person's cells are changed, without changing the cells involved in reproduction, so that the change is not passed on to children.

***Germ-line gene therapy,*** In germ line gene therapy, genetic changes would be applied, which the individual's children may inherit.

Because of important ethical concerns, as well as some technical problems, germ line methods are not undergoing research, and are banned in many countries.

Gene therapy for PD has a number of different approaches. In one of these, an attempt is made to bypass the problem, and genes for dopamine production from tyrosine (a common amino acid in the body) are inserted into the cells of the striatum [Chen et al, 2005].

## Stem Cell Therapy

The rationale behind the use of cells as therapeutic modalities for neurodegenerative diseases in general, and in PD in particular, is that they will improve patient's functioning by replacing the damaged cell population. It is reasoned that these cells will survive, grow neurites, establish functional synapses, integrate best and durably with the host tissue mainly in the striatum, renew the impaired wiring, and lead to meaningful clinical improvement. To increase the generation of dopamine, researchers have already transplanted non-neuronal cells, without any genetic manipulation or after introduction of genes such as tyrosine hydroxylase, in animal models of PD. Because these cells were not of neuronal origin, they developed without control, did not integrate well into the brain parenchyma, and their survival rates were low. Clinical experiments using cell transplantation as a therapy for PD have been conducted since the 1980s. Most of these experiments used fetal dopaminergic cells originating in the ventral mesencephalic tissue obtained from fetuses. Although it was shown that the transplanted cells survived and some patients benefited from this treatment, others suffered from severe *dyskinesia*, probably caused by the graft's excessive and uncontrolled production and release of dopamine. It is now recognized that cell-replacement strategy will be effective in PD only

if the transplanted cells have the same abilities, such as dopamine synthesis and control release, reuptake, and metabolizing dopamine, as the original dopaminergic neurons. Recent studies on embryonic and adult stem cells have demonstrated that cells are able to both self-renew and produce differentiated tissues, including dopaminergic neurons. These new methods offer real hope for tissue replacement in a wide range of diseases, especially PD [Levy et al, 2004].

A new optimism that PD can be defeated is energizing the research community and patient advocates. This hope is fueled by the accelerating pace of discovery in neuroscience research generally, by advances in understanding what causes PD, and by a wide range of new treatments on the horizon including stem cell transplants, precision surgical repair, chronic brain stimulation, and natural growth factors to name a few. Optimism is tempered by the recognition that we cannot yet cure any major neurodegenerative disorder, and defeating PD requires crossing a major frontier of medicine.

## 1.5 Pathology: Lewy Bodies

Pathologically, PD is characterized by the loss of the pigmented dopaminergic neurons from the substantia nigra pars compacta (SNpc) (Fig. 1.4), the presence of extracellular melanin (a dark pigment), released from degenerating neurons, reactive gliosis (increase in numbers of glial or support cells), and pink-staining cellular inclusions known as *Lewy Bodies* (LBs) in the remaining SNpc neurons [Lewy, 1912].

The LB, which was first described by Frederick Lewy in 1913, is present in essentially all cases of PD [Lewy, 1912]. Without its

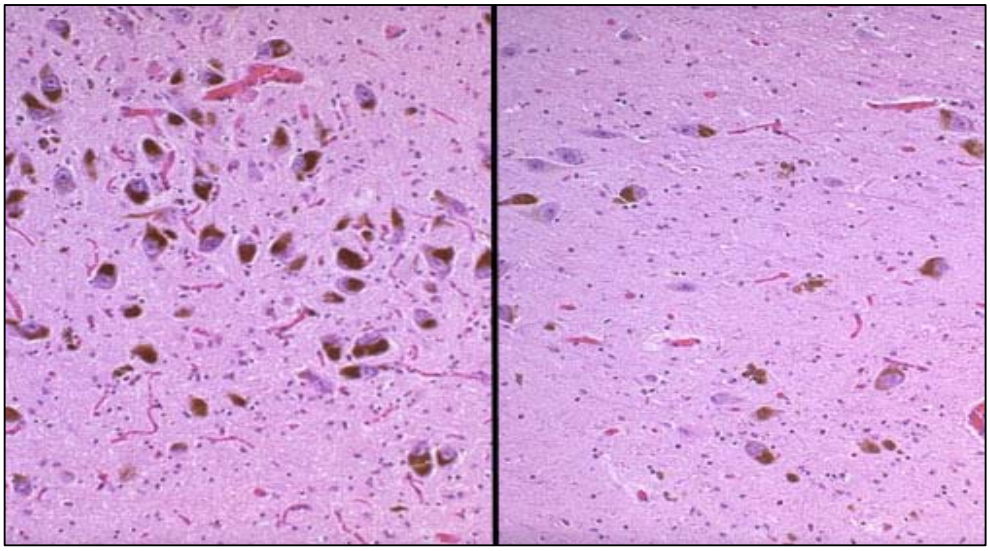


**Figure 1.4**
**Loss of pigmentation in substantia nigra in PD brain.**
At the left, normal numbers of neurons in the SN are pigmented. At the right, there is loss of neurons and loss of pigmentation with PD. (picture**.** Courtesy, International Parkinson's disease research Foundation website, 2003).

presence in the post-mortem tissue, most experts would deny the diagnosis of PD. In other words, the detection of LBs in Snpc neurons of the postmortem brain is required to establish a definite diagnosis of PD.

In addition to the dopaminergic neurons of the SNpc, other populations of neuromelanin-containing and non-melanin containing neurons in the brainstem and basal forebrain degenerate and accumulate LBs similar to those found in the SNpc. Some experts suggest that the loss of other neuronal types are responsible in part for many of the secondary features in PD, including autonomic nervous system instability (low blood pressure, constipation, bladder difficulties, perspiration, seborrhea), sleep disturbances and possibly dementia.

## 1.6 Causes of PD

The exact causes of PD remain undetermined. The causes are likely to include both genetic and environmental factors. A variety of mechanisms that are believed to cause accelerated cell death have also been suggested, including oxidative stress, excitotoxicity and mitochondrial dysfunction.

Most experts in the field share the opinion that PD is caused by a combination of genetic and environmental factors, and other contributing mechanisms of cell death. There are many theories about the causes of PD. Researchers have reported families with apparently inherited PD for more than a century. Until recently, however, the prevailing theory held that one or more environmental factors caused the disease [Fernandez and Calne, 2002].

### *Genetic risk factors*

During the past decade, genetic approaches to the study of PD has resulted in major insights. The number of genes implicated in the pathogenesis of PD has been constantly increasing, and includes genes encoding for α-synuclein, parkin, DJ-1 and PINK1 [Hofer and Gasser, 2004]. These genes are thought to be involved in the proteasomal protein degradation pathway, in the cell's response to oxidative stress, and in mitochondrial function, respectively [Hofer and Gasser, 2004]. Over the last few years, several genes for rare, monogenically inherited forms of PD have been mapped and/or cloned. In dominant families, mutations have been identified in the gene for alpha-synuclein. Generally, about 15-25% of Parkinson's patients report would have a relative with PD. There appears to be a 2-3 fold increased risk of PD in first degree relatives compared to matched control populations. However, the majority of cases of PD still appear to be sporadic. However, for several decades there has

been a controversy on the contribution of genetic factors to the pathogenesis of sporadic idiopathic PD. The identification of families in which typical parkinsonism is inherited as an autosomal dominant or recessive trait sheds light on genes that cause phenotypes resembling sporadic PD [Kruger, 2004]. Although most people do not inherit PD, studying the genes responsible for the inherited cases is advancing our understanding of both common and familial PD. Identifying genes that can cause PD is crucial for understanding the disease process, revealing drug targets, improving early diagnosis, and developing animal models that accurately mimic the slow nerve cell death in human PD.

### *Environmental risk factors*

The study of environmental risk factors for PD is difficult for several reasons. Because, environmental exposures and gene-environment interactions may occur well before the onset of clinical manifestations and remain undetected years later. Moreover, the severe neurodegenerative changes that underlie the symptoms of PD may be the result of additive or synergistic effects of multiple exposures and, over the years these effects could be compounded by increased vulnerability of the aging nigrostriatal system to toxic injury. Despite these complexities, the results of studies of environmental factors may have far-reaching implications, including the development of preventive strategies and policies that could identify individuals at risk and limit exposure to harmful agents [Di Monte, 2001].

Evidence has accumulated to support the view that PD can originate from long-term, subclinical damage to the nervous system caused by environmental toxins [Corti et al, 2005; Farina et al, 1994; Calne et al, 1986; 1987]. In fact, several studies have implicated

such environmental factors as pesticides, herbicides, and heavy metals in the PD origin [Tanner, 1989; Hirsch et al, 1991; Good et al, 1992; Yasui et al, 1992; Hertzman et al, 1994; Gorell et al, 1999; Altschuler, 1999; Le Couteur et al, 1999]. The major suspected environmental factors implicated in PD are presented in Table 1.1.

### *Metals*

Metals, especially iron and copper have been postulated to have important roles in damage in the brain, because of their potential to catalyze oxidative reactions [Zecca and Swartz, 1993]. The possible involvement of heavy metals in the etiology of PD follows primarily from the results of epidemiological studies. Analysis of the PD mortality rates in Michigan (1986-1988) with respect to potential heavy metal exposure revealed that countries with an industry in the paper, chemical, iron, or copper related-industrial categories had significantly higher PD death rates than countries without these industries [Rybicki et al, 1993]. An epidemiological study (1987-1989) of Valleyfield, Canada, established that an increased risk for PD is associated with occupational exposure to manganese, iron and aluminum, especially when the duration of exposure is longer than 30 years [Zayed et al, 1990]. A population based case-control study suggested that chronic occupational exposure to manganese or copper, individually or to dual combinations of lead, iron, and copper, is associated with PD [Gorell et al, 1999; Gorell et al, 1997].

Postmortem analysis of brain tissue from patients with PD gives further confirmation for the involvement of heavy metals in this disorder, in that a considerable increase in total iron, zinc and aluminum content of the PD SN was observed when compared with control tissues [Hirsch et al, 1991; Dexter et al, 1989a; Riederer et al, 1989; Dexter et al, 1991]. Moreover, analysis of Lewy bodies in the

PD substantia nigra revealed high levels of iron and the presence of aluminum [Hirsch et al, 1991; Dexter et al, 1991]. Interestingly, the SN of PD brain is characterized by a shift in the $Fe^{2+}$/ $Fe^{3+}$ ratio in favor of $Fe^{3+}$ and a significant increase in the $Fe^{3+}$-binding protein, ferritin [Riederer et al, 1989]. Finally, it was shown that unilateral injection of $FeCl_3$ into the SN of adult rats resulted in a substantial selective decrease of striatal dopamine (95%), supporting the assumption that iron initiates dopaminergic neurodegeneration in PD [Youdim et al, 1991].

There is increasing evidence that iron is involved in the mechanisms that underlie pathogenesis of PD. Conditions such as neuroferritinopathy and Friedreich ataxia are associated with mutations in genes that encode proteins that are involved in iron metabolism, and as the brain ages, iron accumulates in regions that are affected by PD. High concentrations of reactive iron can increase oxidative-stress induced neuronal vulnerability, and iron accumulation might increase the toxicity of environmental or endogenous toxins. By studying the accumulation and cellular distribution of iron during ageing, we should be able to increase our understanding of these neurodegenerative disorders and develop new therapeutic strategies [Zecca et al, 2004a; 2004b].

***Pesticides***

Pesticides are another class of agents that are of interest in PD; evidence of their role as neurotoxins is provided by clinical and experimental work. The epidemiological studies have found that pesticide exposure was associated with a high risk of PD [Seidler et al, 1996; Semchuk et al, 1992; Liou et al, 1997; Gorell et al, 1998; Petrovitch et al, 2002]. The results were consistent with a dose-dependent effect; in agricultural workers, risk was increased with

duration of pesticide use [Liou et al, 1997; Gorell et al, 1998]. Studies suggest that bipyridyl (paraquat), organochlorine and carbamate derivatives could have a causal role in PD [Seidler et al, 1996; Semchuk et al, 1992; Liou et al, 1997]. However, information about exposure to specific pesticides and risk of developing PD is limited. Moreover, the available data are not equivocal and more detailed information about association between pesticide exposure and risk of PD is needed.

Reports have also linked the development of Parkinsonism to drugs used in medical settings and to industrial workers who were exposed to chemicals, especially exposure to industrial solvents [Smargiassi et al, 1998; Kim et al, 2000]. In other cases an infection, such as a virus, might alter the protein. A third possibility is some other kind of environmental insult or toxin that twists the protein into an abnormal shape and block normal protein breakdown. These possible environmental causes have not yet been firmly identified.

**Table 1.1**
**Environmental factors positively associated with PD**

| Factors | PD risk | Reference |
|---|---|---|
| Rural residence | + | Liou et al, 1997; Marder et al, 1998; Morano et al, 1994. |
| Farming | + | Liou et al, 1997; Gorell et al, 1998; Semchuk et al, 1993; Fall et al, 1999 |
| Well-water drinking | + | Marder et al, 1998; Morano et al, 1994 |
| Pesticide exposure | + | Liou et al, 1997; Gorell et al, 1998; Semchuk et al, 1993; Fall et al, 1999 |
| Metal exposure | + | Gorell et al, 1997; Kuhn et al, 1998 |

***Cross-talk of Environment and Genome***

There is interaction between the environment and the genome; in some disorders inheritance establishes susceptibility and environment triggers pathology [Fernadez and Calne, 2002]. Hence, the recent trend to study PD is to look at the interplay or *Cross-talk* between genetics and environmental triggers (Fig. 1.5). Hence, beyond single genes, we must unravel the complex interactions between genetic predisposition and environmental influences that cause most cases of PD [Corti et al, 2005].

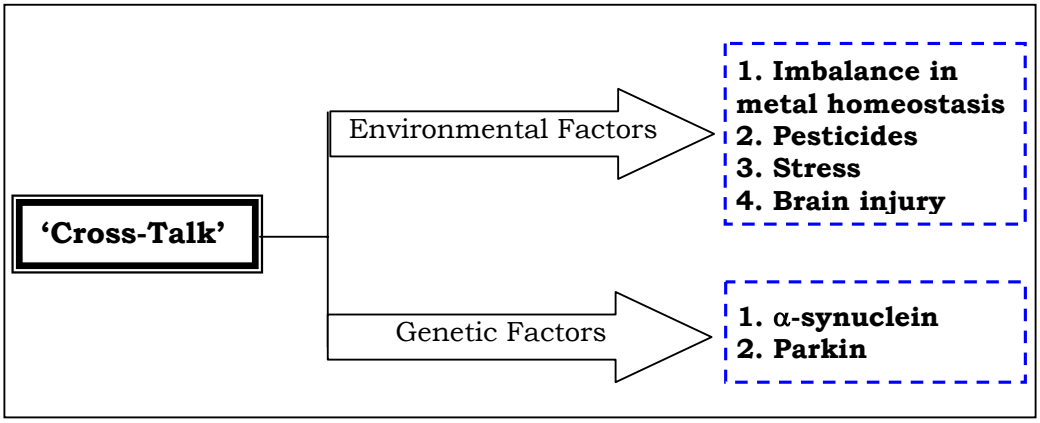



**Figure 1.5**
**The '*Cross-Talk*' or interplay of environmental and genetic causative factors for Parkinson's disease**

While the debate concerning environmental factors and genetics as causative factors in PD continues, there has been extensive investigation of the mechanisms involved in the cell death process. A number of cell death concepts have been put forward including, oxidative stress, mitochondrial dysfunction and excitotoxicity [Jancovic, 2005].

## 1.7 Oxidative stress in PD

Oxidative stress is a deleterious condition that results from insufficient scavenging of reactive oxygen species, which are generated by a myriad of biochemical reactions [Lotharius and Brundin, 2002]. Normally these species are eliminated by intracellular antioxidant systems, which might be impaired as a result of the ageing process or in disease states [Zecca et al, 2003].

Nigral dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine gives rise to various molecules that can act as endogenous toxins if not handled properly [Lotharius and Brundin, 2002; Graham, 1978]. In PD, nigral cells seem to be under a heightened state of oxidative stress, as indicated by elevations in by-products of lipid, protein and DNA oxidation, and by compensatory increase in antioxidant systems [Jenner, 1998; Sofic et al, 1998; Saggu et al, 1989; Dexter et al, 1989b; Alam et al, 1997]. The levels of melondialdehyde and lipid hydroperoxides, which are markers of oxidized lipids, were found to be up to ten-fold higher than normal in the SN of PD [Dexter et al, 1989a; Dexter et al, 1989b].

**Table 1.2**
**Evidences for involvement of oxidative stress in PD**

| Parameter | Percentage of control | Reference |
|---|---|---|
| Total iron level | 129 % | Sofic et al, 1988 |
| Superoxide dismutase | 133 % | Saggu et al, 1989 |
| Lipid peroxidation | 135 % | Dexter et al, 1989b |
| Protein oxidation | ~ 200 % | Floor & Wetzel, 1998 |
| DNA oxidation | 238 % | Alam et al, 1997 |

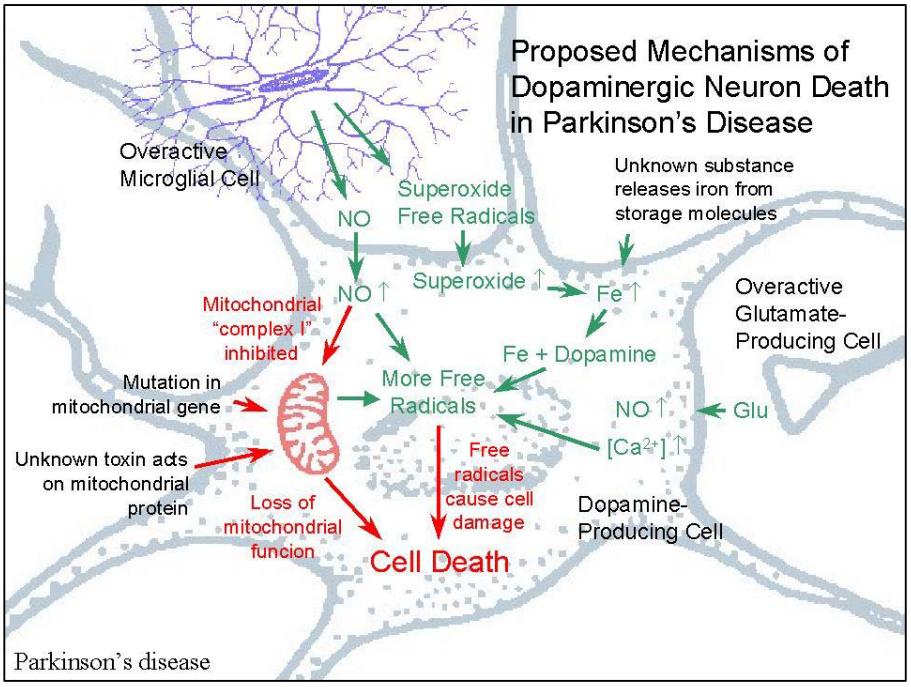



**Figure 1.6**

**The oxidative reactions leading to cell death in Parkinson's disease**

The proposed mechanisms of dopaminergic neuronal cell death in PD involves oxidative stress complexities in one or the other way. (Courtesy: International Parkinson's disease research Foundation website, 2003).

Immunocytohemical detection of 8-hydroxyguanosine revealed a marked increase in oxidized RNA and DNA in nigral neurons from PD [Alam et al, 1997; Zhang et al, 1999]. Further, the levels of '*protein carbonyls*', which are used as markers of protein oxidation, were reported to be two-fold higher in the SN than in other brain regions [Floor and Wetzel, 1998; Alam et al, 1997]. Also, a preferential increase in the activity of superoxide dismutase was detected in nigral tissue in PD [Saggu et al, 1989; Marttila et al, 1988]. The level of iron, which is significantly higher in the normal SN than in other regions owing to its binding affinity to

neuromelanin, was further increased in the SN of PD [Mecocci et al, 1993; Dexter et al, 1989a; Ben-Shachar et al, 1991; Riederer et al, 1989; Jellinger et al, 1992]. The evidences for oxidative stress in SN of PD have been listed in Table 1.2. The complexity of oxidative events associated with PD is represented in Fig. 1.6.

## 1.8 Central pathway of disease causation- Proteins and Parkinson Disease

Recent evidence indicates that diverse neurodegenerative diseases like PD, might have a common pathological mechanism- the misfolding, aggregation, and accumulation of proteins in the brain, resulting in neuronal apoptosis [Soto, 2003]. Hence, these disorders are now being considered as *'protein conformational disorders'* [Eriksen et al, 2005; Snyder and Wolozin, 2004]. The *hallmark* feature of protein conformational disorders is that a particular protein can fold into a stable alternative conformation, which in most cases results in its aggregation and accumulation in tissues as fibrillar deposits [Carrell and Lomas, 1997; Dobson, 1999; Soto, 2001].

## 1.9 α-Synuclein family proteins and Parkinson's disease

α-Synuclein is a highly conserved protein of unknown function, which has been implicated in the pathology of several neurodegenerative diseases, including PD, Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [Hashimoto et al, 2004; Martin et al, 2004; Goedert, 2001; Spillantini et al, 1997; Baba et al, 1998; Wakabayashi, 1998]. The protein accumulates in intracellular inclusions and abnormal neuritis (Lewy

bodies and Lewy neuritis) that are characteristic of PD, the second common neurodegenerative disorder [Baba et al, 1998]. However, the role of α-synuclein in neuropathology leading to degeneration of neurons is not clearly understood. The synucleins are a family of proteins whose function in normal cell is not well understood. The first synuclein protein was described in 1988 and it is known as α-synuclein. The name 'synuclein' was chosen because the protein was found in both synapses and nuclear envelope [Maroteaux et al, 1988]. Later, it was also named the non-amyloid component (NAC) of plaque precursor protein. The NAC peptide was isolated from amyloid-rich senile plaques (SPs) of brains of patients with AD. Amyloid plaques are one of the hallmarks of AD. NAC peptide was shown to be identical to a certain part of α-synuclein. The second member of the synuclein family is known as β-synuclein. Both these proteins are found in the presynaptic terminals of neurons and many researchers believe they may be involved in synaptic function. The third member of synuclein family is gamma-synuclein. All synucleins have in common a highly conserved alpha-helical lipid-binding motif with similarity to the class-A2 lipid – binding domains of the exchangeable apolipoproteins [George, 2002]. Synuclein family members are not found outside vertebrates, although they have some conserved structural similarity with plant 'late-embryo-abundant' proteins. All synucleins were shown to be extremely conserved among distantly related species [George et al, 1995; Jakes et al, 1994]. The α- and β-synuclein proteins are found primarily in brain tissue, where they are seen mainly in pre-synaptic terminals. The γ-synuclein protein is found primarily in the peripheral nervous system and retina, but its expression in breast tumors is a marker for tumor progression. While α- synuclein has been implicated in neurodegenerative disorders mainly PD, until recently there has been no evidence to suggest a role for the other synucleins. α-synuclein

forms fibrillar aggregates known as Lewy bodies in PD brain, these insoluble protein aggregates are morphologically similar to the amyloid fibrils found in AD neuritic plaques and in deposits associated with other amyloidogenic diseases [Conway et al, 2000a; El-Agnaf et al, 1998].

Since 1997, a remarkable paradigm shift in PD research has resulted from the following six lines of converging evidence that directly implicate α-synuclein in mechanisms underlying the onset/ progression of PD [Trojanowski and Lee, 2001]:

1. Missense mutations in the α-synuclein gene (A53T, A30P) cause familial PD in rare kindreds [Kruger et al, 1998; Polymeropoulos et al, 1997].

2. Recently, a novel third mutation (E46K) has been identified in a Spanish family with an autosomal-dominantly inherited form of DLB [Zarranz et al, 2004].

3. Antibodies to α-synuclein specifically detect LBs, and dystrophic Lewy neuritis in PD as well as in other synucleinopathies characterized by filamentous intra-neuronal α-synuclein inclusions such as DLB, the LB variant of AD and other forms of AD [Baba et al, 1998; Irizarry et al, 1998; Lippa et al, 1999; Lippa et al, 1998; Mezey et al, 1998; Spillantini et al, 1998; Spillantini et al, 1997; Takeda et al, 1998; Wakabayashi et al, 1997].

4. Epitope specific antibodies detect protein domains throughout the amino- to carboxy-terminal extent of α-synuclein in LBs and LB filaments [Baba et al, 1998; Spillantini et al, 1998;

Spillantini et al, 1997; Giasson et al, 2000].

5. Insoluble α-synuclein filaments can be recovered from DLB brains, LBs purified from DLB brains contain abnormally aggregated α-synuclein and insoluble forms of this protein are detected in brain regions with abundant LBs [Baba et al, 1998; Lippa et al, 1998].

6. Recombinant A53T and A30P mutant as well as wild-type α-synucleins assemble into LB-like filaments with distinct morphologies under defined *in vitro* conditions [Conway et al, 1998; Crowther et al, 1998; Giasson et al, 1999; Hashimoto et al, 1999; Narhi et al, 1999].

## 1.10 What is known about α-synuclein structure?

Despite the intense interest in α-synuclein generated by its potential role in PD, the structural properties of this protein have only been characterized at low resolution, using circular dichroism (CD) or other optical techniques. The reason for this is that α-synuclein does not appear to possess an intrinsic well-defined native structure [Weinreb et al, 1996], making it a member of a class of proteins referred to as intrinsically unstructured proteins. In such cases, neither X-ray diffraction nor NMR spectroscopy, the two classical high-resolution structure determination techniques, can be used to yield a unique structure. X-ray studies of non-crystalline solutions are largely limited to the determination of geometrical properties such as radius of gyration or the distance distribution function of a solute molecule. NMR spectroscopy, on the other hand, is uniquely capable of providing high resolution, residue or atom-

specific information on the structural and dynamic properties of proteins in solution even in the absence of a unique native structure.

α-Synuclein is a relatively small (140 amino acids), natively unfolded protein, exhibiting a random coil secondary structure in normal physiological conditions [Weinreb et al, 1996]. At neutral pH it is calculated to have 24 negative charges (15 of which are located in the last third of protein sequence) leading to a strong electrostatic repulsion, which contributes to the lack of folding of α-synuclein [Uversky et al, 2001a; 2001b]. The primary structure of α-synuclein is represented in Fig. 1.7. α-Synuclein from various organisms possesses a high degree of sequence conservation. Human α-synuclein differs from rat and mouse α-synuclein by only 2 amino acids in the first 93 amino acids [Clayton and George, 1998]. Based on the amino acid sequence, three distinct domains have been identified in the 140 amino acid sequence of α-synuclein:

(i) The basic N-terminal amphipathic region, containing residues 1-60, rich in basic amino acids with a high propensity for α-helix formation. This region contains four 11-aminoacid imperfect repeats with a highly conservative hexamer motif (KTKEGV).

(ii) The central hydrophobic region (amino acids 61-95) comprising of the highly amyloidogenic NAC sequence. It contains two additional KTKEGV repeats. And

(iii) The residues 96-140 constitute the acidic C-terminal region in which most of the negatively charged amino acids and prolines are located [Davidson et al, 1998; Manning-Bog et al, 2002].

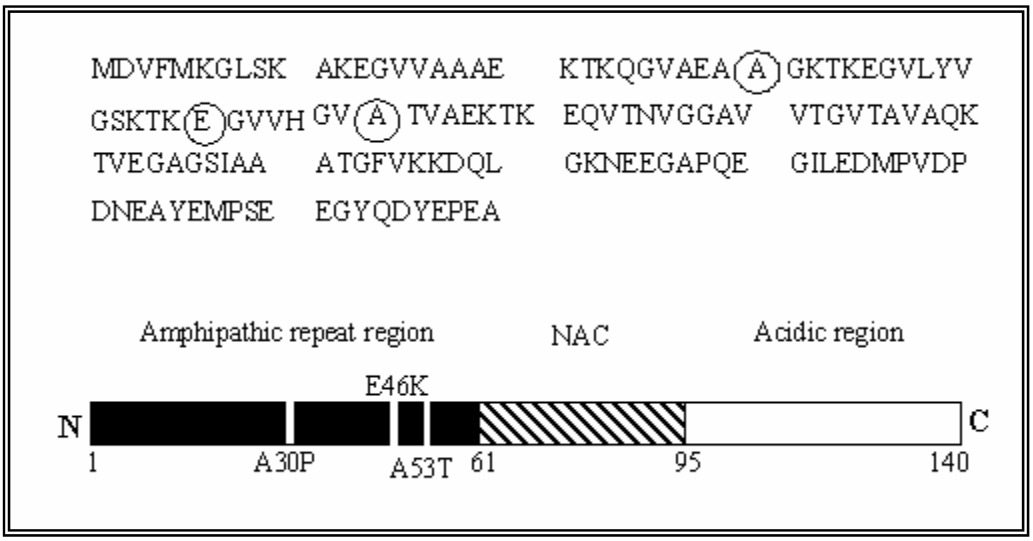



**Figure 1.7**
**α-Synuclein primary structure**

The amino acid sequence and schematic organization of α-synuclein. The two sites of early onset PD-linked mutations (positions 30 and 53) are indicated. α-synuclein contains three major regions including the amino-terminal amphipathic α-helical domains, the non-Aβ component (NAC) of the insoluble fibrillar core of the Alzheimer's disease senile plaques, and the acidic carboxy-terminal region.

The three highly conserved tyrosine residues, which are considered as a family signature of α- and β-synucleins are present in this region. The protein undergoes aggregation leading to fibrillar structures in PD brain, which adopts a β-sheet secondary structure [Serpel et al, 2000]. It is unclear how an essentially unfolded protein with little or no ordered structure is transformed in to highly ordered fibrils in PD brain. Eliezer et al, [2001] applied modern multi-dimensional hetero-nuclear NMR spectroscopy to the characterization of residual structure in free monomeric α-synuclein. A propensity for helical structure was observed throughout the N-

terminal portion of the protein, with a stronger helical signature in one specific region. Such residual structure may play a role in inter-molecular folding events leading to amyloid fibril formation.

Three missense mutations in the α-synuclein gene have been reported to be associated with families susceptible to inherited forms of PD [Polymeropoulos et al, 1997; Kruger et al, 1998; Zarranz et al, 2004]. These mutations cause alterations in the amino acid sequence of α-synuclein (at residues Ala30Pro or Ala53Thr or Glu46Lys) in regions predicted to influence the secondary structure of α-synuclein. The substitutions may disrupt the structure of α-synuclein, rendering the protein more prone to self- aggregation [Vogel, 1997; Heintz and Zoghbi, 1997].

An important feature of α-synuclein primary structure is six imperfect repeats within the first 95 residues. This brings the similarity of α-synuclein with the amphipathic lipid-binding α-helical domains of apolipoproteins [Clayton and George, 1998; George et al, 1995], which show variation in hydrophobicity with a strictly conserved periodicity of 11. α-Synuclein shares the defining properties of the class A2 lipid-binding helix, distinguished by the clustered basic residues at the polar-apolar interface, positioned $\pm 100^0$ from the center of apolar face; predominance of lysines relative to arginines among these basic residues; and several glutamate residues at the polar surface [Segerst et al, 1990; Segerst et al, 1992; Perrin et al, 2000]. In agreement with the above structural features, α-synuclein binds specifically to synthetic vesicles containing acidic phospholipids [Davidson et al, 1998; Perrin et al, 2000]. Further, this binding was shown to be accompanied by a dramatic increase in α-helix content.

Recently, attempts have been made to analyze the structure of α-synuclein using NMR studies [Bussell and Eliezer, 2003; Chandra et al, 2003; Eliezer et al, 2001]. It was shown that the conformation of α-synuclein consists of two α-helical regions that are interrupted by a short break [Chandra et al, 2003]. NMR study of free monomeric α-synuclein revealed that the first 100 residues in N-terminus region of free α-synuclein have an overall preference for helical structure and there may be the presence of a transient helical structure from residues 6 to 37. In contrast, the final 40 residues of free α-synuclein exhibited secondary shifts indicative of highly unfolded and extended form [Eliezer et al, 2001). NMR data of α-synuclein in presence of unilamellar vesicles suggested that the N-terminal region is responsible for lipid binding and the boundary for this region occurs between residues 102 and 103. The shifts in $C^{\alpha}$ chemical shifts clearly indicated that there is the formation of helical structure upon α-synuclein association with unilamellar vesicles. It was noted that it is only the N-terminal region of the protein containing the amphipathic apolipoprotein helical motifs, which binds and adopts a helical conformation. The C-terminal region remains in the same conformation as in the free α-synuclein and does not bind to the lipid vesicle surface [Eliezer et al, 2001].

## 1.11 Theories and hypotheses on α-synuclein functions

The exact role of α-synuclein in normal cell functioning is not known to date. Understanding the role of α-synuclein in normal cell life might be critical importance since disruption of its normal function might result in neurodegeneration [Lucking and Brice, 2000]. Several hypotheses for the normal function of α-synuclein have been proposed, based on its structure, physical properties,

subcellular localization and interacting partners. The primary site of α-synuclein function is most likely pre-synaptic, as it can be isolated from synaptic-membrane fractions and localizes predominantly near and around the vesicles of the pre-synaptic terminal [Clayton and George, 1999; Nakajo et al, 1990; Withers et al, 1997]. *In vivo*, it binds to rat brain vesicles via the first four 11-mer N-terminal repeats [Jensen et al, 1998], and also binds to monolayer phospholipid membranes, acquiring α-helical secondary structure [Davidson et al, 1998]. Interestingly, α-synuclein binds exclusively to acidic phospholipids, especially phosphatidic acid and to vesicles with small diameters. This may target the protein to specific subpopulations of membranes or vesicles [Davidson et al, 1998]. In vitro, α-synuclein is phosphorylated on Serine-129 by casein kinase 1 and 2. Phosphorylation and dephoshporylation of α-synuclein seem to be tightly regulated in vivo [Okochi et al, 2000] and might influence its binding to lipid membranes or to phospholipase D2 [Jenco et al, 1998].

Though it appears lipid binding is a broadly distributed property of α-synuclein [McLean et al, 2000; Perrin et al, 1999], not all cellular α-synuclein seems to be linked to membranes, since it can also be purified from the cytosol and nucleus as reported earlier [George et al, 1995; Maroteaux et al, 1988; El-Agnaf et al, 1998]. The physical and functional homology of α-synuclein with 14-3-3 chaperone proteins suggest that α-synuclein may play a role in cell signaling pathways [Osterova et al, 1999]. α-Synuclein stimulates Protein kinase A, that phosphorylates the Ser262 and Ser356 residues of tau protein [Jensen et al, 1999], which is the major constituent of insoluble paired helical filaments found neurofibrillary tangles and plaque neuritis in AD [Grundke-Iqbal et al, 1986a;

Grundke-Iqbal et al, 1986b]. Since the phosphorylation of Ser262 inhibits tau binding to microtubules, α-synuclein may modulate tau function. α-Synuclein was detected in axons and developing pre-synaptic terminals after their formation in rat embryonic hippocampal cells in culture, suggesting a possible role in synaptic development and maintenance [Withers et al, 1997]. α-Synuclein also seems to contribute to neuronal differentiation. The arguments in this direction are (a) In vivo, α-synuclein is localized in the cell body of neuronal precursors during early embryonic development in mice [Hsu et al, 1998] and humans [Bayer et al, 1999], but in pre-synaptic terminals in postnatal and adult cortex, and (b) a sustained increase in α-synuclein levels was observed when rat pheochromocytoma cells (PC12) were treated with nerve growth factor, which induces a neuronal phenotype [Stefanis et al, 2001]. The involvement of α-synuclein in synaptic plasticity and neuronal differentiation may be mediated by the selective inhibition of Phospholipase D2 by α-synuclein [Jenco et al, 1998], since isoforms of phoshpolipase D were shown to be implicated in cell growth and differentiation [Klein et al, 1995].

However, the involvement of α-synuclein in neuronal and synaptic development could not be confirmed in mice lacking the α-synuclein gene homozygously, since these mice were behaviorally normal and showed neither macroscopic nor microscopic changes in their nervous system [Abeliovich et al, 2000]. Hence, inactivation of the α-synuclein gene does not lead to a significant neurological phenotype, although changes in dopaminergic electrophysiology may reflect a specific function related to neurotransmitter release. When α-synuclein expression was markedly reduced in cultured rat neurons [Murphy et al, 2002] or abolished in α-synuclein knock out mice [Cole and Murphy, 2002], the number of vesicles in the distal

pool of the pre-synaptic terminal is reduced indicating a role for α-synuclein in vesicular dynamics. According to Cole and Murphy [2002] α-synuclein's involvement in lipid metabolism cannot be ruled out, given its propensity to bind molecules with high hydrophobic content or exposed hydrophobic domains. Recently, Tanji et al, [2002] reported that α-synuclein was also expressed in cultured human astrocytes and its levels were increased by stimulation with interleukin-1β, suggesting that it may be involved in inflammatory processes and immune responses. Though several studies reported the presence of α-synuclein in nucleus of cultured cells, there is no data to suggest the function of α-synuclein in nucleus. The presence of nuclear phospholipids and related proteins demonstrates phosphatidylinositol-linked activities in nucleus [Leng et al, 2001]. The authors further suggest that α-synuclein may regulate processes in the Phosphatidyl Inositol (PI)-cycle in the nucleus as it appears to in cytoplasm and plasma membrane. Table 1.3 gives the potential normal functions of α-synuclein and important basis for the given function.

The major difficulty in understanding α-synuclein function has been its inherent flexibility in native structure and its altered conformation in presence of lipids. It is not known whether the molecules that bind to α-synuclein in vivo, do so in its membrane-associated (modular) or free (natively unfolded) state, or both [Cole and Murphy, 2002]. It also remains to be investigated whether the interactions of α-synuclein with membranes influence its self-assembly and filament formation. Further, the unstructured acidic C-terminus of α-synuclein is a likely site for interaction with additional proteins and other molecules, and hence, non-specific electrostatic interactions are bound to lead to false avenues of investigation.

**Table 1.3**

**Potential normal functions of α-synuclein,** Important basis for the given function is indicated.

| |
|---|
| ***Synaptic Plasticity:*** Interaction of α-synuclein with brain vesicles and phospholipid membranes. |
| ***Synaptic development and maintenance:*** α-synuclein was detected in developing pre-synaptic terminals. |
| ***Neuronal differentiation:*** Treatment of PC12 cells with nerve growth factor caused increase in α-synuclein levels |
| ***Lipid metabolism:*** α-synuclein has a high propensity to bind molecules with high hydrophobic content or exposed hydrophobic domains. |
| ***Vesicular dynamics****:* Number of vesicles in pre-synaptic terminal is reduced when α-synuclein expression was markedly reduced in cultured rat neurons or abolished in α-synuclein knock out mice. |
| ***Inflammatory processes and immune responses:*** α-synuclein levels were increased by stimulation with interleukin-1β in cultured human astrocytes. |
| ***Modulation of tau function:*** The ability of α-synuclein to phosphorylate Ser262 of tau, which inhibits tau binding to microtubules. |

Thus persuasive evidence of a role of α-synuclein in any pathway or function requires multiple approaches [Cole and Murphy, 2002]. The structure, expression and functions of α-synuclein has been recently reviewed by Dev et al, [2003].

## 1.12 α-Synuclein and neurodegeneration-Genetic basis

The two prominent features in neurodegenerative disorders are a movement disorder (typified by PD) and a memory disorder (typified by AD). The population of patients with a late-life dementia with mild to moderate motor symptoms and memory disturbances is referred to as dementia with Lewy bodies (DLB). Both PD and DLB

are characterized pathologically by abundant neocortical and subcortical LBs and LNs in brain [Spillantini et al, 1997; Wakabayashi et al, 1998; Arima et al, 1998].

Of the three synucleins, only α-synuclein is associated with filamentous inclusions of PD and DLB. Using specific antibodies, β- and γ-synucleins have been shown to be absent from these inclusions [Spillantini et al, 1997; Spillantini et al, 1998]. In 1997, five families with hereditary PD were reported to have the illness because of a mutation that changed a Glycine to an Alanine at position 209 (G209A) in the α-synuclein gene, on chromosome 4q21, resulting in an A53T exchange [Polymeropoulos et al, 1997]. Subsequently a second mutation in the α-synuclein (nucleotide change G88C, resulting in an A30P exchange) was described in another family indicating that these mutations are involved in the pathogenesis of PD [Lynch et al, 1997]. This was followed by the discovery that the LBs found in typical non-familial PD contain the protein α-synuclein. The LBs and LNs contained both partially truncated α-synuclein and insoluble aggregates of both full-length and truncated protein [Baba et al, 1998; Culvenor et al, 1999]. α-Synuclein was also screened for mutations in other neurodegenerative diseases like AD, however, no mutations have been detected in the α-synuclein gene in AD [Campion et al, 1995].

The finding of insoluble protein-containing materials in different neuronal and glial cell populations in a broad range of syndromes suggests that many of these disorders have something in common. Even though these syndromes express different symptoms and lesions, the mechanisms underlying filament formation may be

similar. The assembly of normally soluble protein subunits into insoluble filaments in these diseases does not normally occur in healthy brain. Hence, another way to approach these disorders is to consider the disease state as one of an abnormality in protein metabolism. Future research efforts will pursue molecular analyses of shared protein abnormalities across several disorders. This approach should provide insights into disease mechanisms underlying one or more degenerative disorders characterized by abundant filamentous lesions. In this manner, preventive and potentially curative strategies for these disorders may be possible.

## 1.13 α-Synuclein aggregation- Unraveling the destructive role of α-synuclein

The major etiological factors for PD are environmental (metals and pesticides) and genetic factors (mutations). There are few studies on the role of these factors in promoting α-synuclein aggregation (Fig 1.8). However, very few cases of PD have pure genetic or environmental etiology, while in vast majority both genetic and environmental factors are involved. Understanding this "Cross-talk" between genetic and environmental factors is important in PD research. The major unanswered question is how an essentially random coiled α-synuclein go to β-sheet and aggregates in PD. In the context of inherited PD, the immediate question is how mis-sense mutations of α-synuclein gene can cause degeneration of dopamine nerve cells in SN [Goedert, 2001]. Also, it is important to know the relevance of α-synuclein dysfunction to the neurodegenerative process in all cases of Lewy body diseases. Because of its 'natively unfolded' structure, α-synuclein might be especially prone to self-aggregation or to cause the aggregation of other proteins or intracellular structures [Lucking and Brice, 2000]. Both wild-type

(WT) and mutant α-synucleins (A30P and A53T) were capable of time-, temperature-, pH- and concentration-dependent self-aggregation in to fibrils, when present in supersaturating conditions [Hashimoto et al, 1997; Hoyer et al, 2002]. The fibrils were similar to those isolated from LBs of patients with PD [Conway et al, 1998]. Fibrillization was accompanied by a change in secondary structure of α-synuclein from an unfolded random coil to an antiparrellel β-sheet [Conway et al, 2000b; Narhi et al, 1999]. Fibril formation from WT and mutated (A30P and A53T) α-synuclein involves nucleation dependent elongation [Conway et al, 2000b; Wood et al, 1999] in which the protein aggregates in to seeds and then accelerates fibril formation in a dose-dependent manner.

As previously reported [Weinreb et al, 1996; Uversky et al, 2001a] α-synuclein at neutral pH has a far-UV-CD spectrum typical of an unfolded polypeptide chain, and reflecting the lack of ordered secondary structure under these conditions. Aggregation or self-association is a characteristic property of a partially folded (denatured) proteins and most aggregating protein systems probably involve a transient partially folded intermediate as the key precursor of fibrillation [Fink, 1995]. It has also been shown that in some cases the self-association induces additional structure and stability in the partially folded intermediates. The natively unfolded character of α-synuclein arises from its low intrinsic hydrophobicity and high net charge at neutral pH (pI 4.7) [Uversky et al, 2001a].

Thus the conditions that decrease the net charge and that increase the hydrophobicity would be expected to result in partial folding. This partially folded conformation is characterized by an increased amount of ordered secondary structure and by the appearance of solvent accessible hydrophobic clusters.

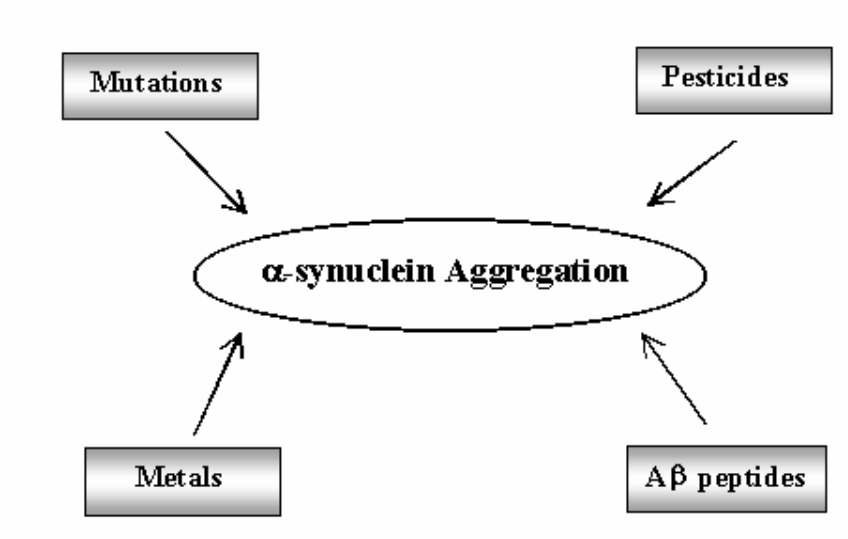



**Figure 1.8**
**Factors influencing α-synuclein folding and accelerating aggregation.** The major etiological factors for PD environmental: Metals and Pesticides, and genetic: Mutations and Aβ. However, in majority of PD cases a "cross-talk" of environmental and genetic factors is prevalent.

Importantly, the strong correlation observed between the degree of protein folding and its efficiency of fibril formation suggests that this intermediate partially folded form can be a precursor of fibrils [Uversky et al, 2001a]. This is because, in contrast to an unfolded protein, a partially folded intermediate is anticipated to have contiguous hydrophobic patches on its surface which are likely to foster self-association and hence potentially fibrillation. There are two interesting features of the α-synuclein partially folded intermediate: (i) It has some β-structure, which is the major type of secondary structure in α-synuclein fibrils, and (ii) It is relatively unfolded (i.e. more similar to random-coil conformation than a native tightly folded globular conformation). Khurana et al, [2001] have recently observed that a partially folded intermediate of IgG light

chains that form amyloid fibrils is relatively unfolded and they have also predicted that it could be a common feature in amyloid fibril formation that the critical monomeric partially folded intermediate is relatively unfolded. The intracellular factors that lead to a shift in the equilibrium position between the natively unfolded state and the partially folded intermediate will increase the likelihood of α-synuclein fibril formation. The recent arguments suggest that the misfolding of proteins leading to aggregation as a common pathological mechanism in diverse neurodegenerative diseases [Soto, 2003]. Several genetic and environmental factors have been associated with protein misfolding and aggregation. The familial PD mutations also probably destabilize the native protein conformation, favoring misfolding and aggregation. Besides the mutations, environmental factors that might catalyze α-synuclein misfolding include changes in the levels of metal ions, pathological chaperone proteins, pH or oxidative stress, macromolecular crowding and increases in the concentration of misfolding protein [Soto, 2003; Teplov, 1998]. Many of these changes are associated with ageing and late onset of neurodegenerative disease.

Though the A30P and A53T mutations did not change the secondary structure of the protein [Conway et al, 1998], the mutants are more prone for aggregation compared to WT [Narhi et al, 1999]. The role of metal ions in the aggregation of α-synuclein has been studied in some detail [Paik et al, 1999; Hirsch et al, 1991]. Cations like copper, iron and aluminium have been shown to accelerate the kinetics of aggregation [Paik et al, 1999]. α-Synuclein was shown to bind to amyloid peptides in AD brain [Jensen et al, 1997] and Aβ 25-35 fragment triggers α-synuclein aggregation in vitro [Paik et al, 1997]. However, the Aβ 25-35 has not been described in vivo, thus the relevance of this fragment for the pathological process remains to

be established [Lucking and Brice, 2000].

## 1.14 Our Hypothesis-mechanism of α-synuclein toxicity

α-Synuclein is in random coil conformation in aqueous solutions *in vitro* and also in cytoplasm in vivo. After translation however, majority of α-synuclein in cell is carried to its most likely functional site namely plasma membrane and vesicular membranes. In association with membranes α-synuclein is in α-helix conformation. This is the case with normal or non-neurodegenerative brains cells.

However in PD, a totally different cytological scenario exists. Several studies have reported higher levels of iron and other transition metals in PD brain [Dexter et al, 1989a]. In the presence of these metals α-synuclein acquires a misfolded or partially folded conformation as described above. Especially iron and aluminium initially induce a partially folded structure in random coil α-synuclein and eventually promote aggregation in vitro [Uversky et al, 2001a]. We hypothesize that the partially folded or misfolded α-synuclein induced by metals may not bind to vesicle membrane lipids as it does in normal brain (Fig 1.9). It was observed that the A30P mutation completely abolished membrane-binding property of α-synuclein [Clayton and George, 1999]. Hence, the disruption in membrane binding resulting from increase in metals and mutations in familial PD would result in the increase in free α-synuclein (in the form of partially folded or unfolded native conformation) levels in the cytoplasm, which may be prone to oligomerization and subsequent aggregation *in vivo*.

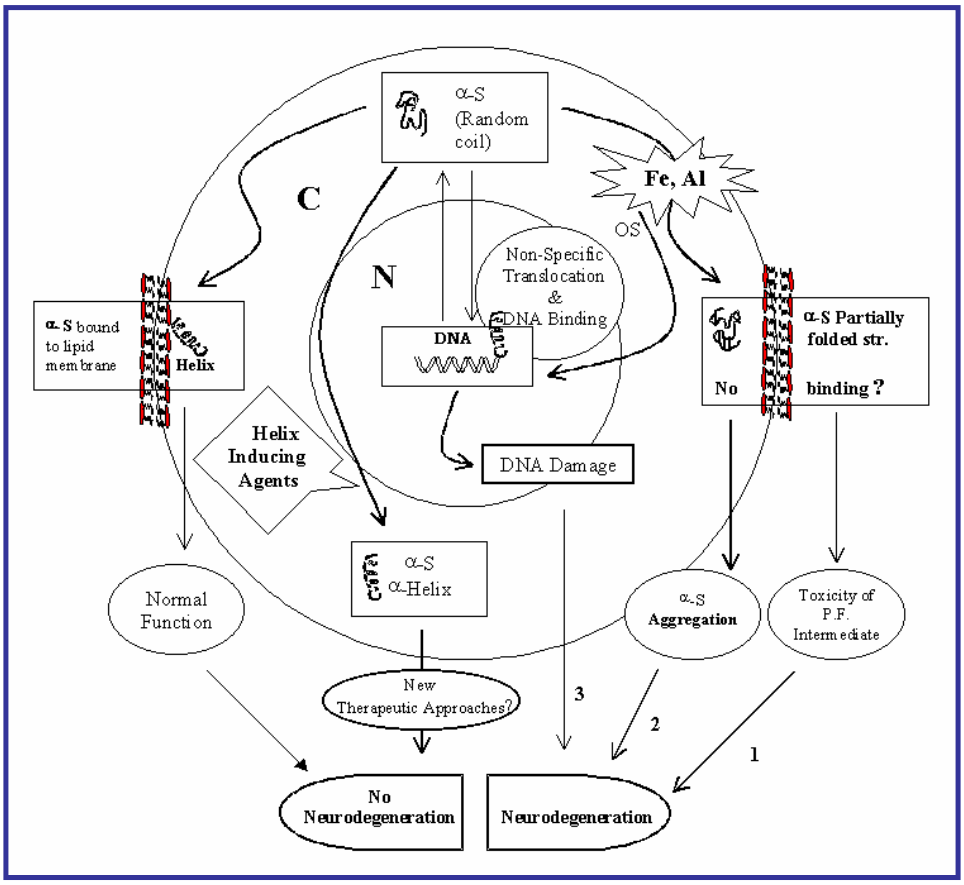



**Figure 1.9**

**Three possible ways of protein and metal induced neurodegeneration in Parkinson's disease and a new insight in to a potential therapeutic approach.** This is a new argument on what possibly triggers the increase in precursor for α-synuclein aggregation in PD. Legends: α-S: α-synuclein; P.F: Partially folded; N: Nucleus; C: Cytoplasm; OS: Oxidative stress.

This is a new argument on what possibly triggers the increase in precursor for α-synuclein aggregation in PD. The protein aggregates having multiple shapes can create mechanical injury to cell system and because of the large solid size of the aggregates may interfere with the normal functions of the cell and may ultimately

lead to neurodegeneration.

Another school of thoughts suggests that the misfolded or partially folded α-synuclein is more cytotoxic than the protein aggregates. The intermediate partially folded or misfolded form may be entropically rich in energy and may bind to other components in the cell and may be a cause for neurodegeneration. In that sense, the formation of aggregates could be a protective measure adapted by the cell against the toxicity of this intermediate. However, it is still a matter of debate regarding the toxic form of the protein (monomeric or oligomeric?) in neurodegenerative disorders.

Further, based on the several evidences for localization in nucleus and the primary sequence of α-synuclein, where it has the positively charged amino acids clustered towards N-terminal end, we hypothesized that α-synuclein may be having a DNA binding role in nucleus. The α-synuclein appears to be stabilized in the form of α-helix [Davidson et al, 1998]. Because formation of helix may prevent the β sheet secondary structure in α-synuclein and thereby prevent or delay the aggregation. We advocate that the helix inducing agents could become important tools in newer therapeutic approaches against PD.

## 1.15 New directions in recent times

Research studies are exploring the basic biomechanisms of PD and looking for ways to stop the degenerative process that cause the disorder. Clinical trials across the country are investigating new medications and therapies to find ways to improve the lives of people with PD. For example:

- Studies are looking at how Lewy bodies - the characteristic dense clumps of abnormal protein that are found in the neurons of people with PD - are involved in the disease process. Some researchers believe Lewy bodies are the cause of neuron death, others see them more as a byproduct of the neurodegenerative process, and still others believe they are a protective mechanism employed by the neuron to lock away abnormal and harmful proteins. Discovering how Lewy bodies work, and why, may open doors to potential interventions that could prevent the cascade of biological events that cause the neurodegeneration of PD.

- Several groups of researchers are exploring new opportunities for drug development by studying neurotrophins, molecules in neurons that support growth and survival. Understanding how neurotrophins work could lead to the development of drugs that stop the degeneration of dopamine cells and heal those that are already damaged. Another avenue of exploration involves neuroprotective substances in cells - molecules that protect neurons from harm.

- Cell transplantation is one way of repairing the damage PD causes in the brain. In laboratory experiments, researchers are studying embryonic stem cells, adult stem cells, and other types of promising cells to see if they can be coaxed into turning into dopamine-producing neurons. In studies using animal models, researchers are looking at what these new neurons need to survive, make the proper connections, and become fully functional cells in the brain.

**Recent advances in PD research prompt a new research agenda to elucidate mechanisms underlying all synucleinopathies particularly PD, and to discover new therapies:**

The goals need to be accomplished are:

1. Elucidate the normal functions and metabolism of α-synuclein in neurons and glia of the developing and mature nervous system.
2. Characterize the normal interactions of α-synuclein with other proteins as well as with organelles in neurons and glia in addition to the biological significance of these interactions.
3. Determine the effects of A53T, A30P and E46K familial PD α-synuclein gene missence mutations on the functions and properties of αsynuclein and the mechanisms underlying these effects.
4. Understand the mechanisms leading to the aggregation and fibrillation of α-synuclein and the pathological consequence there off.
5. Develop informative *in vivo* (e.g. transgenic mice) and *in vitro* (e.g. transfected cultured cells and recombinant proteins) model systems for mechanistic studies of the normal and abnormal biology of synucleins
6. To elucidate the role of α-synuclein conformation, aggregation and fibrillation in the onset/ progression of PD.
7. Screening novel therapeutics for PD and other synucleinopathies based on stabilizing the non-toxic form of α-synuclein.

## AIMS AND SCOPE OF THIS STUDY

Recent studies showed that oxidative stress, DNA damage, chromatin condensation, and altered expression of genes [Enochs et al, 1994] are also associated with neurodegeneration in PD like AD. Further, our lab for the first time evidenced that the genomic DNA undergoes a helicity change in AD from B-form to left handed Z-DNA form [Anitha et al, 2002a]. These observations and the fact that α-synuclein has DNA-binding property suggest that topological changes in the genetic material may be involved in the pathogenesis of PD also. So far no studies have been reported on the DNA structure and conformation in PD affected brain cells**.** In this perspective we made an attempt to map the helicity and stability pattern of genomic DNA isolated from different regions of PD affected human brain.

Recent observations showed that α-synuclein is localized in the chromatin region of nuclei in the brain (Torpedo and rat) possibly due to non-specific translocation [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004]. Moreover, the presence of the majority of the lysine residues in the N-terminal region of α-synuclein suggests a possible DNA binding role for α-Synuclein. Hence, it is interesting to investigate the DNA binding property of α-synuclein and study the effect of DNA binding on α-synuclein folding/conformation and aggregation properties. The osmolytes induced conformational changes in α-synuclein and influence of osmolytes on aggregation properties of α-synuclein was also investigated and this was used as a model system to understand the mechanism of DNA induced folding in α-synuclein.

Metals are well established to be risk factors for PD, though the significance of metals in the aetiopathogenesis of PD is still unknown. Several studies have shown decreased copper, increased zinc and iron in the *substansia nigra* and increased copper concentrations in the cerebrospinal fluid of PD affected patients [Dexter et al, 1991]. Though much work has been done on metal homeostasis in PD brain, limited data is available on trace elemental levels and their inter-relationships in serum of Parkinson's patients [Jemenez et al, 1998]**.** We provided a comprehensive database on metal levels in moderate and severe PD serum and their inter-relationship with severity of PD.

## Objectives

1. To study the helicity and stability (topology) of genomic DNA isolated from selected regions of Parkinson disease affected human post-mortem brain (*Chapter* 2)

2. To study the mechanism of DNA binding and nicking property α-synuclein (*Chapter* 3)

3. To study the conformation/aggregation of α-synuclein in presence of DNA and understanding the mechanism using the chaperon property of naturally occurring osmolytes (*Chapter* 4)

4. To map trace elemental homeostasis in the serum samples Parkinson's patients (*Chapter* 5)

☼ ☼ ☼ ☼ ☼

# CHAPTER 2

# *Studies on Genomic DNA topology and stability in selected brain regions of Parkinson's disease*

## 2.1 Introduction

Parkinson's disease (PD) is a neurological disorder of the extrapyramidal system characterized clinically by akinesia, rigidity and resting tremor [Enochs et al, 1994] and pathologically by extensive and progressive loss of the melanized dopaminergic neurons projecting from the substantia nigra pars compacta to the striatum in the midbrain to the striatum (caudate nucleus and putamen) [Greenfield and Bosanquet, 1953; Pakkenberg and Brody, 1965; Jellinger, 2000]. PD is associated with widespread occurrence of Lewy bodies (LBs) in the neurons of central and autonomic nervous system along with multiple biochemical deficits as the basis of clinical symptoms [Jellinger, 2000]. In both PD and normal aging, the pigmented neurons in midbrain are most susceptible to degeneration [Pakkenberg and Brody, 1965; Graham, 1979; Mann and Yates, 1979, 1983; Hirsch et al, 1988].

Causes/ mechanisms of cell death in PD are still poorly understood [Jellinger 2000]. Substantial evidence implies that redox imbalance or oxidative stress following overproduction of reactive oxygen/nitrogen species overwhelming the protective defense mechanism of cells contributes to the pathogenesis of PD [Berg et al, 2004; Migliore et al, 2001; Jellinger, 2000; Hirsch et al, 1999; Cohen, 1986; Youdim et al, 1989; Olanow, 1992; Dexter et al, 1992]. Oxidative stress in PD could result from several mechanisms, such as generation of hydrogen peroxide by normal and abnormal metabolism of dopamine [Cohen, 1983; 1986; Graham, 1984]; increased amount of redox active metal ions, principally iron, [Sofic et al, 1988; Zecca and Swartz, 1993; Zecca et al, 2001] reduction of hydrogen peroxide to potentially highly toxic hydroxyl radical by redox metals [Halliwell, 1989; Adams and Odunze, 1991; Dexter, 1992; Riederer et al, 1992; Ben-Shachar et al, 1992; Enochs et al, 1994]; reduced antioxidative

capacity [Jenner, 1992; Jenner et al, 1998]; and the presence of increased products of lipid peroxidation [Jenner, 1992; Jenner et al, 1998]. One of the consequences of redox imbalance is apoptosis and/or necrosis (programmed vs passive cell death) which are associated with neurodegeneration in PD [Burke and Kholodilov, 1998; Kingsbury et al, 1998; Ziv and Meland 1998; Tatton et al, 1998; Jenner and Olanov et al, 1998; Ziv et al, 1997; Robbins, 1987]. Studies have also shown that the levels of the nucleoside, 8-hydroxy - 2'-deoxyguanosine (8-OHdG), a product of free radical attack on DNA were generally increased and differentially distributed in PD brains with highest levels in caudate, putamen, SN and cerebral cortex [Alam et al, 1997].

Features of apoptosis based on histochemical methods to mark endonuclease-induced DNA fragmentation by in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling - TUNEL/ISEL [Gavrieli et al, 1992] or in situ nick translation [Gold et al, 1994] have been reported in SN in PD [Anglade et al, 1996, 1997a, 1997b; Tompkins et al, 1997; Tatton et al, 1998; Olanow et al, 1998; Kingsbury et al, 1998; Hirsch et al, 1999; Tatton and Olanow, 1999]. However, there have been many conflicting reports on the presence/incidence of DNA fragmentation in SN neurons in PD [Jellinger, 2000; Kingsbury et al, 1998; Kosel et al, 1997]. Also little information is available on DNA integrity in other regions of a parkinsonian brain. Further, we have shown an altered DNA conformation in hippocampus of brains of Alzheimer's disease [Anitha et al, 2002a]. However, such information on the topology and conformations of DNA in PD brain is lacking.

In this investigation, genomic DNA was isolated from eight regions in the human brain, namely frontal, temporal and occipital

cortex, hippocampus, caudate/ putamen, thalamus, cerebellum and midbrain collected at post-mortem from cases of PD and controls and were analyzed for single and double strand breaks in DNA, and their conformations and topology.

## 2.2 Materials and Methods

### 2.2-1. Chemicals

Radiolabelled $^{3}$[H]-TTP (Sp. Act. 40Ci/nmol) from Amersham radiochemicals, UK. Ribonuclease A (RNAse A), Proteinase K, Deoxyribonuclease I (DNAse I), dATP, dTTP, dCTP, dGTP, Low melting agarose, Cacodylate buffer, *E. Coli* DNA Polymerase I, Terminal deoxynucleotidyl transferase enzymes, 1Kb and 100bp DNA ladders, Lamda DNA ladder were purchased from Bangalore Genei, India. Ethidium bromide (EtBr), HEPES and Tris buffers were purchased from Sigma Chemicals (USA). All other chemicals were of analytical grade and were purchased from Sisco Research Labs, Mumbai, India.

### 2.2-2. Brain Samples

Six normal and five PD affected human brain samples were obtained from the 'Human Brain Tissue Repository for Neurological Studies' (HBTR), National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India. Autopsies were performed on donors from whom written informed consent was obtained either from the donor or direct next of kin. The control human brains (age 46–64 yrs) were collected from road traffic accident victims, who had no history of long-term illness, movement disorders, dementia, or neurological disease prior to death. The PD-affected (50–71 yrs) brains were postmortem specimens from clinically well-documented and neuropathologically confirmed cases who were under long term follow up at the Neurological

services at NIMHANS, Bangalore, India. The average post-mortem interval between the time of death and collection of brain and freezing was ≤ 8 hrs. With in one hour after death, the body was kept in refrigerator maintained at $4^0$C. Dissected brain tissue was stored frozen at -$80^0$C till the analysis.

### 2.2-3. Isolation of DNA from brain tissue

Genomic DNA was isolated from various regions (frontal, temporal and occipital cortex, hippocampus, caudate/ putamen, thalamus, cerebellum and midbrain) of frozen brain tissue by standard 'phenol-chloroform extraction' method after Sambrook et al, [1989] with some modifications to prevent DNA fragmentation during isolation. The method involved the following steps:

1. Brain tissue was cut into small pieces and a weighed amount of tissue was transferred into an autoclaved porcelain mortar and pestle. All glass wares, mortar, pestle etc were autoclaved in order to avoid bacterial contamination.

2. Liquid nitrogen was poured into the mortar and the tissue was allowed to freeze.

3. Tissue was ground thoroughly with pestle with frequent additions of liquid nitrogen.

4. Sufficient quantity of liquid nitrogen was poured into the mortar and was swirled. Tissue homogenate was transferred into a sterile tube and the liquid $N_2$ was allowed to evaporate (a sterile spatula was used to transfer the powdered tissue into a graduated tube)

5. Lysis buffer (50mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 100mM NaCl) was added into the tube along with 15µg per mL of proteinase K and 2% SDS final volume. (1 mL of lysis buffer was used for every 500 mg of tissue. (Note1: Lysis buffer should be pre warmed, 2: Add Proteinase K after first 2 h, optimum: 3 h).

6. The homogenate was incubated at $37^0$ C in a water bath for 12-16 h or over night.

7. After the completion of incubation, the incubated lysate was transferred to an autoclaved 50 mL conical flask and equal volume of tris-saturated phenol (pH 8.0) and mixed thoroughly, either manually or mechanically for 10min.

8. The lysate was centrifuged for 10min at 10,000 rpm at $13^0$C.

9. The supernatant was collected into a fresh autoclaved 50 mL conical flasks and ½ volume of Tris-saturated phenol and chloroform: isoamyl alcohol was added and mixed thoroughly. (1 part phenol: 1 part chloroform (C) and isoamylalcohol (IA) mixture (C:IA=23:1). (Note: Tris-saturated phenol was stored in amber colored bottles at low temperature to avoid oxidation of phenol).

10. The supernatant and Tris-saturated phenol-chloroform mixture was centrifuged at 5000 rpm at $4^0$C.

11. The upper aqueous layer was collected into a fresh tube and $1/30^{th}$ volume of sodium acetate (pH 5.5) and equal volume of chilled absolute ethanol were added.

12. DNA was precipitated by slowly swirling the tube manually. (Note: Pre-cooled tubes were used and DNA was transferred into another tube containing 70% alcohol for washing).

13. DNA was washed twice with 70% alcohol and once with absolute alcohol to remove excess salt and vacuum dried.

14. The vacuum dried DNA was dissolved in 1mL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The DNA isolated from cells also contains RNA which was removed by digesting the preperation with RNAse enzyme. RNAse solution was kept in boiling water for 10min before use so as to inactivate any DNAse, because the RNAse may also contain DNAse.

***Precautions taken in order to prevent in vitro DNA damage during phenol/chloroform genomic DNA extraction:***

1. Employed proper microbiological aseptic technique when working with DNA.

2. Wore latex gloves when isolating and handling DNA to prevent nuclease contamination from the surface of the skin and to minimize the activity of endogenous nucleases.

3. Used sterile, disposable plasticware. Autoclaved pipette tips and 1.5 mL microcentrifuge tubes were used for additional protection against nucleases. Pipettes were wiped with DNAse-removal solutions (diethylpyrocarbonate) when transitioning between handling crude extracts to handling more purified material.

4. Pipette tips with blunt ends (the tips cut with blade) were used as a precaution to prevent DNA fragmentation.

5. Liquid nitrogen was used to keep the frozen tissue hard before grinding. This step will prevent activation of endonucleases.

### 2.2-4. DNA Concentration and Purity

The concentration of DNA was measured by noting absorbance at 260nm and the purity of DNA preparation in terms of any protein contamination was checked by recording the ratio of absorbance at 260nm/280nm which should be ideally between 1.6-1.8.

DNA was quantified by the following equation:

OD at 260 nm x 50 x Dilution factor = μg/mL of DNA

Low salt and alkaline buffer were used in order to achieve reproducible absorbance at 260nm.

### 2.2-5. Neutral and alkaline agarose gel electrophoresis

The integrity or damage in genomic DNA was assessed by running neutral and alkaline gel electrophoresis. The migration pattern in neutral gels reflects the double strand breaks present in the DNA and the migration pattern in alkaline gels shows both single and double strand breaks [Sutherland and Shih, 1983]. Neutral gels were electrophoresed on 1·5% agarose gels in Tris-acetate-EDTA buffer [pH 8.0) at 4V/cm for 4 hrs. 3μg of DNA was loaded in each well.

For alkaline gels, the DNA samples were mixed with alkaline stop mix solution consisting of 1N NaOH and 2 x BCG dye. BCG dye consists

of 0.25% Bromocresol green and 50% (v/v) glycerol. 3 μg of DNA sample was mixed with the alkaline stop mix and loaded on to the gel and run with alkaline running buffer consisting of 30mM NaOH and 2mM EDTA for 4 hrs at 4V/cm. After the run the alkaline gel was neutralized in 100mM Tris buffer, pH 8.0 for 30 minutes and then stained with EtBr for 15 minutes. The stained gels were analyzed in UV gel documentation system. DNA ladders (1Kb and 100bp) and lamda DNA Hind III digest were used as molecular weight markers.

## 2.2-6. Estimation of single strand and double strand breaks in genomic DNA isolated from control and PD affected post mortem brain samples

***Single strand breaks (SSBs)-*** SSBs were calculated through incorporation of $^{3}$[H]-TMP in to DNA samples when incubated with *E. Coli* DNA polymerase I (Klenow Fragment) in a nick translation assay. DNA polymerase I adds nucleotides at the 3´-OH end of a SSB, generated by various means, using the other strand as template. When one of the deoxynucleotide triphosphates is labeled, then the incorporation of radioactivity into substrate DNA would be proportional to the number of SSBs present in the DNA sample. During the standardization of the assay conditions with a plasmid DNA (Cos T fragment of λ phage) having known number of SSBs, it was found that an average of 1500 nucleotides were added at each of the 3'-OH group. From this it was inferred that each picomole of TMP incorporated was equivalent to 1.6 X $10^{9}$ 3'-OH groups or SSBs [Mandavilli and Rao, 1996]. In a total reaction volume of 50 μl, the assay mixture consisted of: 40mM Tris-HCl, pH 8.0, 1mM β-mercaptoethanol, 7.5mM $MgCl_2$, 4mM ATP, 100μM each of dATP, dCTP and dGTP and 25μM of dTTP, 1μCi of $^{3}$[H]TTP and 1μg of genomic DNA

and 1 unit of *E. coli* DNA polymerase I.

SSBs were estimated by following the incorporation of $^{3}$[H]-thymidine into DNA. After incubation of DNA (treated with Aβ peptides) with *E.coli* polymerase I for 30mins, the DNA was precipitated by adding 10% TCA containing 10 mM Na pyrophosphate in the presence of 100μg of Calf Thymus DNA and bovine serum albumin (BSA) act as carrier molecules. Pellet was separated after centrifugation at 12000 rpm for 15 sec and dissolved in 400 μl NaOH (0.2 N) by vortexing. DNA was reprecipitated in 10% Trichloro acetic acid (TCA) containing 10 mM Na pyrophosphate, which was separated by centrifugation at 6000 rpm for 5 min. The DNA precipitated was washed twice with 5% TCA, and 95% ethanol on Glass Fibre (GF 6). The filters were dried at room temperature for ~12 h and counted for radioproperty in BRAY'S mixture (containing 4gms/litre of PPO, 200 mg/ liter POPOP in Dioxan) in Beckman LS1800 Liquid scintillation counter. The values were expressed as DPM incorporated into the 1μg of DNA.

***Double strand breaks (DSBs)***- Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides to the 3´ termini of DNA and does not need direction from template strand. Here, 3´ ends of duplex DNA also serve as substrates. Similar conditions to incubate DNA with Terminal transferase as in the case of *E. coli* poymerase I assay were used. The incorporation of the $^{3}$[H]-dTTP into DNA would be proportional to the number of DSBs in the DNA. From the conditions of incubation [Mandavilli and Rao, 1996; Deng and Wu, 1983] it was assumed that about 50 TMP residues were added at each of the 3'-ends of the duplex DNA. From this it was calculated that each femtomole of TMP incorporation would be equivalent to 1.2 X $10^{7}$ 3´ ends or half that number minus one DSBs.

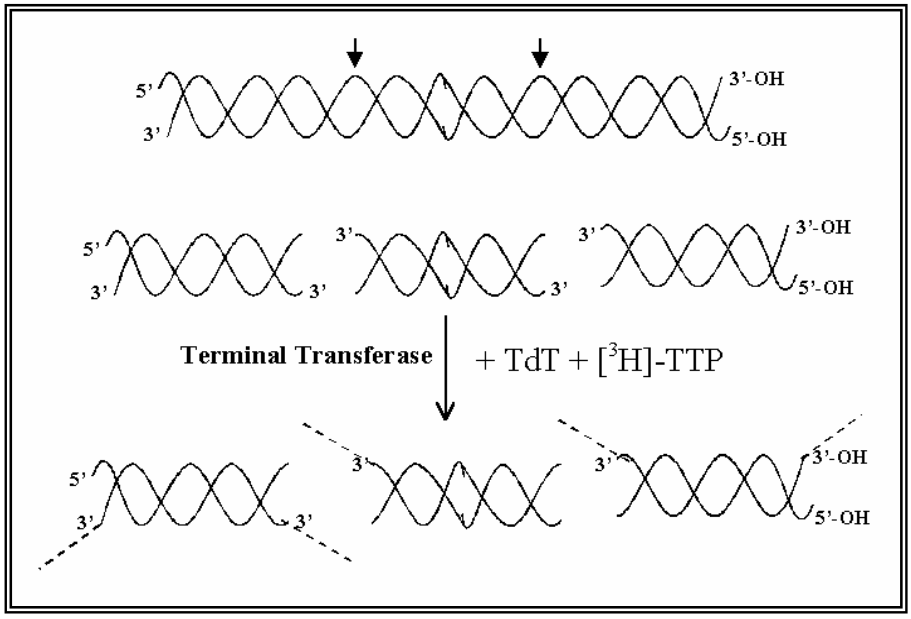



**Figure 2.1**
**Nick Translation Strategy**

**Strategy for calculating the number of DSBs present in DNA using terminal transferase activity.** Intact linear double-stranded DNA molecule has two terminal 3'-OH groups. Supposing that two DSBs occurred in that molecule, as shown by arrows, the result would be three pieces of double-stranded DNA with the generation of four more terminal 3'-OH groups. The number of such terminal 3'-OH ends can be assessed by labeling those ends with a radioactive nucleotide using terminal transferase activity. As can be seen, the DSBs that are present in the DNA sample would be equivalent to half the number of terminal 3'-OH groups minus one.

The assay mixture for Terminal transferase reaction consisted in a total volume of 50μl: 100mM sodium cacodylate buffer, pH 7.0, 1mM $CoCl_2$, 0.2mM DTT, 1 μCi of $^3$[H]-dTTP, 1 μg DNA and 1 unit of the enzyme. The schematic representation of Nick Translation strategy for terminal transferase assay is given in Fig. 2.1.

## 2.2-7. Melting temperature and hyperchromicity of the genomic DNA

In order to determine the physical state of the DNA in PD brain, the melting profiles of DNA isolated from different regions of PD brains and corresponding control groups were examined. DNA was dissolved either in HEPES buffer, 0.01M, pH 7.4 or in sodium cacodylate buffer, pH 7.4. The DNA was used at a concentration of 20 μg/mL. The melting profiles (Tm) of the control and PD DNA were recorded in spectrophotometer, equipped with a thermoprogrammer and data processor (Amersham, Hongkong). The hyperchromicity changes of the DNA was recorded from $30^0$C - $95^0$C with $1^0$C increment/ minute. The temperature point at which there is a 50% hyperchromic shift was taken as Tm of the DNA sample. Tm values were determined graphically from the hyper-chromicity versus temperature plots. The precision in Tm values estimated in triplicate was ± 0.05 $^0$C.

## 2.2-8. Ethidium bromide (EtBr) binding studies

The quantification of EtBr bound in moles per base pair of genomic DNA was measured in 0.01M HEPES, pH 7.4 [Chatterjee and Rao, 1994] using HITACHI F-2000 Fluorescence Spectrophotometer. The fluorescence was measured using a constant amount of DNA with increasing EtBr against the blank containing no DNA. The measurements were performed keeping excitation at 535 nm and emission at 600 nm with 10 mm path-length.

The maximum amount of EtBr bound per bp DNA was calculated using Scatchard plots of 'r' versus 'r/Cf', in the DNA-EtBr reaction mixture at various titration intervals when increasing amount of EtBr was titrated to constant amount of DNA [Scatchard,

1949].The concentration of bound EtBr in 1.0 mL dye-DNA mixture (Cb') was calculated using the equation:

Cb'=Co'[(F-Fo)/(VxFo)]

Where, Co=EtBr (pmoles) present in the dye-DNA mixture
F=observed fluorescence at any point of dye-DNA mixture
Fo=observed fluorescence of EtBr with no DNA
V=experimentally derived value, ratio of bound EtBr/free EtBr at saturation point

The concentration of free dye (Cf') was then calculated by the relation

Cf'=Co'-Cb'

Where, Cf', Co' and Cb' were expressed in pmoles.

The amount of bound EtBr/base pair 'r' was calculated by
r=Cb' (pmoles) / DNA concentration (pmoles of basepair)

A plot was made for r vs r/Cf and the point where the straight line intersects the axis r was defined as the maximum amount of dye bound per base pair (n), where Cf=Cf'x$10^{15}$ M.

## 2.2-9. Circular dichroism (CD) studies

The CD spectra (190–330 nm) were recorded for genomic DNA in 0.01M HEPES buffer (pH 7.4) on a JASCO-J 700 Spectropolarimeter. The cell length and width was 1 mm each. Each spectrum is the average of quadruplicate recordings. 20 μg of DNA from each sample was used. The DNA conformations were characterized from the CD spectra as per Gray et al, [1978].

### 2.2-10. Deoxyribonuclease I digestion of genomic DNA

The DNA from PD and control brain samples were treated with DNAse I for limited time (0.1µg/mL for 5 min) and were analyzed by 1% agarose gel. The DNAse concentration was 0.1µg/mL. The enzymatic reaction was stopped by adding SDS. The DNA digestion pattern was analyzed by running the DNA samples on 1% agarose gel electrophoresis and visualized under UV light after staining the gel with EtBr.

### 2.2-11. Statistical analysis

All the data obtained in this study were statistically treated and the significance of differences between control and PD DNA groups were calculated according to Student's *t*-test. The statistical analysis was carried out using Microsoft Excel 2000 soft-ware.

## 2.3 Results

The clinical characteristics of patients and controls are summarized in Table 2.1. There was no significant difference in the mean age at death or postmortem interval (PMI) between controls and patients with PD. DNA was isolated from eight different regions namely frontal, temporal, occipital cortex, hippocampus, caudate/ putamen, thalamus, cerebellum and midbrain from six control and five PD brains.

### 2.3-1. Agarose gel electrophoresis

Fig. 2.1 shows the neutral gels of genomic DNA from various regions of control and PD affected postmortem brains. In each gel

**Table 2.1**

**Clinical features of control and PD-patient groups.** Data of quantitative variables are expressed as mean ± standard deviation

| **Features** | **Control** | **PD** |
|---|---|---|
| Age (yrs) | 57.6 ± 13.5 | 60.2 ± 11.1 |
| Sex (M/F) | 6 / 0 | 2 / 3 |
| Postmortem interval (PMI) (hrs) | 10.4 ± 5.3 | 4.8 ± 3.3 |
| Diagnosis | No neurological illness | Parkinson's disease |

DNA isolated from five PD brains and a representative control is given.

It was observed that midbrain showed more damage in genomic DNA in PD than other anatomical regions and controls (Fig. 2.1H) as indicated by more diffuse band in agarose gel. The next highest damage was found in caudate nucleus + putamen (Fig. 2.1G) and hippocampus (Fig. 2.1B). Thalamus (Fig. 2.1D) and cerebellum (Fig. 2.1C) also showed more damage compared to controls. Among the control samples, the frontal cortex (Fig. 2.1A. lane1) showed more DNA damage than other regions while temporal and occipital cortex showed least damage (Fig. 2.1E. lane1, F. lane1).

Fig. 2.2 shows the mobility of DNA in alkaline gel of PD and control brains. One representative DNA sample from each brain region is shown. It was observed that midbrain, caudate/ putamen, thalamus and hippocampus showed more strand breaks than the corresponding controls. Among the controls, frontal cortex showed more damage than other brain regions as observed in neutral gel.

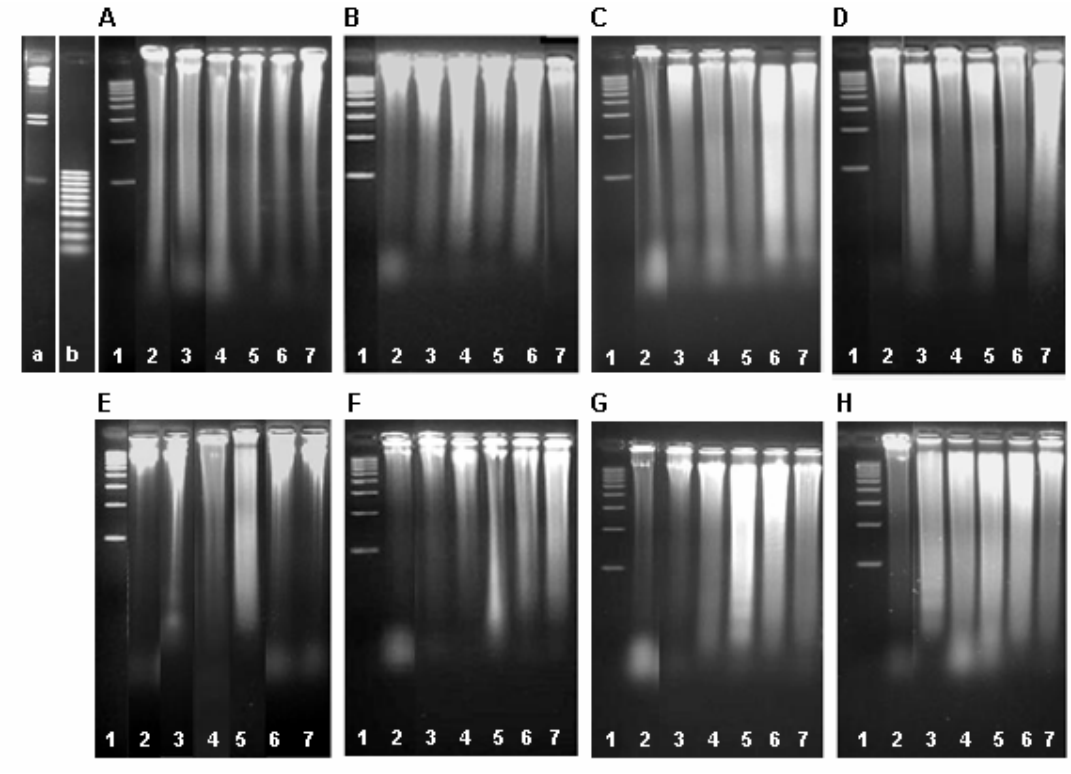



**Figure 2.1**

**Neutral agarose gel electrophoresis pattern of genomic DNA isolated from PD and age-matched control brains.** Neutral gels were run on 1.5% agarose. 3 μg of each DNA sample was used for gel studies. A, frontal cortex; B, cerebellum; C, hippocampus; D, thalamus; E, temporal cortex; F, occipital cortex; G, caudate nucleus/ putamen; H, midbrain. In A-H, lane 1 is 1 Kb DNA ladder, lane 2 is control DNA and lanes 3-7 are DNA from PD affected post-mortem brains. In Gel A, lane a and b represent Hind III digest of lamda DNA and 100bp ladder respectively. Gel studies were done on 5 samples of neuropathologically confirmed PD cases and 6 controls (one DNA sample from control is represented). The results indicate that DNA from midbrain, caudate nucleus/ putamen, thalamus and hippocampus showed more DNA fragmentation (double strand breaks) in neutral gels compared to control DNA. The damage was very prominent in midbrain DNA. There was little difference in DNA gel pattern for frontal cortex, cerebellum, temporal and occipital regions.

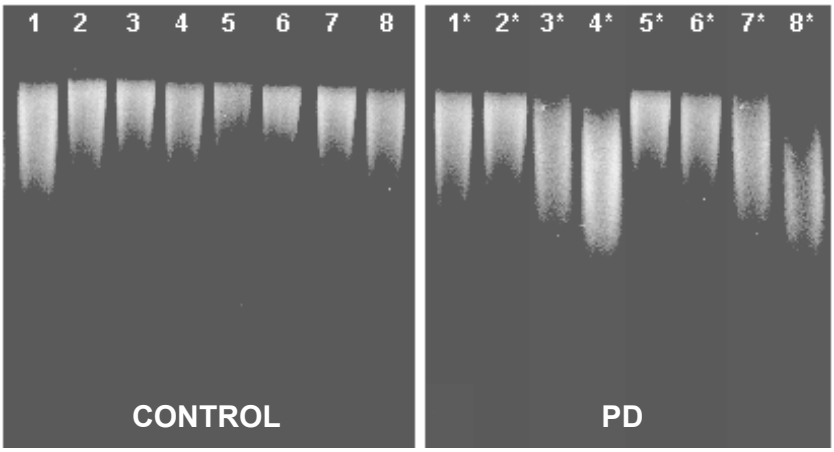



**Figure 2.2**

**Alkaline gel electrophoresis for observation of SSB and DSB in DNA.** Alkaline gel was run on 0.5 % agarose gels and 3 µg of each DNA was used. Lanes 1 to 8 represent DNA from control brain and lanes 1* to 8* represent DNA from PD brain. 1/1*, frontal cortex; 2/2*, cerebellum; 3/3*, hippocampus; 4/4*, thalamus; 5/5*, temporal; 6/6*, occipital; 7/7*, caudate nucleus/ putamen; 8/8*, midbrain. Alkaline gel studies were done on 6 control and 5 PD samples in each brain region and one DNA sample from each group has been represented in the figure. DNA isolated from midbrain, caudate nucleus/ putamen, thalamus and hippocampus showed significant fragmentation in PD than controls.

## 2.3-2. Single strand breaks (SSB)

The most prevalent type of DNA damage in mammalian cells is the SSBs. Single stranded breakage is the end point of several types of structural insults inflicted [Rao, 1993] on the genome by both endogenous and exogenous agents. Figure 2.3 shows number of SSBs per µg of genomic DNA isolated from various brain regions. Accumuation of SSBs was more frequent in PD than in controls in the following regions. The results showed that midbrain ($p<0.01$), caudate/ putamen ($p<0.01$), thalamus ($p<0.05$) and hippocampus ($p<0.01$) accumulated considerable number of SSBs compared to controls from respective areas (Fig. 2.3). In the control brain, DNA

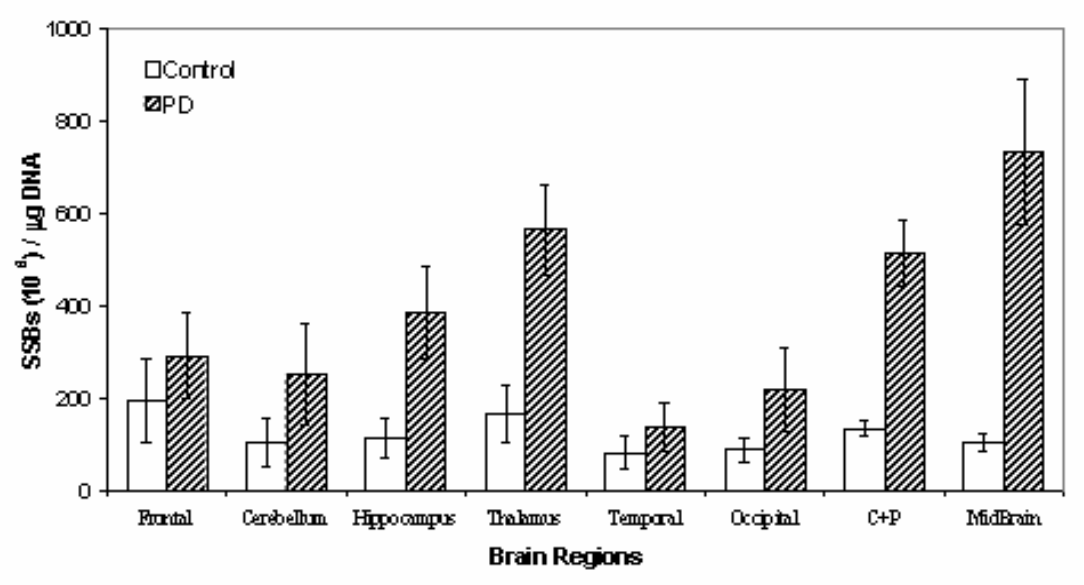



**Figure 2.3**

**Assessment of single strand breaks (SSB) in DNA isolated from different regions of PD and control brains.** The SSBs in DNA were determined through nick translation type of incubation with *E. Coli* DNA-polymerase I. The bar-chart represents SSB values ± SD for 6 control and 5 PD brain DNA samples. PD Midbrain DNA accumulated significantly more number of SSBs than controls. The other brain regions affected are caudate nucleus/ putamen, thalamus and hippocampus.

samples from frontal cortex showed more number of SSBs while temporal and occipital cortex showed least accumulation of SSBs which was also evident in gel experiments.

***Correlation of Single strand breaks versus Age and Post-mortem interval*** - Correlation of SSBs estimated in DNA isolated from various regions of control, and PD brains with age of the subject at the time of death and post-mortem interval (PMI) was studied. No significant correlation was found between SSBs in DNA and PMI both in control and PD brain ($r<0.5$). Similar observations were made by

earlier studies correlating the DNA damage and PMI [Kingsbury et al, 1998]. There was, however, a significant direct correlation between SSBs and age both in control and PD (r>0.8). A representative correlation graphs for DNA from control and PD midbrain have been represented in Fig. 2.4. Similar correlation pattern was observed for DNA isolated from all other brain regions (data not shown). Our observations are in agreement with other reports which showed accumulation of DNA damage as a function of age [Mandavilli and Rao, 1994; Mandavilli and Rao, 1996; Chetsanga et al, 1977].

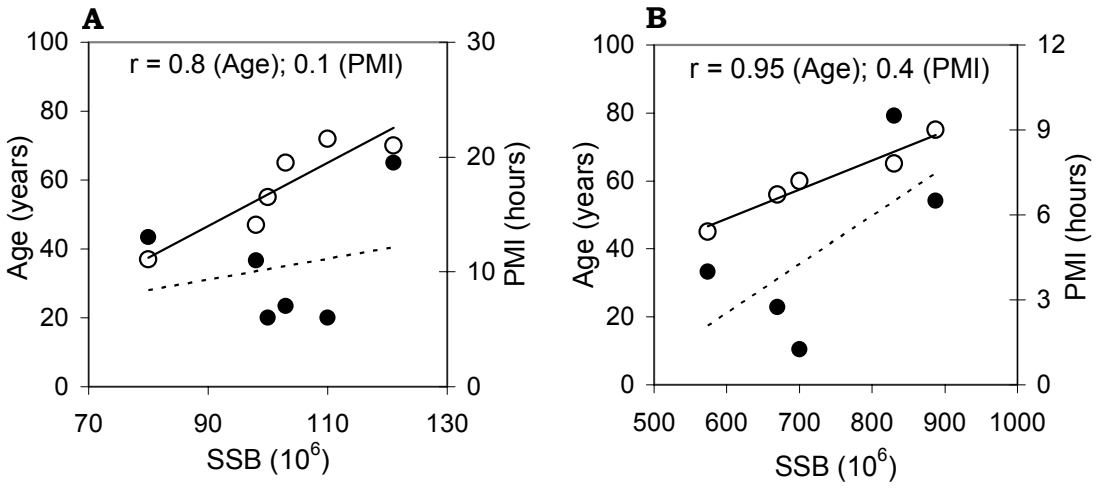



**Figure 2.4**

**Correlation of Single strand breaks (SSB) in midbrain DNA with Age at death of the control (A) and PD (B) cases and with post-mortem interval (PMI).** (○)-Age; (●)-PMI. No significant correlation was found between SSBs determined in DNA and PMI both in control and PD brain (r=0.1 and 0.4 respectively). There was, however, a significant direct correlation between SSBs and age both in control and PD (r=0.8 and 0.95 respectively). Similar trend was observed for DNA isolated from all other brain regions.

### 2.3-3. Double strand breaks (DSBs)

The results are shown in Fig 2.5. The DNA isolated from midbrain, caudate nucleus + putamen, thalamus and hippocampus showed significant number of DSBs than respective controls ($p<0.01$) (Fig. 2.5). As observed in agarose gels and SSB assay frontal cortex showed more DNA damage among the control brain samples. Hence it was observed that there is a notable difference in the accumulation of DNA damage in the form of both SSBs and DSBs among different brain regions in normal humans also.

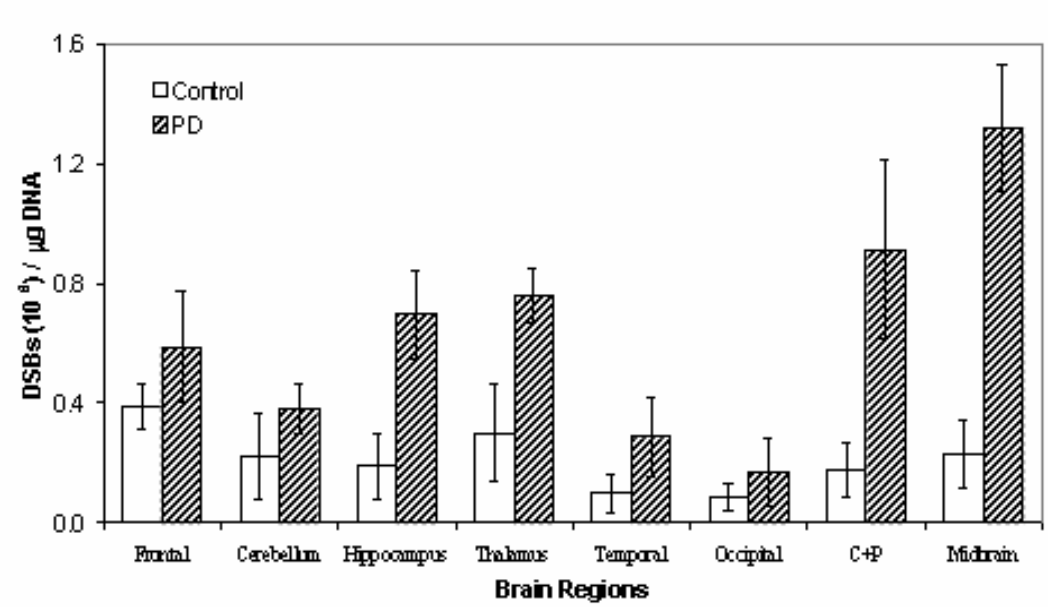



**Figure 2.5**

**Assessment of double strand breaks (DSB) in DNA isolated from different regions of PD and control brains.** The DSBs in DNA were measured using terminal transferase assay. The bar-chart represents mean of DSB values ± SD for 6 control and 5 PD brain DNA samples. Similar to the data on SSBs, Midbrain, caudate nucleus/ putamen, thalamus and hippocampus DNA accumulated significantly more number of DSBs in PD compared to controls. No significant difference in DSBs were observed in DNA from frontal cortex, cerebellum, temporal and occipital regions.

## 2.3-4. Melting temperature and hyperchromicity of DNA isolated from PD and control brain samples

Heating leads to the destruction of double-stranded hydrogen-bonded regions of DNA. The process of denaturation is accompanied by increase in absorbance as double-stranded DNA is converted to single strands. Melting of double stranded DNA is co-operative. The denaturation of the ends of the DNA will destabilize adjacent regions of helix, leading to a progressive and concerted melting of the DNA at

**Table 2.2**

**Melting temperature (Tm) of DNA isolated from control and PD affected post-mortem human brains.** Values are as degrees centigrade with percent hyperchromicity given in bracket. All the values are mean ± SD. The melting temperature (Tm) was calculated as the point of 50% hyperchromic shift. The Tm and % hyperchromicity were significantly decreased for DNA isolated from midbrain, caudate nucleus + putamen, thalamus and hippocampus of PD brain compared to controls.

| Brain Regions | In 0.01 M HEPES buffer | | In 0.01 M Na Cacodylate | |
|---|---|---|---|---|
| | Control | PD | Control | PD |
| Frontal | 60.3±0.6<br>(44.2±3.1) | 61.8±1.0<br>(42.9±1.9) | 78.6±0.1<br>(29.0±2.3) | 79.0±0.3<br>(26.2±0.8) ** |
| Cerebellum | 61.4±0.4<br>(36.3±0.8) | 60.4±0.7<br>(35.7±1.6) | 78.6±0.0<br>(25.2±0.3) | 78.3±0.2<br>(24.9±0.7) |
| Hippocampus | 64.3±0.6<br>(42.4±2.4) | 62.1±0.3 **<br>(38.5±0.9) * | 78.6±0.1<br>(26.6±1.3) | 77.2±0.5 **<br>(24.0±0.3) ** |
| Thalamus | 65.7±0.2<br>(38.7±1.3) | 63.8±0.9 *<br>(34.6±2.1) ** | 79.0±0.3<br>(26.4±0.7) | 77.5±0.6 **<br>(20.6±1.2) * |
| Temporal | 65.1±1.1<br>(42.1±3.1) | 63.7±0.8<br>(39.5±3.4) | 79.3±0.2<br>(29.5±2.0) | 79.0±0.5<br>(27.0±2.2) |
| Occipital | 64.1±0.8<br>(37.4±1.1) | 63.4±1.0<br>(36.8±0.7 | 79.0±0.1<br>(29.9±0.5) | 78.8±0.4<br>(28.0±0.4) |
| Caudate/ putamen | 63.2±0.5<br>(32.0±0.9) | 60.4±0.8 *<br>(26.1±1.8) * | 78.6±0.0<br>(25.7±0.2) | 77.3±0.2 *<br>(22.3±1.0) * |
| Midbrain | 60.5±0.6<br>(34.3±2.1) | 57.6±1.0 *<br>(26.1±2.3) * | 78.6±0.1<br>(26.4±1.2) | 76.6±0.6 *<br>(19.5±1.6) * |

* These values are significantly different from the corresponding control values at $p<0.01$

** These values are significantly different from the corresponding control values at $p<0.05$

Values in parentheses represent percent hyperchromicity ±SD

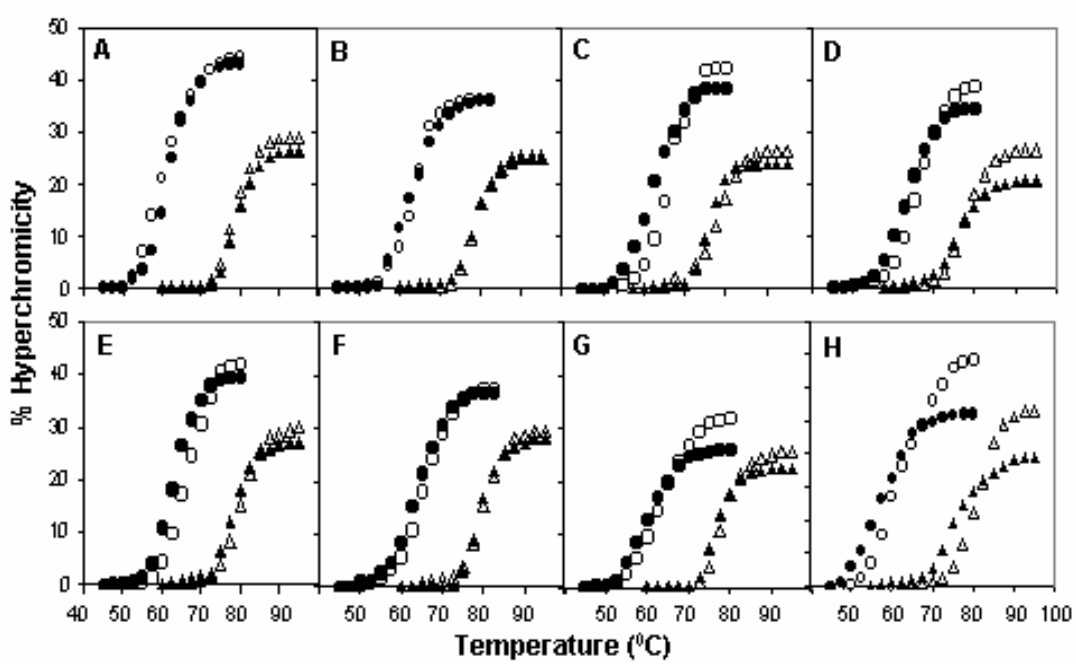



**Figure 2.6**

**Melting profiles of DNA isolated from different regions of PD affected post-mortem human brain with reference to control.** DNA samples were taken at a concentration of 15µg/mL of either in 0.01 M HEPES, pH 7.4 or 0.01 M sodium cacodylate buffer, pH 7.4. (○)-control; (●)-PD for DNA dissolved in HEPES buffer and (Δ)-control; (▲)-PD for DNA dissolved in sodium cacodylate buffer. The legends A-H correspond to: A, frontal cortex; B, cerebellum; C, hippocampus; D, thalamus; E, temporal; F, occipital; G, caudate nucleus/ putamen; and H, midbrain. Optical density at 260 nm at temperature ranging from 30-95$^0$C was recorded continuously using Amarsham spectrophotometer equipped with a thermoprogrammer set for 1$^0$C rise per every minute. Mean temperature of denaturation, 'Tm' was calculated graphically as the point of 50 percent hyperchromic shift.

a well-defined temperature corresponding to the mid-point of the smooth transition, known as the melting temperature (Tm).

Table 2.2 shows the Tm and hyperchromicity values of genomic DNA dissolved either in 0.01 M HEPES buffer, pH 7.4 or 0.01 M sodium cacodylate buffer, pH 7.4. The data showed that the Tm and percent hyperchromicity are significantly low for PD DNA

from midbrain ($p<0.01$), caudate/ putamen ($p<0.01$), thalamus (Tm at $p<0.01$, % hyperchromicity at $p<0.05$) and hippocampus ($p<0.05$) compared to controls indicating DNA damage or decrease in stability. No significant difference was observed either in Tm or percent hyperchromicity values for DNA isolated from frontal, temporal, occipital cortex and cerebellum from control or PD brains. The trend in Tm and hyperchromicity changes were essentially same in both HEPES and cacodylate buffers, though the difference is greater in HEPES than cacodylate buffer. Fig. 2.6 shows the pattern of % hyperchromicity changes with temperature for DNA isolated from control and PD affected brain regions. The decrease in Tm and percent hyperchromicity of DNA in midbrain, caudate/ putamen, thalamus and hippocampus indicated more strand breaks and resulting destabilization of such DNA.

## 2.3-5. EtBr binding studies

The amount of EtBr molecules bound per basepair (bp) of DNA is represented in Table 2.3. A representative Scatchard plot of 'r vs r/Cf' for control and PD midbrain and frontal cortex DNA is shown in Fig. 2.7.

The EtBr binding data showed (Table 2.3) that DNA isolated from mid-brain, caudate/ putamen, thalamus and hippocampus bound less EtBr/ bp in PD compared to corresponding controls at a statistical significance of $p<0.05$. EtBr bound / bp to DNA from frontal, temporal, occipital cortex and cerebellum did not differ significantly between PD and control groups. To understand the significance of low EtBr binding to DNA from PD brain, a saturated reaction mixture of mid-brain DNA from control brain and EtBr (1:1 w/w) was treated with DNAse I (0.5μM/mL) and the fluorescence emission at 600 nm was

monitored with time at excitation level of 535 nm. It was observed that the EtBr fluorescence decreased with time as the DNA was digested with DNAse I (Fig. 2.8).

**Table 2.3**

**EtBr binding assay.** Bound EtBr per basepair (bp) of DNA from PD and control samples were calculated using Scatchard plots [Scatchard, 1949; Chatterjee and Rao, 1994]. Values are as mean of number of EtBr molecules bound per bp DNA ± SD. These values with asterisk (*) are significantly different from the corresponding control values at $p<0.05$. For representative Scatchard plots see Fig. 2.7.

| Brain regions | Control | PD |
|---|---|---|
| Frontal | 0.51± 0.11 | 0.54 ± 0.15 |
| Cerebellum | 0.43± 0.16 | 0.51 ± 0.13 |
| Hippocampus | 0.49 ± 0.06 | 0.31 ± 0.03* |
| Thalamus | 0.44 ± 0.05 | 0.25 ± 0.04* |
| Temporal | 0.41 ± 0.08 | 0.36 ± 0.03 |
| Occipital | 0.45 ± 0.1 | 0.35 ± 0.08 |
| Caudate/ putamen | 0.42 ± 0.06 | 0.27 ± 0.03* |
| Midbrain | 0.44 ± 0.14 | 0.19 ± 0.07* |

This shows that partially damaged DNA binds less EtBr than the intact DNA. In light of this, the above results confirm the presence of more number of strand breaks in DNA isolated from midbrain, caudate/ putamen, thalamus and hippocampus of PD compared to controls. The amount of EtBr bound in moles per base pair of DNA was found to be significantly low for cerebellum, frontal and temporal cortex while it was high for DNA from hippocampus in PD brain compared to controls.

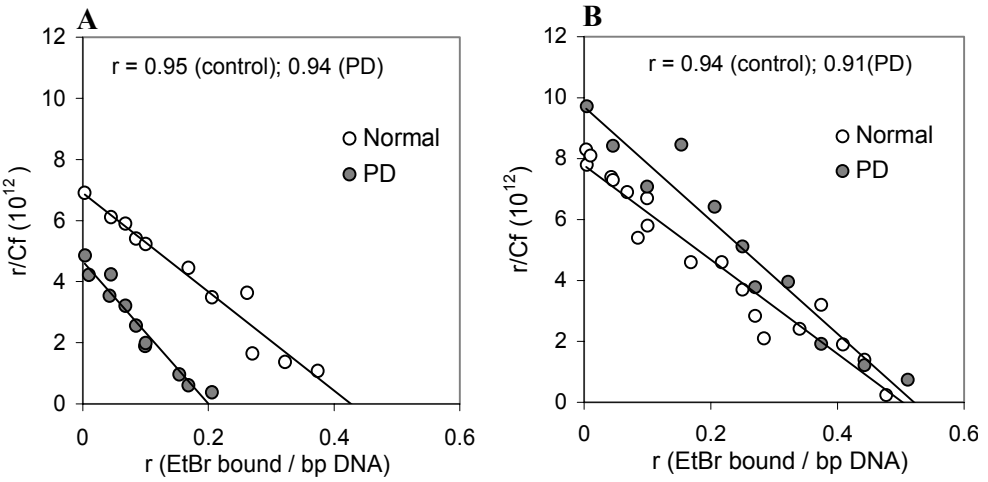



**Figure 2.7**

**Scatchard plot of ethidium bromide binding to DNA isolated from midbrain and frontal cortex.** A, midbrain DNA; B, frontal cortex DNA. Increasing amounts of EtBr were added to a fixed concentration of DNA in a 1mL reaction mixture (0.01 M HEPES pH 7.4). Fluorescent measurements were done at room temperature setting excitation at 535nm and emission at 600nm. The Scatchard plot was drawn using least square method.

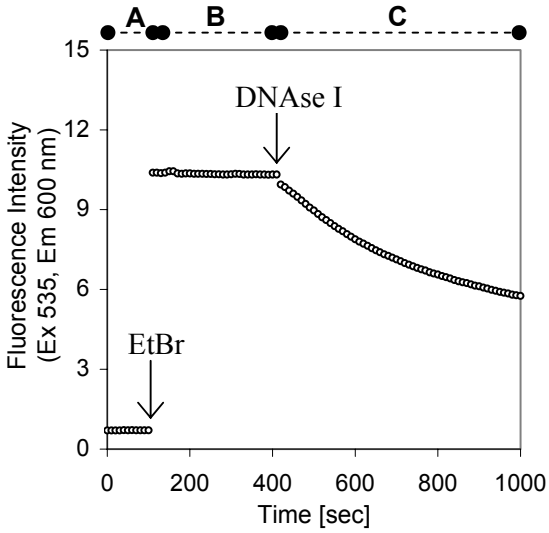



**Figure 2.8**

**Effect of DNAse I treatment on EtBr binding to DNA.** A saturated reaction mixture of control midbrain DNA and EtBr (1:1 w/w) was treated with DNAse I (0.5µg/mL) and fluorescence was monitored with time at excitation at 535 nm and emission at 600nm. In the graph section A, B and C represent DNA alone, DNA + EtBr and DNA + EtBr + DNAse I respectively. EtBr fluorescence decreases with time as the DNA is digested with DNAse I.

## 2.3-6. Circular dichroism (CD) studies

The CD studies showed that the conformation of DNA isolated from frontal, temporal and occipital cortex and cerebellum did not differ from corresponding controls. A representative CD spectra of control and PD DNA from frontal cortex is shown in Fig. 2.9. However, the DNA CD signal for midbrain, caudate+putamen and hippocampus differed from respective controls. The negative CD intensity at ~245 nm was consistently low for PD DNA compared to control (Fig. 2.10). The change in CD signal was very prominent for midbrain DNA in PD where the negative peak at 245 nm nearly disappeared (Fig. 2.10 D).

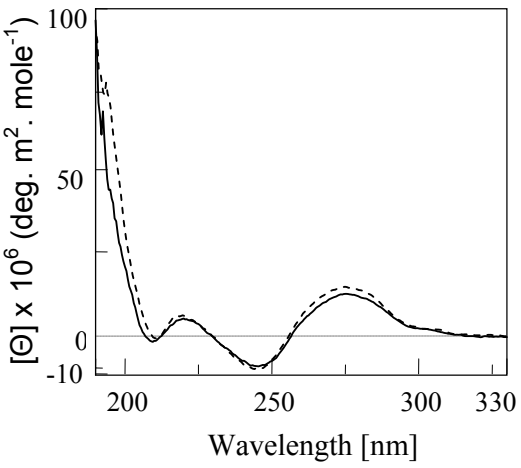



**Figure 2.9**

**Circular dichroism (CD) spectra of DNA isolated from frontal cortex.** Solid line represents control DNA and dotted line represents PD DNA. The recordings were performed in 0.01 M HEPES buffer, pH 7.4. Each spectrum represents average of 3 recordings. CD was performed on 6 control and 5 PD DNA samples and one spectrum from each group has been represented. Both control and PD DNA from frontal cortex showed B-DNA conformation. Similarly DNA from cerebellum, temporal and occipital regions showed no CD spectral difference between control and PD.

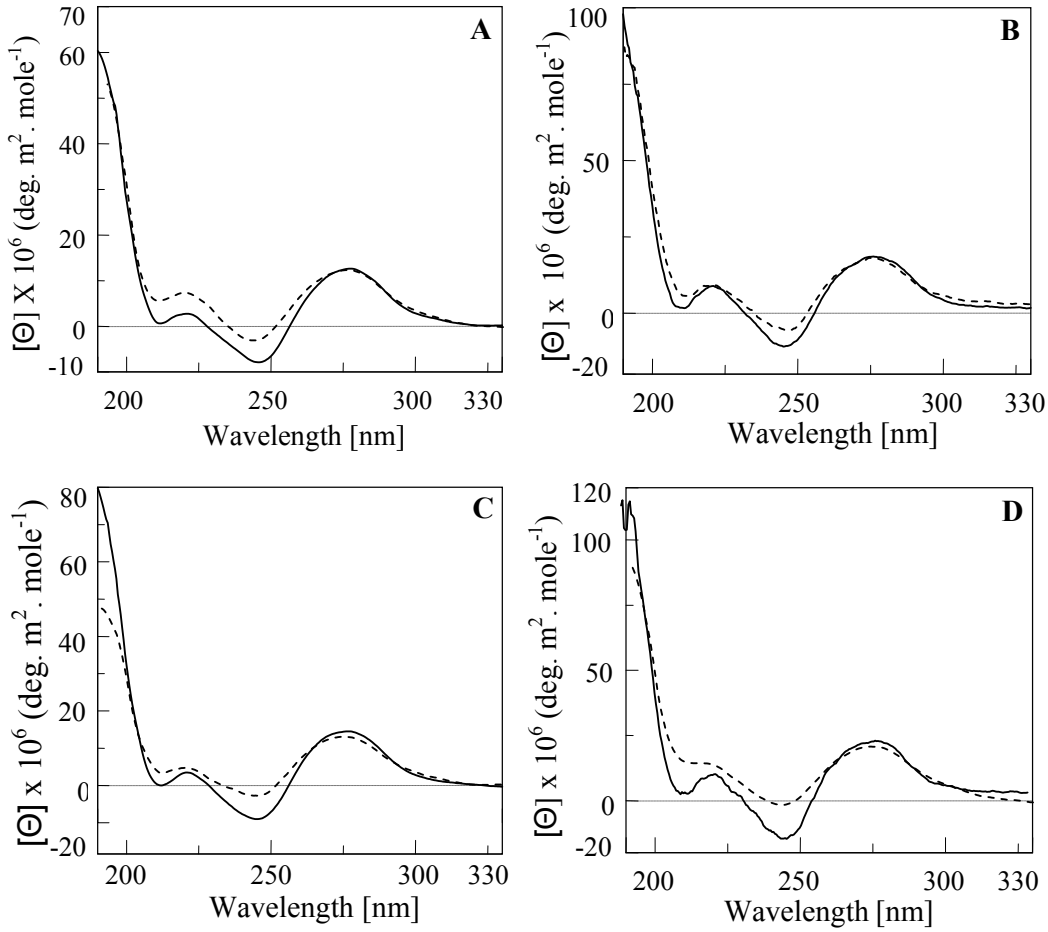



**Figure 2.10**

**Circular dichroism (CD) spectra of DNA isolated from A, hippocampus; B, thalamus; C, caudate nucleus/ putamen and D, midbrain.** Solid line represents control DNA and dotted line represents PD DNA. The recordings were performed in 0.01 M HEPES buffer, pH 7.4. Each spectrum represents average of 3 recordings. CD was performed on 6 control and 5 PD DNA samples and one spectrum from each group has been represented. The negative CD signal around 245nm was decreased for PD DNA from the above 4 brain regions compared to controls. The change was very prominent for midbrain DNA in PD where the negative 245nm signal almost disappeared.

This could indicate DNA damage and low DNA helical integrity as CD signal around 245 nm comes from base pairing between the two DNA helices. The same trend was observed more or less in all the four brain regions which showed DNA damage (strand breaks) in the above studies.

## 2.3-7. DNAse I digestion

It was observed that genomic DNA isolated from midbrain, caudate/ putamen, hippocampus, thalamus and occipital cortex of PD brain were more susceptible to DNAse I than their respective controls (Fig. 2.11). DNA from frontal and temporal cortex and cerebellum did not show significant difference in DNAse I digestion pattern compared to controls.

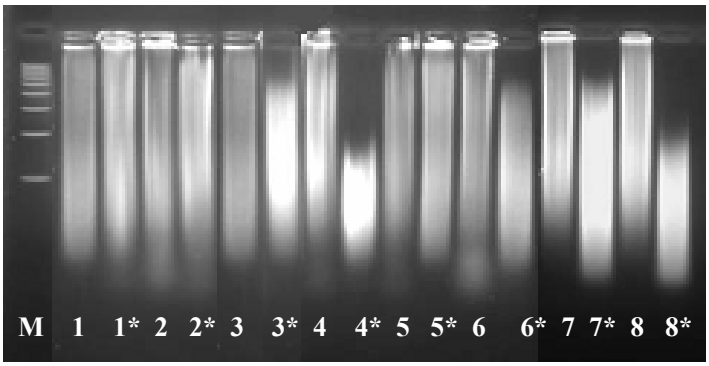



**Figure 2.11**

**DNAse I treatment of genomic DNA isolated from control and PD affected post-mortem brains.** 1-8 represents control DNA and 1* - 8* represent PD DNA samples. M, 1Kb molecular weight marker; 1/1*, frontal cortex; 2/2*, cerebellum; 3/3*, hippocampus; 4/4*, thalamus; 5/5*, temporal; 6/6*, occipital; 7/7*, caudate nucleus/ putamen and 8/8*, midbrain. DNA samples were treated with DNAse I (0.1µg/mL) for 5 minutes and run on 1% agarose gel. The experiment was performed for 6 control and 5 PD DNA samples and one DNA sample from each group is represented in the figure.

A representative agarose gel electrophoresis pattern of DNAse I digestion of DNA from each region of control and PD brain is given in Fig. 2.11.

## 2.4 Discussion

Accumulation of the DNA damage and decrease in the DNA repair capacity is one of the causes for aging and age related neurodegenerative disorders (Gensler and Bernstein, 1981; Hart and Setlow, 1974]. Genomic integrity is very essential for the survival of any organism as any damage to it will ultimately lead to the death of the organism. Recent studies showed that oxidative stress, DNA damage, chromatin condensation, and altered expression of genes in dopaminergic neurons are associated with neurodegeneration in PD.

The present study assessed the topology and damage of isolated DNA in PD brains and corresponding age-matched non-parkinsonian controls. DNA from midbrain in PD was observed to be most severely damaged accumulating significant number of single and double strand breaks compared to controls. The other brain regions like caudate/ putamen, hippocampus and thalamus also showed more DNA damage than controls. The Tm and EtBr intercalation studies also supported the above results. It was observed that the DNA from the above regions were more susceptible for endonuclease (DNAse I) digestion indicating lesser stability than control brain DNA. The CD spectral studies showed an altered CD signal for DNA isolated from midbrain, caudate/ putamen, hippocampus and thalamus in PD indicating a distorted/imprecise DNA conformation possibly owing to unstable strand pairing in such DNA. Frontal, temporal and occipital cortex and cerebellum showed no significant difference either in SSB/DSB or in stability and conformations between PD and controls.

It is interesting to explore the physiological relevance of CD and DNAse I digestion analysis of extracted genomic DNA, since conformation of extracted "naked" DNA does not reflect conformation of DNA *in vivo*. However, as both control and PD brain DNA were extracted by the same procedure, shows that these changes were specific to few regions of DNA from PD affected brain samples. It was previously shown that the genomic DNA extracted from severe AD hippocampus is predominantly in Z-DNA conformation rather than the usual B-DNA conformation [Anitha et al, 2002a] and it was assumed that the change in conformation of DNA may also be relevant under cell system. It appears that the increased DNA fragmentation may be crucial to the DNA conformational modulation and the susceptibility to DNAse I as these two parameters directly correlated in the present study. It was earlier shown that DNA fragmentation reduces the high activation energy barrier required to induce the conformational and topological changes in DNA.

The above results suggested that the structural integrity and topology of genomic DNA is altered in many regions of PD brain. The changes include DNA damage in the form of single strand and double strand breaks and DNA instability as indicated by the CD, Tm, EtBr binding and DNAse I treatment experiments. Interestingly, the changes observed appear to be region specific in the brain. The DNA isolated from frontal, temporal occipital cortex and cerebellum were largely unaffected while midbrain, caudate/ putamen, hippocampus and thalamus showed more strand breaks. It is however, evocative to understand the implications of the above findings in relevance to PD pathogenesis.

It is interesting to note that our results in neutral agarose gel electrophoresis showed no classical apoptotic 'DNA laddering'

pattern. However, significant DNA fragmentation up to 200 base pair (bp) fragments were observed in midbrain (most damaged), caudate/ putamen, hippocampus and thalamus. According to Wyllie et al, [1980] the 'DNA laddering' on gel electrophoresis which is the main criterion of apoptosis is relatively a late event. However, in many instances cell death is preceded by DNA fragmentation by $Ca^{2+}Mg^{2+}$-dependent DNAse into 180 and 200 bp fragments with endonuclease activation, occurring early in the process of cell death [Wyllie et al, 1980; Kerr et al, 1995; Clarke, 1999]. Further, it was suggested by Jellinger [2000] that mechanisms distinct from classical apoptosis play a central role in the pathogenesis of PD and related neurodegenerative diseases.

Our results also showed that the strand breaks in DNA increase significantly with age of the person at death both among controls and PD brain regions among the samples studied (Fig. 2.5). The direct correlations between DNA strand breaks and aging has been established in many reports [Mandavilli and Rao, 1996; Hart and Setlow, 1974; Bernstein and Bernstein, 1991; Rao, 1993; Grossman and Wei, 1994; Bohr et al, 1989; Shigenaga et al, 1994]. The DNA damage/repair theory of aging predicts the accumulation of genomic damage as the basic cause leading to senescence and death of cells [Mandavilli and Rao, 1996; Rao, 1993; Evans et al, 1995]. The statistically significant correlation between age and strand breaks in our study ($r>0.8$) supported this hypothesis, though this is not the primary goal of the present investigation.

The influence of perimortem conditions, antemortem hypoxia on DNA fragmentation in post-mortem tissue have been demonstrated in some previous studies [Kingsbury et al, 1998]. However, we evaluated our results on DNA stability/damage by

several approaches with matching case-controls and established that postmortem delay related DNA damage does not account for the changes in PD and control brains.

Damage or alteration of the conformation of DNA appears to be a universal phenomenon both in normal aging and age related disorders [Rao, 1997]. The etiology of PD includes many endogenous and exogenous factors such as synuclein and other proteins, neuromelanin, trace metals, environmental toxins, products of oxidative stress events, mitochondrial insufficiency etc [Lotharius and Brundin, 2002]. DNA is very much subject to damage by both endogenous and exogenous events resulting in the modification or loss of bases, the production of mismatched base pairs, strand breaks, DNA-DNA cross-links and cross-links between DNA and proteins implicated in neurodegeneration [Lotharius and Brundin, 2002]. The manner in which DNA can be damaged has been extensively reviewed by Friedberg [1985], Bernstein and Bernstein [1991] and Rao [1997]. The major modes of DNA damage have been represented in Fig. 2.12. Recently, we have shown that α-synuclein, which is a prime protein implicated in PD, binds and nicks DNA [Hegde and Rao, 2003; Hegde et al, 2004b]. Many of these insults may potentially lead to single strand and double strand breaks. Another plausible reason for accumulated DNA fragmentation in PD brain could be limited basal DNA repair capacity of the neurons in PD. DNA damage responses or DNA repair systems are essential for the maintenance of genomic integrity. Under the conditions of PD with environmental insults causing oxidative stress and genotoxic stress, the genomic DNA's structural integrity is under constant threat [Davydov et al, 2003]. Hence any insufficiency in the machinery to counteract the damage lead to accumulation of DNA breaks [Rao, 2003]. It was reported that genetic defects in genes are

associated with a range of neurodegenerative phenotypes in man [Rolig et al, 2000]. A decline in DNA stability signifies a shift between DNA damage and repair. Protracted cellular damage will eventually affect DNA integrity and repair mechanisms. Obviously, this question has puzzled researchers for a long time, and it is difficult to think of any stringent analysis of cause and effect to settle this issue, though. It appears that the DNA damage might just be one of the markers of cellular integrity among others.

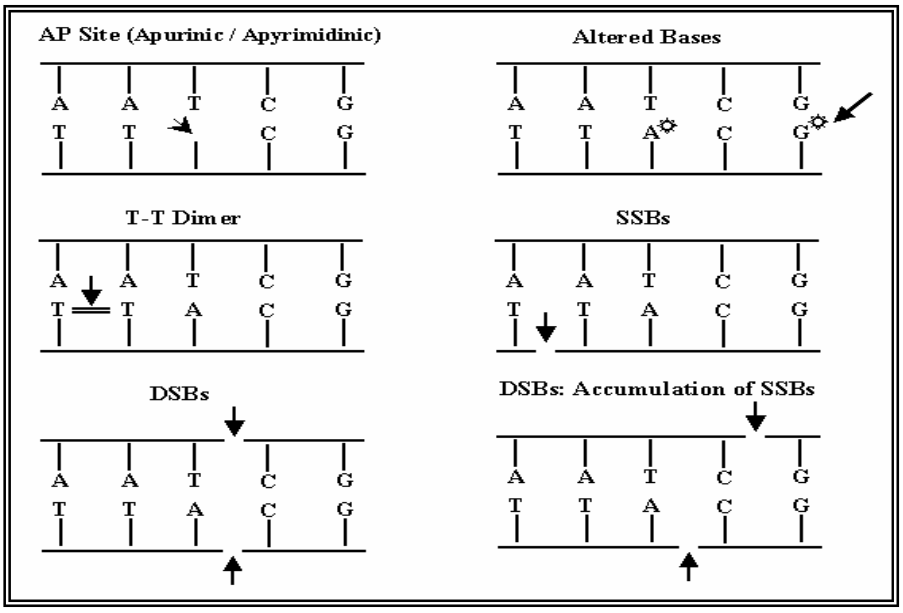



**Figure 2.12**
**The major modes of DNA damage**
Single strand break (SSB) is the most frequent consequential end points of various damages, while double strand break (DSB) is an occasional end of oxidative DNA damage.

Generally, midbrain (nigral) dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine gives rise to various molecules that can act as endogenous

toxins if not handled appropriately. This is evidenced by the presence of higher levels of cytotoxic hydroxyl radicals in SN than in other regions of brain [Lotharius and Brundin, 2002]. Alam et al, [1997] observed striking rise in oxidative DNA damage in SN in PD. Increased iron levels have also been reported in SN in PD [Dexter et al, 1989a; Riederer et al, 1989]. Oxidative DNA damage was also reported in caudate/ putamen, globus pallidus in PD [Alam et al, 1997]. In the present study, we observed changes in DNA base stacking integrity or conformations and damage in the form of strand breaks in midbrain, caudate nucleus + putamen, hippocampus and thalamus in PD brain. The presence of DNA strand breaks were prominent in midbrain than in other regions.

However, it is interesting to understand the cellular origins of the DNA undergoing fragmentation and topological changes in the present study. We have used total tissue from each brain region to derive homogenates, which comprises glial cells, neurons and astrocytes. Apart from neuronal dysfunctions, glial and astrocyte pathology was also shown to be associated with PD recently [Banati et al, 1998; Ouchi et al, 2005; Hishikawa et al, 2005; Mori et al, 2005; Dervan et al, 2004; Teismann and Schulz, 2004]. Activation of microglia, as well as to a lesser extent reactive astrocytes, were found in the regions associated cell loss, possibly contributing to the inflammatory process by the release of pro-inflammatory prostaglandins or cytokines [Teismann and Schulz, 2004]. However, no study has clearly established DNA damage in non-neuronal cells in PD. Immunohistochemical characterization of a common product of nucleic acid oxidation, 8-OHdG revealed intense cytoplasmic 8-OHdG immunoreactivity in neurons of substantia nigra, and only occasionally in glia [Zhang et al, 1999]. According to Banati et al, [1998], apoptotic DNA fragmentation was not seen in either neurons

or glia in dopaminergic cells in PD showing glial pathology, however, cells positively labeled for DNA fragmentation were identified in both neurons and glia in cases of multiple system atrophy. Love, [2001] observed significantly TUNEL-positive neurons, sparse TUNEL-positive glia nuclei in substantia nigra of Lewy body disease. The above studies clearly indicate that nuclear DNA damage was observed only occasionally in few glial cells in PD brain, where as no DNA fragmentation in astrocytes has been shown so far. Hence, it may be assumed that the major, primary targets of toxic insults and mechanisms associated with PD are neurons. Moreover, there is little information available till date on the topology and stability patterns of genomic DNA in PD brain. In the present study, we have extracted DNA from total tissue from different brain regions of PD evidently assuming that the changes in DNA observed should come predominantly from neuronal cells. The occasional DNA fragmentation observed in glial cells in previous studies could have only little or negligible contributions to results obtained in this study.

Further, increasing evidence is implicating deficits in mitochondrial DNA in neurodegenerative disorders [Brown et al, 1996]. Oxidative damage to mitochondrial DNA, may be especially very damaging to neurons as the mitochondrial DNA repair systems are much less efficient than those of nuclear DNA [Clayton, 1992]. Moreover, the somatic mutation theory of aging is having at present a resumption: mutations (in particular at mitochondrial level) may accumulate with age in the brain and thus play a role in aging and neurodegenerative diseases including PD, Mitochondrial point mutations and a 4977-bp 'common deletion' both of which are related to oxygen radical attack, have been reported in some studies to occur with increased frequency in midbrain from patients with PD

[Brown et al, 1996; Ozowa et al, 1991; Mayer-Aohlfart et al, 1997; Ozowa et al, 1990]. However, subsequent studies could not confirm these results; rather, it was suggested that the mitochondrial point mutations and common deletion are associated with aging and not PD [Bandmann et al, 1997; Kosel et al, 1997]. Zhang et al, [1999] showed that oxidative damage to nucleic acid in midbrain of PD patients is present largely in the nigral neurons, possibly within mitochondria.

However, in the present study, the DNA extraction method employed tend to eliminate mitochondrial DNA contamination with nuclear DNA and hence the changes observed appear to be specific to nuclear genomic DNA from predominantly neurons and to a small extent glia.

The study provides a comprehensive database on DNA damage/fragmentation and conformations in eight regions of PD affected brain samples with reference to age-matched controls. It remains to be seen whether the DNA instability and fragmentation observed in PD brain is due to endogenous/exogenous toxins interactions with DNA or it is a part of apoptotic pathway.

Previously, much attention was not given to nucleic acid metabolism either in normal or diseased conditions. However, some information was available on changes in protein synthetic capacity, nucleolar volume and cytoplasmic RNA content in PD [Mann, 1982; Mann and Yates, 1983]. Our study leaves us with few questions whether DNA damage and loss of stability observed in PD is the cause or consequence of the disease, (defects are primary/ secondary), and neurodegenerative disorders like PD can be solely because of defect in DNA repair capacity. The present study and

other similar studies indicated that DNA damage may be the final path for cell death in PD, but whether this plays a central pathogenic role in causing the disease needs to be further investigated.

✡ ✡ ✡ ✡ ✡

# CHAPTER 3

# *α-synuclein nicks DNA: An evidence for a new toxic role*

## 3.1 Introduction

α-Synuclein is a highly conserved protein of unknown function, which has been implicated in the pathogenesis of several neurodegenerative diseases, including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [Goedet, 2001; Spillantini, 1997; Baba, 1998; Wakabayashi, 1998]. The protein accumulates in intracellular inclusions and abnormal neuritis (Lewy bodies and Lewy neuritis) that are characteristic of PD, the second common neurodegenerative disorder [Baba, 1998], however, the role of α-synuclein in neuropathology leading to degeneration of neurons is not clearly understood.

α-synuclein is a natively unfolded protein, exhibiting a random coil secondary structure in aqueous solution [Weinreb, 1996]. At neutral pH, it is calculated to have 24 negative charges (15 of which are located in the last third of protein sequence) leading to a strong electrostatic repulsion, which contributes to the lack of folding of α-synuclein [Uversky et al, 2001a]. Based on the amino acid sequence, three distinct domains have been identified in α-synuclein: (i) the basic N-terminal amphipathic region, rich in basic amino acids with a high propensity for α-helix formation, (ii) the central hydrophobic region (amino acids 61-95) and (iii) the acidic C-terminal region in which most of the negatively charged amino acids are located [Davidson et al, 1998; Manning-Bog, 2002]. The protein undergoes aggregation leading to fibrillar structures in PD brain, which adopts a β-sheet secondary structure [Serpel, 2000]. Three missense mutations in the α-synuclein gene have been reported to be associated with families susceptible to inherited forms of PD [Polymeropoulos et al, 1997; Kruger, 1998; Zarranz et al, 2004]. These mutations cause alterations in the amino acid sequence of α-

synuclein (at residues Ala30Pro, Ala53Thr and E46K) in regions predicted to influence the secondary structure of α-synuclein. The substitutions may disrupt the structure of α-synuclein, rendering the protein more prone to self- aggregation [Vogel, 1997; Heintz, 1997].

α-synuclein is a relatively small protein (140 amino acids), which is highly expressed both in nucleus and cytoplasm [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004]. Although α-synuclein was originally believed to be a presynaptic protein and its accumulation was predominantly cytosolic, several recent studies have shown its immunoreactivity in nuclei of neuronal cell lines expressing α-synuclein [McLean et al, 200l; Gomez-tortosa et al, 2002]. Tanji et al, [2002] have shown the presence of α-synuclein in both nucleus and cytoplasm of human macrophage cell lines. Besides these evidences for the expression of α-synuclein in nuclei, the highly oxidative cytological environment in PD brain, because of increase in paramagnetic ferrous and other free radical generating metals, are known to disrupt the biological membranes leading to translocation of α-synuclein in to the nucleus. Sangchot et al, [2002] have provided new evidences for nuclear membrane disruption by lipid peroxidation caused by increase in iron and consequent translocation of α-synuclein aggregates in to perinuclear and endonuclear regions of human dopaminergic neuroblastoma SK-N-SH cell lines. Leng et al, [2001] also observed α-synuclein both in monomeric and oligomeric forms in nuclear fractions of human dopaminergic neuroblastoma SH-SYSY cell cultures. Although the above observations do not suggest what the function of α-synuclein in nucleus is, Leng et al, [2001] predicted that α-synuclein may play a role in regulating processes in the PI-cycle in the nucleus and a phosphatidyl inositol-linked activities may

also occur in nucleus.

With the evidences for localization in nucleus [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004] and the knowledge of primary sequence of α-synuclein, where it has the positively charged amino acids clustered towards N-terminal end, we hypothesized that α-synuclein may be having a DNA binding role in nucleus [Hegde and Rao, 2003]. The present study demonstrates that α-synuclein binds to DNA *in vitro*, and interestingly it has a significant DNA nicking activity. These clear evidences for direct DNA damage by α-synuclein, provides a new role for α-synuclein in neurodegeneration in PD. The results also raise the argument that whether the α-synuclein aggregation is pathological feature of the disease or it is an adaptation to counter the toxicity of soluble form of α-synuclein (in terms of DNA damage).

## 3.2 Experimental procedures

### Materials

pUC 18 plasmid supercoiled DNA (scDNA), Cesium chloride purified, 90% supercoiled structure), lamda DNA, Calf thymus DNA, single stranded circular DNA (M13 phage), double stranded circular DNA (M13 phage), DNA molecular weight markers, *E. coli* DNA polymerase I, terminal deoxynucleotidyl transferase, Eco R I restriction enzyme, Tris and HEPES buffers were purchased from Bangalore Genei, India. Poly d(GC).d(GC) and poly d(AT).d(AT), aurintricarboxylic acid (ATA), diethylpyrocarbonate (DEPC), Anti α-synuclein antibody (rabbit), Copper grids (300 mesh size) for electron microscopy were purchased from Sigma Chemical Company USA. α-synuclein was purchased from rPeptides, USA. [$^3$H]-dTTP was

purchased from Amersham, UK. Uranyl acetate was purchased from B.D.H. laboratory chemicals division. Sybre gold gel staining dye was procured from Molecular Probes. All other chemicals sodium chloride (NaCl), sodium acetate, sodium dodecyl sulphate (SDS), EDTA, were of analytical grade and were purchased from Sisco chemical laboratories, Mumbai, India.

## Expression and purification of α-synuclein

The recombinant plasmid pT7-7, encoding α-synuclein, was kindly provided by the laboratory of Peter Lansbury. The plasmid was transformed into *Escherichia coli* BL21 (DE3), and α-synuclein expression was induced with 1mM isopropyl-1-thio-β-D-galactopyranoside. The cell pellet was collected by centrifugation at 4500X g, resuspended in lysis buffer (10mM Tris-HCl, pH 8, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, 1mM dithiothreitol), freeze-thawed three times, and sonicated. DNA was removed by precipitation with streptomycin (10 mg/ml) and centrifugation at 22,000 X g (rotor JA-20, Beckman Avanthi J-25 centrifuze) for 30 min at $4^0$C. The supernatent was collected, incubated in a boiling water bath for 20 min, and centrifuged at 22,000Xg. α-synuclein was precipitated by adding ammonium sulfate to the supernatent (final concentration, 361mg/ml) and centrifuged at 22,000Xg. The residue was subjected to anion-exchange and gel-permeation chromatographic purification methods.

## Anion-exchange chromatography

The precipitate was resuspended in 25mM Tris-HCl, pH 8.0, applied to Poros HQ column on a Biocad gel perfusion chromatographic system (Applied Biosystems), and eluted with a NaCl gradient (final concentration, 300mM) in same buffer (Fig. 3.1).

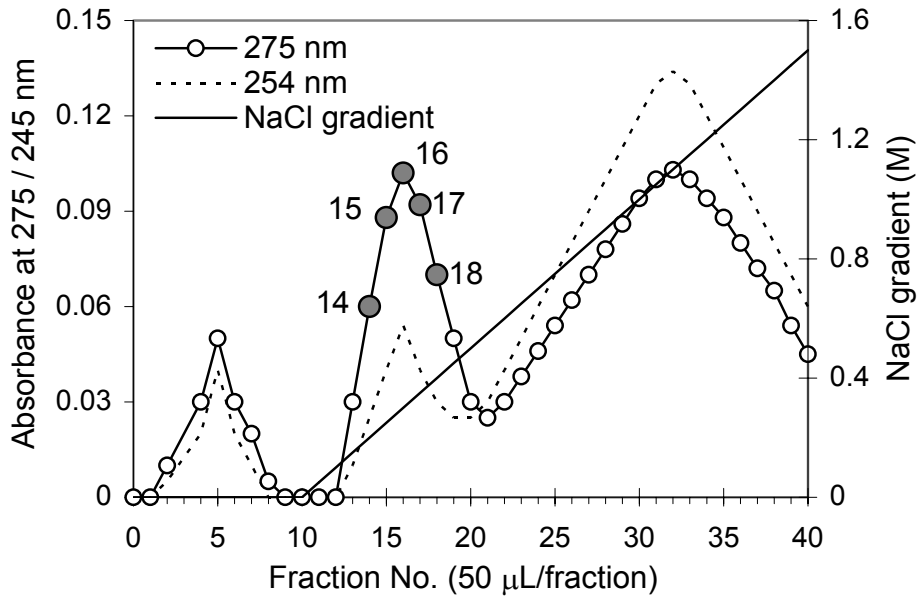



**Figure 3.1**

**Anion exchange chromatography for purification of α-synuclein**. BL-21 cell line was transformed with pT7-7 plasmid containing α-synuclein gene and the protein was overexpressed. The purification was carried out on HQ column using BIOCAD system. α-synuclein was eluted with NaCl gradient. Fractions as indicated on the chromatography profile by filled circles (14-18) were pooled and dialyzed to remove salt. The protein was then concentrated using Centricon filters.

The protein fractions were collected, dialyzed against 10mM Tris-HCl, pH 7.0, and concentrated with Millipore Centricon filters. The protein was quantitated spectroscopically using a molar extinction coefficient at 275nm of 5600 $M^{-1}$ $cm^{-1}$ [Weinreb et al, 1996].

## Gel-permeation chromatography (GPC)

Analytical GPC of α-synuclein was carried out by FPLC using Superdex 75 PC 3.2/30 column (3.2 x 300 mm, 13 μm particle size; Pharmacia Biotech, Uppsala, Sweden) using 10 mM Tris-HCl, pH 7.0 containing 50 mM NaCl as the mobile phase. Superdex-75 has an

exclusion limit of 100,000 Da and separation range of 3000-70,000 Da for globular proteins and peptides.

### Linearization of pUC 18 DNA by Eco R1 treatment

Linear form of supercoiled DNA was prepared using Eco R1 restriction enzyme. The reaction was carried out in 50 mM Tris chloride buffer (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol. 1 μg of DNA was treated with one unit of enzyme in a 50 μL reaction volume (one unit of Eco R1 is defined as the amount of enzyme required to produce a complete digest of 1μg of lamda DNA in a reaction volume of 50 μL in 60 min under optimal conditions of salt, pH and temperature). After incubating the reaction mixture for 60 min at $37^0$C, the reaction was stopped by heating the reaction mixture at $60^0$C for 20 min which inactivates the Eco R1.

### Circular Dichroism Studies

The CD spectra (190–330 nm) were recorded for scDNA in the presence / absence of increasing concentrations of α-synuclein in 0.01M HEPES buffer (pH 7.4) on a JASCO-J 700 Spectropolarimeter. The cell length was of 1 mm width and 1 mm length. Each spectrum was the average of four repetitions. The CD contributions from synuclein alone was substracted in the DNA-synuclein complex spectra. 20 μg of scDNA from each sample was used. The DNA conformations were characterized from the CD spectra using the reference of Gray et al, [1978; 1992].

### Agarose Gel Studies

DNA in the presence/absence of α-synuclein was electrophoresed on 1% and 1.2% agarose gel at 4V/cm to assess the DNA damage induced by α-synuclein. For kinetics studies different

concentrations of α-synuclein ($1x10^{-6}$ M to $20x10^{-6}$ M) were incubated for various time intervals at $37^0$ C with scDNA and subjected to agarose gel electrophoresis (1.2% agarose) at 4V/cm at room temperature. DNA concentration loaded in all lanes was 50ng. The samples were stained with either Sybre gold or ethidium bromide and photographed using gel documentation system.

**Polyacrylamide gel electrophoresis (PAGE)**

SDS-PAGE for α-synuclein was carried out using Laemmli's discontinuous buffer system [Laemmli, 1970].

**Dot-Immunoblot analysis**

Dot Immunoblot Analysis was carried out to confirm the presence of α-synuclein using Anti-α-synuclein antibody (From Sigma, USA, raised in rabbit). The presence of α-synuclein was identified using a secondary antibody (Anti goat-rabbit) coupled to alkaline phosphatase enzyme. The color development on addition of substrate NBT (Nitro blue tetrazolium) along with BCIP (5-bromo, 4-chloro, 3- indolyl phosphate) indicated the presence of α-synuclein.

**Thioflavin T Fluorescence for α-synuclein aggregation**

α-synuclein in the presence and absence of scDNA (50 μM) was incubated in 0.01M Tris-HCl, pH 7.4, at $37^0$ C with vigorous stirring (magnetic bar) in glass vials. scDNA (50nM) was diluted in to protein solutions in the same buffer conditions. Aliquots were removed from the incubation mix at different time intervals and diluted to appropriate concentrations for CD and Fluorescence measurements to assess the formation of β-sheet and aggregates. CD spectra were recorded on a JASCO 700 spectropolarimeter equipped with a Peltier

temperature controller. For Thioflavin T (Thio T) assay, the protein solutions were diluted 40-fold with 5μM ThioT in 10mM Tris-HCl, pH 8.0. Fluorescence was measured on a Varian Cary Eclipse spectrofluorometer in 1-cm path length quartz curettes. Emission spectra (470-650nm) were recorded for excitation at 450nm, using a 5-nm band pass for both excitation and emission. The contribution of unbound Thio T and Thio T bound to DNA were measured separately under the similar conditions and subtracted.

## Transmission Electron Microscopy

An aliquot was withdrawn from the incubation mixture and placed on to a glow-discharged carbon film attached to an EM grid. Carbon films, 30 to 4-cm thick, were pretreated by glow ~150 millitorr; discharge current, 2-3mA; duration of discharge, 30 s) as described elsewhere [Dubochet et al, 1971]. The adsorption continued for 1-2 min, after which the grids were rinsed with few drops of 2% aqueous uranyl acetate, blotted with a filter paper, and dried. The samples were examined with a Philips CM12 electron microscope. The negatives were scanned with a DuoScan T2500 scanner (Agfa) at 1200 dots per inch. Micrographs were measured using Image software (National Institutes of Health) modified for Windows. For printing, images were flattened using a high pass filter with a radius of 250 pixels and subsequently adjusted for contrast/brightness using Adobe Photoshop.

## Estimation of single strand and double strand breaks in scDNA treated with α-synuclein

### *Single strand Breaks (SSB)*

sscDNA induced by α-synuclein were determined through a Nick Translation type of reaction using E. coli DNA Polymerase I

(Klenow Fragment). DNA polymerase I is known to add nucleotides at the 3'-OH end of a single strand break, generated by various means, using the other strand as template.

***Double strand Breaks (DSB)***

The DSBs in DNA in presence of α-synuclein was estimated by terminal transferase assay [Mandivilii and Rao, 1996]. (For detailed methodology for the estimation of SSBs and DSBs refer methodology section of Chapter 2).

## 3.3 Results

### 3.3-1. α-synuclein binds to DNA and alters DNA conformation

Based on the several evidences for localization of α-synuclein in nucleus [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004] and the primary sequence of α-synuclein, where it has the positively charged amino acids clustered towards N-terminal end [Hegde and Rao, 2003], we hypothesized that α-synuclein may be having a DNA binding role in nucleus. While investigating this hypothesis we found that α-synuclein binds to DNA *in vitro*. Fig. 3.2 indicates the CD spectra of α-synuclein-scDNA complex. The spectra indicated a strong binding of α-synuclein to scDNA, causing a conformational change from the B-form of DNA to an altered B-form [Hegde and Rao, 2003]. This is the first report on DNA binding property/ability of α-synuclein and presents an interesting curiosity about the implications of this property in PD.

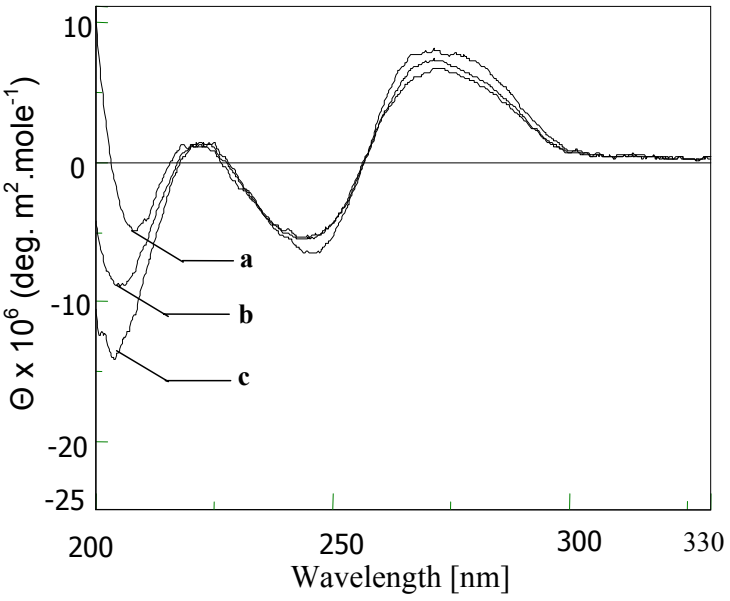



**Figure 3.2**

**Circular dichroism spectra of pUC18 supercoiled DNA-α-synuclein complex.** α-Synuclein binds to DNA and alters the B-DNA conformation of scDNA to altered A-DNA. (a) 25μg scDNA, (b) 0.5μM α-synuclein, and (c) 10μM α-synuclein.

## 3.3-2. α-Synuclein has scDNA nicking property- New evidence

α-synuclein and scDNA interactions were further studied by several techniques like Agarose gel electrophoresis, Transmission Electron Microscopy, Measurement of SSBs and DSBs by Nick Translation strategy. Plasmid scDNA was used to understand α-synuclein-DNA interactions. The reasons for choosing scDNA are based on the evidences for presence of naked superhelical packets of DNA in mammalian genome which are similar to plasmid scDNA. These non-histone superhelical packets are supposed to play an important role during the initiation of gene expressions

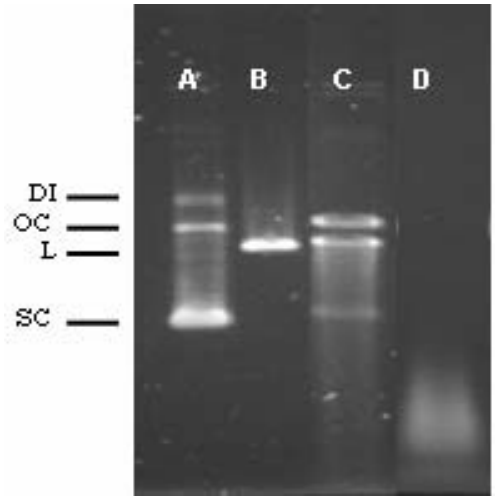



**Figure 3.3**

**Agarose gel showing α-synuclein induced DNA nicking and damage** Lane A represents pUC18 scDNA, lane B represents Linearised pUC18 (EcoR1 restriction digested, EcoR1 has a single restriction site in pUC18) as reference for linear DNA, and lane C and D represent pUC18 DNA treated with 5 μM and 10μM α-synuclein respectively. The nicked circular DNA, linear DNA bands can be seen with 5μM synuclein. The positions of various forms of scDNA (SC, supercoiled; L, Linear; OC, open circular; DI, dimers of scDNA) is indicated on the left side of the gel.

[Bauer et al, 1980]. Hence the results can be justifiably interpreted for the human *in vivo* system in PD.

Interestingly, it was observed in agarose gel electrophoresis experiments that ***α-synuclein nicks scDNA and mimics a DNA degrading nuclease enzyme***. Fig 3.3 shows the nicking activity of wild-type α-synuclein on scDNA. The scDNA (50 ng) was converted in to open circular and linear forms at 5 μM (Fig. 3.3, lane C) and DNA was fragmented in to apparently small oligos as a sheared staining of DNA was observed lower down the lane at 10 μM α-synuclein concentrations (Fig. 3.3, lane D). This is a new and interesting

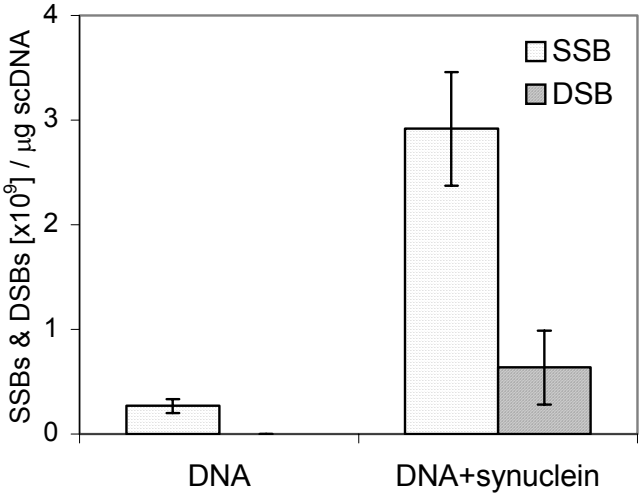



**Figure 3.4**

**Quantification of Single strand breaks (SSBs) and Double strand breaks (DSBs)**. The strand breaks were estimated in scDNA and sc DNA incubated with α-synuclein at 37$^0$C for 12 hrs using Nick translation strategy for SSBs and terminal transferase assay for DSBs. In scDNA alone, few SSBs were recognized by polymerase I enzyme (which is in keeping with the agarose gel pattern of scDNA where a faint band for nicked circular DNA was observed, Fig 3.3). However there was no DSBs in pUC 18 DNA. On incubation with 10 µM α-synuclein there was considerable increase in both SSBs and DSBs. The number of SSBs was ~ 5 folds more than DSBs.

finding on α-synuclein. The mechanism and significance of the DNA nicking activity of α-synuclein are studied and discussed further.

DNA nicking property of α-synuclein was further confirmed by quantitating the number of SSBs and DSBs using Nick Translation method. It was observed that both SSBs and DSBs were formed in scDNA on incubation with α-synuclein at 37$^0$C for ~12 hrs (overnight) (Fig. 3.4). However, the number of SSBs were several fold higher (~5 times) than DSBs.

The TEM images of scDNA in the presence and absence of α-synuclein clearly showed conversion of scDNA into open circular and linear forms caused by α-synuclein. Fig. 3.5 shows the negatively stained electron micrographs providing visible evidence on α-synuclein induced DNA damage.

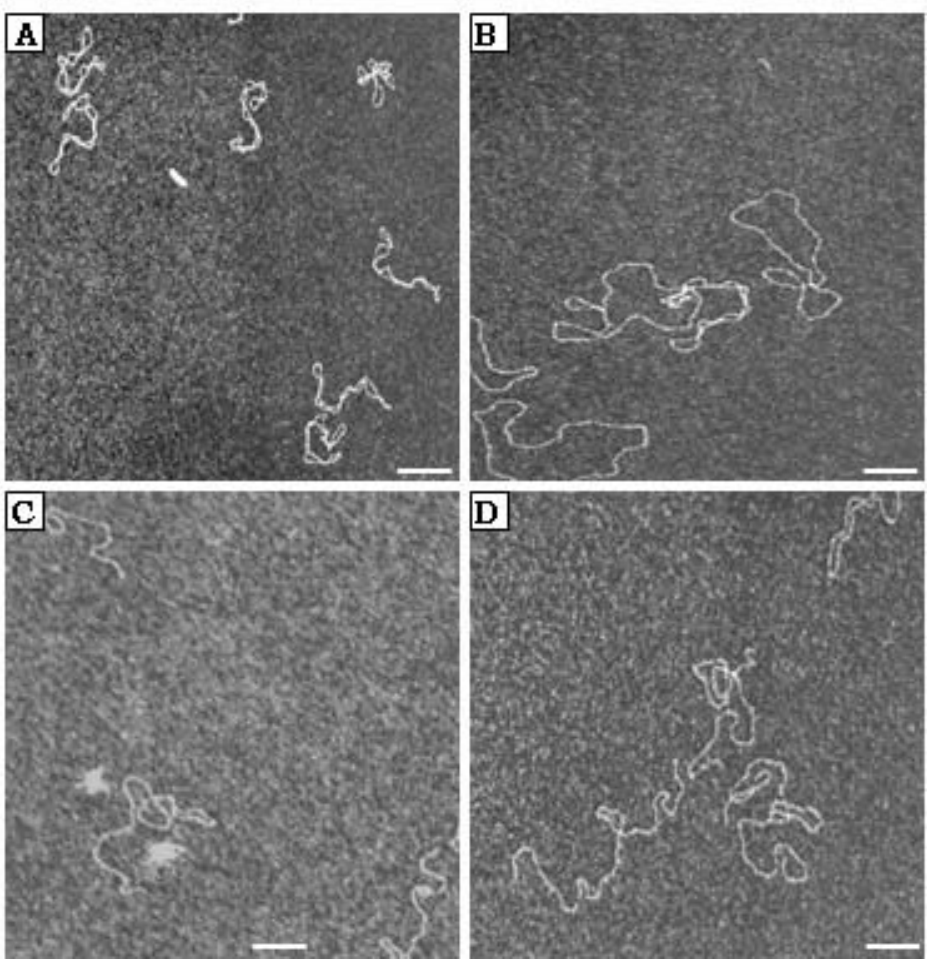



**Figure 3.5**

**Electron Microscopy images of supercoiled DNA and scDNA treated with α-synuclein.** A, sc DNA alone; B, nicked circular form; C and D, linear pUC 18 caused by α-synuclein. scDNA was incubated with α-synuclein (5 μM) at $37^0$C for ~4 hrs (B) and ~12 hrs (C, D). Scale bar represents 100 nm.

The α-synuclein monomers being small in size are not visible in the image. 1 μM α-synuclein was incubated with 1μg of scDNA at 37$^0$C for ~12hrs and imaged by electron microscopy after negative staining with uranyl acetate.

The DNA damage by α-synuclein was also confirmed by monitoring fluorescence due to Thio T binding to scDNA treated with α-synuclein. It was observed that the Thio T binds to scDNA with maximum fluorescence emission at 482 nm when the Thio T containing solution is excited at 446 nm.

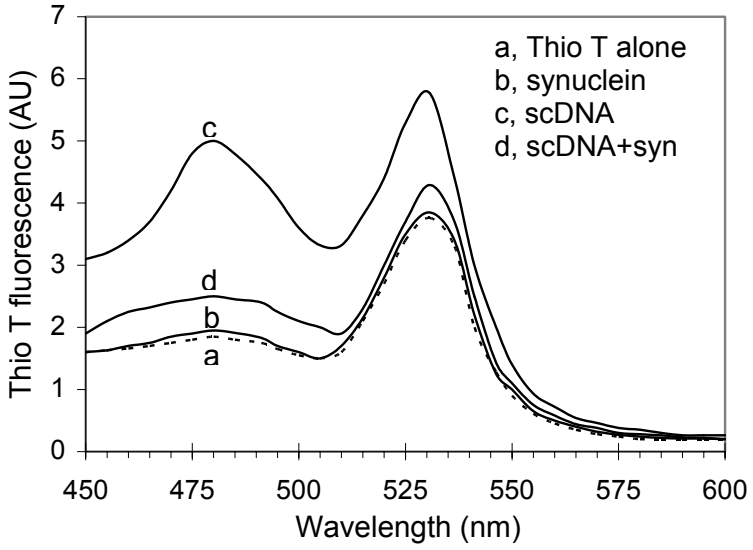



**Figure 3.6**

**Thioflavin T fluorescence for scDNA-α-synuclein complex.** A, Thio T alone; b, α-synuclein (50μM); c, scDNA; d, scDNA + α-synuclein (50μM) incubated at 37$^0$C for 1hr. Thio T fluorescence was recorded with excitation at 446 nm and emission range from 450 to 600nm. The Thio T binds to scDNA and shows signal. The decrease in Thio T signal in case of scDNA-α-synuclein complex could be either because of fragmentation of scDNA by synuclein or due to complexation of scDNA with synuclein making free scDNA unavailable for Thio T binding. There may be contributions from both these effects also.

The Thio T fluorescence of scDNA was decreased to less than half when it was treated with α-synuclein for 1 hr at $37^0$C indicating release of bound Thio T from DNA possibly due to formation of strand breaks or damage by α-synuclein (Fig. 3.6). The α-synuclein alone in monomeric native unfolded form did not show much Thio T binding (Fig. 3.5 b).

### 3.3-3. Effect of Nuclease inhibitors

To understand the mechanism of α-synuclein induced DNA nicking and to see if it is mediated through histidine amino acid residue of α-synuclein, the effect or influence of classical nuclease inhibitors like aurintricarboxylicacid (ATA) and diethylpyrocarbonate (DEPC) on α-synuclein induced DNA nicking were studied. ATA and DEPC are known to derivatize histidine amino acids in the active site of nucleases and thereby abolish protein binding to DNA [Gonzalez et al, 1980]. It was observed that both ATA and DEPC protected scDNA from α-synuclein induced degradation (Fig. 3.7 A and B). This indicates that histidine at 53 position may be playing a crucial role in the DNA nicking property of α-synuclein.

### 3.3-4. Purity of α-synuclein preparation-ruling out any nuclease contamination

Confirming the purity of the protein is very important to characterize the nuclease activity of a protein especially when the protein is expressed and isolated from cell system. The following experimental evidences effectively reveal the purity of α-synuclein and rule out any nuclease contamination in α-synuclein preparation.

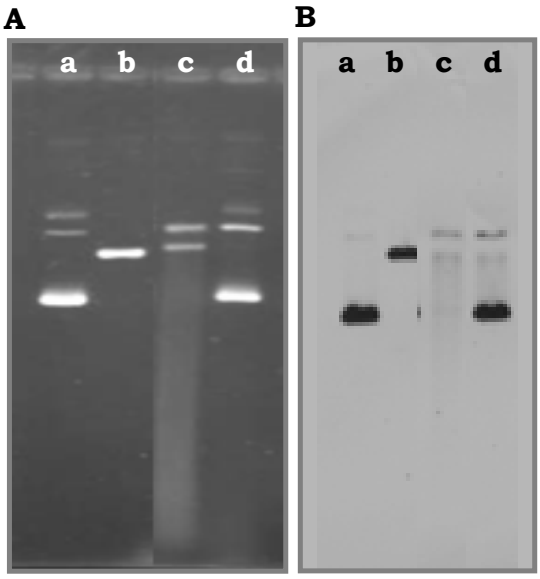



**Figure 3.7**

**Effect of Nuclease inhibitors on scDNA nicking property of α-synuclein.**
**A, Effect of aurintricarboxylic acid (ATA).** a, scDNA alone; b, linearised scDNA (Eco R1 restriction digested) as marker; c, scDNA + 5μM α-synuclein; d, scDNA + 100μM ATA + 5μM α-synuclein.
**B, Diethylpyrocarbonate (DEPC).** a, scDNA alone; b, linearised scDNA (Eco R1 restriction digested) as marker; c, scDNA + 5μM α-synuclein; d, scDNA + 100μM DEPC + 5μM α-synuclein.
Both ATA and DEPC abolished α-synuclein induced scDNA nicking property indicating a role for histidine in the nicking property.

i) The cell lysate was boiled at $100^0$C for 30 min during the isolation of α-synuclein to precipitate other proteins. α-synuclein does not precipitate at this temperature as it is natively unfolded protein. However many of the nucleases would be denatured by this heat treatment except few micrococcal nucleases which are heat stable.

ii) α-Synuclein was purified by anion-exchange chromatography (Fig. 3.1). Most of the typical nucleases have net positive charge at neutral pH making them enable to bind to DNA effectively and hence were not expected to separate in anion-exchange along with α-synuclein.

iii) Further purification of α-synuclein by Gel permeation chromatography showed similar activity [Fig. 3.8].

iv) It was experimentally shown that the DNA nicking property is α-synuclein concentration dependent (Fig. 3.8). In this experiment, the peak and two side fractions from gel permeation chromatography of α-synuclein were collected separately and equal volume (5 μL) of each fraction was incubated with 50 ng of scDNA at $37^0$C for ~12 hrs and electrophoresed on 1% agarose gel at 4V/cm (Fig. 3.8, inset). It was observed that the peak fraction showed more DNA nicking than the side fractions. Also the activity was linearly dependent on α-synuclein concentrations (peak fraction, 64μM; side fractions 32 and 8 μM respectively).

v) The purity was further confirmed by running 5 μg of α-synuclein on 15% polyacryl amide gel electrophoresis (Fig. 3.9 A) where single band was observed on Coomassie blue staining.

vi) The presence of α-synuclein was also confirmed by **dot immuno blot** assay (Fig. 3.9B) using specific antibody for α-synuclein, Anti-α-synuclein antibody (raised in rabbit, SIGMA Chemical Co.) as primary antibody and the alkaline phosphatase linked anti-rabbit-goat antibody was used as secondary antibody.

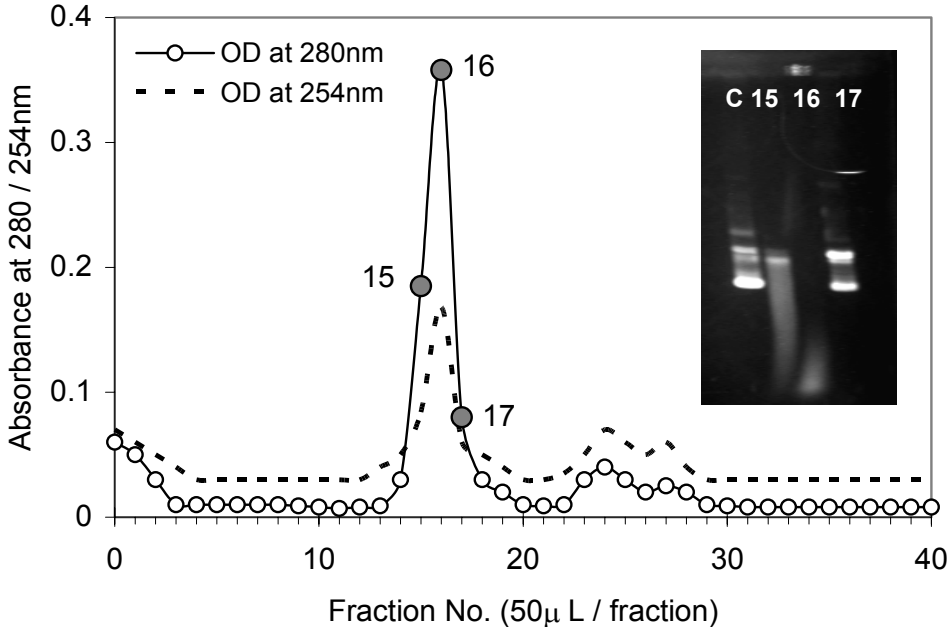



**Figure 3.8**

**GPC (FPLC) Chromatography profile of α-synuclein.** *Column*: Superdex 75 PC 3.2/30 (3.2 x 300 mm, 13 μm particle size: SMART system, Pharmacea Biotech, Uppsala, Sweden); *Sample*: 20 μL of purified α-synuclein (by anion exchange chromatography, see Figure 4) 0.25 mg/mL; *Buffer*: 10 mM Tris-HCl, pH 7.4 containing 50 mM NaCl; *Flow rate*: 0.05 mL/min; *Detection*: UV, 280 nm (protein) and 254 nm (DNA). The 3 fractions from the peak, 15, 16 and 17 were collected separately. The absorbance at 280 and 254 nm represent protein and DNA respectively. The minor peak for 254 nm between fractions 23-28 show elution of small DNA oligonucleotide fragments that separate in anion exchange chromatography along with α-synuclein.

**Inset gel photograph**: The inset is the 1 % agarose gel showing DNA degradation activity of three fractions (Fraction no. 15, 16 and 17) of GPC purified α-synuclein. Concentrations of fraction 15, 16 and 17 were 33, 64 and 8 μM respectively. 5μL of each fraction was incubated with 0.5μg of pUC 18 scDNA at $37^0$ C for 12 hrs. In the gel picture, lane C, scDNA alone; lane 15, scDNA + 5 μl fraction 15 (16.5μM α-synuclein); lane 16, scDNA + 5μl fraction 16 (32μM α-synuclein); lane 17, scDNA + 5μl fraction 17 (4μM synuclein). The figure shows that the DNA nicking activity of the GPC fractions increase with α-synuclein concentrations indicating purity of α-synuclein preparation.

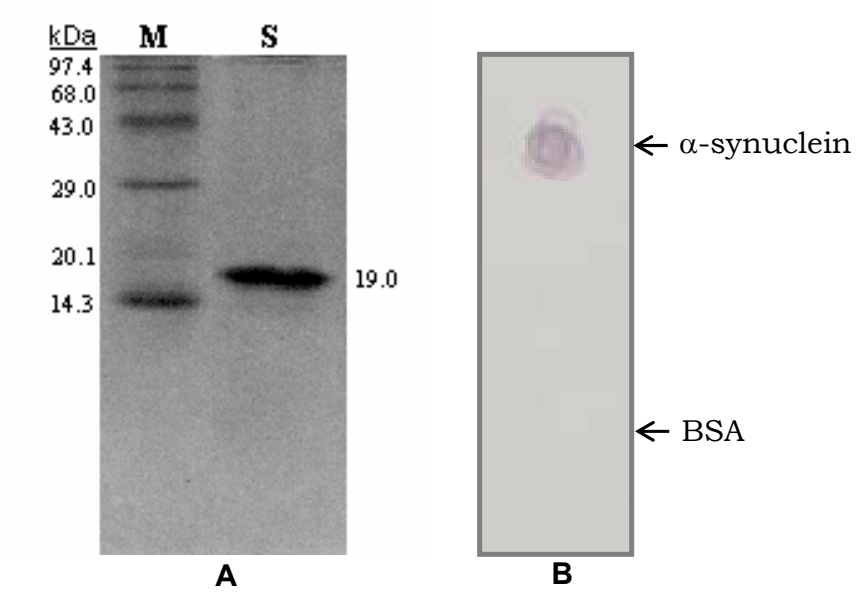



**Figure 3.9**

**Characterization of α-synuclein by polyacrylamide electrophoresis and dot immunoblot analysis.**

**A, Typical SDS-PAGE pattern of α-synuclein** (M, molecular weight marker; S, α-synuclein) Because of its unfolded native structure, α-synuclein monomer migrates in gel electrophoresis to an apparent Molecular mass of ~19 kD. Its actual mass is 14.4kDa. 5μg of protein was loaded on a 15% SDS gel. Bands were visualized using Coomassie Brilliant Blue stain.

**B, Dot Immunoblot analysis of α-synuclein.** Antigens: Control BSA; α-synuclein (purified). The presence of α-synuclein was detected using Anti-α-synuclein of rabbit as primary antibody and peroxidase conjugated anti-rabbit-goat antibody as secondary antibody. BSA was used as control where no immunoreactivity was observed.

## 3.3-5. Effect of divalent metals on α-synuclein induced DNA nicking

Divalent metals like $Mg^{2+}$ were shown to be the co-factors for many nucleases. $Mg^{2+}$ is either an essential requirement or it is required to enhance enzymatic nuclease activity. In the above context, we studied the effect of divalent metal chlorides like $MgCl_2$, $CaCl_2$ and

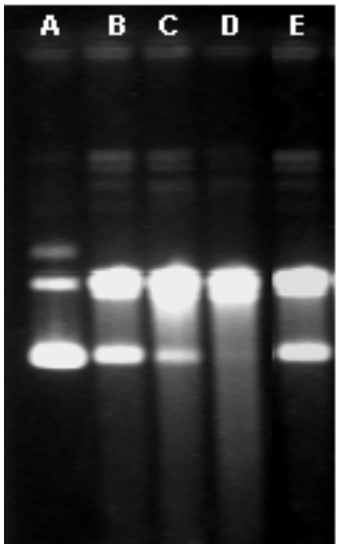



**Figure 3.10**

**Effect of $MgCl_2$, $CaCl_2$ and $ZnCl_2$ on α-synuclein induced DNA nicking**. A, scDNA alone; B, scDNA+ 1 μM α-synuclein; C, scDNA+ 1 μM α-synuclein + 1mM $CaCl_2$ ; D, scDNA+ 1uM α-synuclein + 1 mM $MgCl_2$; scDNA+ 1uM α-synuclein + 1 mM $ZnCl_2$. $Mg^{2+}$ and $Ca^{2+}$ enhanced the nicking activity while $Zn^{2+}$ showed no effect.

$ZnCl_2$ on α-synuclein-DNA interactions. α-synuclein (10 μM) was incubated with scDNA (0.5 μg)) in the presence of these metals at $37^0$C for ~12 hrs and analyzed by running 1% Agarose gel electrophoresis and stained with EtBr (1 μg/mL). It was observed that $Mg^{2+}$ and $Ca^{2+}$ enhanced the DNA nicking activity considerably, while Zn did not show any effect as seen in Fig. 3.10. However, the magnitude of enhancement was several folds more with $Mg^{2+}$ than $Ca^{2+}$.

### 3.3-6. Concentration dependence of α-synuclein-induced DNA nicking property

The concentration dependency of α-synuclein induced DNA nicking property was studied by incubating increasing concentrations of α-synuclein ($1x10^{-8}$ to $10x10^{-6}$ M) with 50 ng of scDNA at $37^0$C for ~12 hrs. The samples were analyzed for disappearance of supercoiled band and appearance of open circular/linear forms and formation of smaller DNA fragments in 1% agarose gel. The nicking activity linearly increased with increasing α-synuclein concentrations (Fig. 3.11).

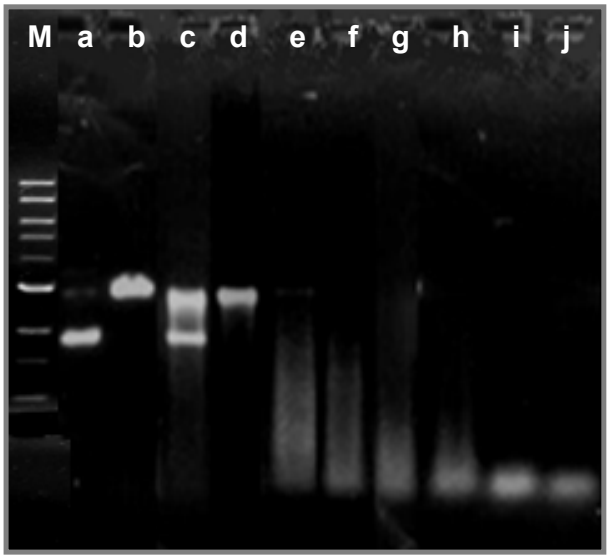



**Figure 3.11**

**Concentration dependence of α-synuclein-induced DNA nicking property.** Agarose gel electrophoresis (1% agarose) run at 4V/cm for 6 hrs. M, 1kb DNA molecular weight marker; a, supercoiled (sc) DNA alone; b, linearised scDNA (Eco R1 restriction digested, as a marker); c to j, scDNA incubated with 0.01, 0.1, 1.0, 2.5, 5.0 10.0, 50.0 and 100 μM α-synuclein respectively at $37^0$C for ~12 hrs. 0.5 μg of scDNA was loaded in each well.

### 3.3-7. Time kinetics of α-synuclein induced scDNA nicking

Time kinetics of DNA nicking activity of α-synuclein was studied using agarose gel electrophoresis and measurement of SSBs and DSBs by Nick Translation method. For agarose gel studies, 10 μM α-synuclein was incubated with 50 ng scDNA at 37$^0$C for various time intervals (0 to 24 hrs) and DNA degradation was analyzed by 1% agarose gel electrophoresis. The DNA nicking activity increased with time (Fig. 3.12).

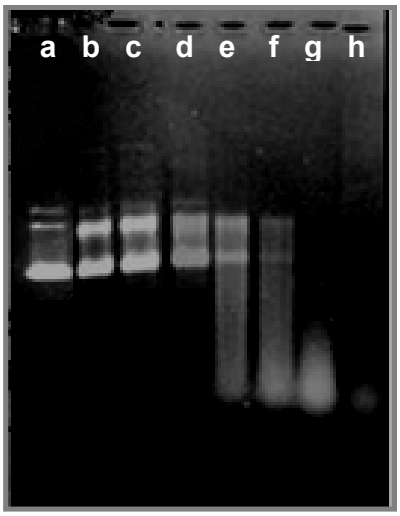



**Figure 3.12**

**Time kinetics of DNA nicking activity of α-synuclein.** Agarose gel electrophoresis (1% agarose) run at 4V/cm for 6 hrs. a, scDNA alone; b to h, scDNA incubated with 10μM α-synuclein at 37$^0$C for 1, 2, 3, 4, 8 and 12 hrs. 0.5 μg of scDNA was loaded in each well.

The Nick Translation studies revealed that the kinetics of formation of DNA SSBs for α-synuclein activity has an initial lag phase of ~8 hrs and then increased exponentially reaching a plateau after ~20 hrs forming a sigmoidal curve pattern (Fig. 3.13 A).

However, in the presence of $MgCl_2$, the time kinetics showed more or less a linear increase pattern with a tendency to slow down after 25-30 hrs. Further, the kinetics of formation of DSBs showed sigmoidal pattern both in the absence and presence of $Mg^{2+}$. However, in the presence of $Mg^{2+}$ the number of DSBs were few folds more compared to that in the absence of it (Fig. 3.13 B). As the sigmoidal pattern of kinetics was obtained for the α-synuclein activity it will be evocative to understand the changes taking place in either DNA or α-synuclein during the lag phase. It may possibly involve destabilization of DNA or changes in the α-synuclein conformation/folding.

The comparison of formation of SSBs and DSBs in scDNA by α-synuclein is represented in Fig. 3.14. Interestingly only SSBs were formed initially till 12 hrs and the DSBs start appearing later. This indicates that α-synuclein damages the scDNA only by the formation of SSBs and the DSBs formed after prolonged incubation could be due to accumulation of more number of SSBs in close vicinity. These results on kinetics of formation of DNA strand breaks caused by α-synuclein indicates that α-synuclein has only DNA nicking property or in other words it forms only SSBs. In this context, it will be interesting to investigate the action of α-synuclein on single and double stranded DNA.

### 3.3-8. α-synuclein interactions with linear double stranded DNA

To study the nicking activity of α-synuclein on double stranded linear DNA, the scDNA was linearized by treating with Eco R1 which has one restriction site in pUC 18 DNA. The Eco R1 treatment converts scDNA in to linear DNA with sticky ends or ends with small single stranded tails.

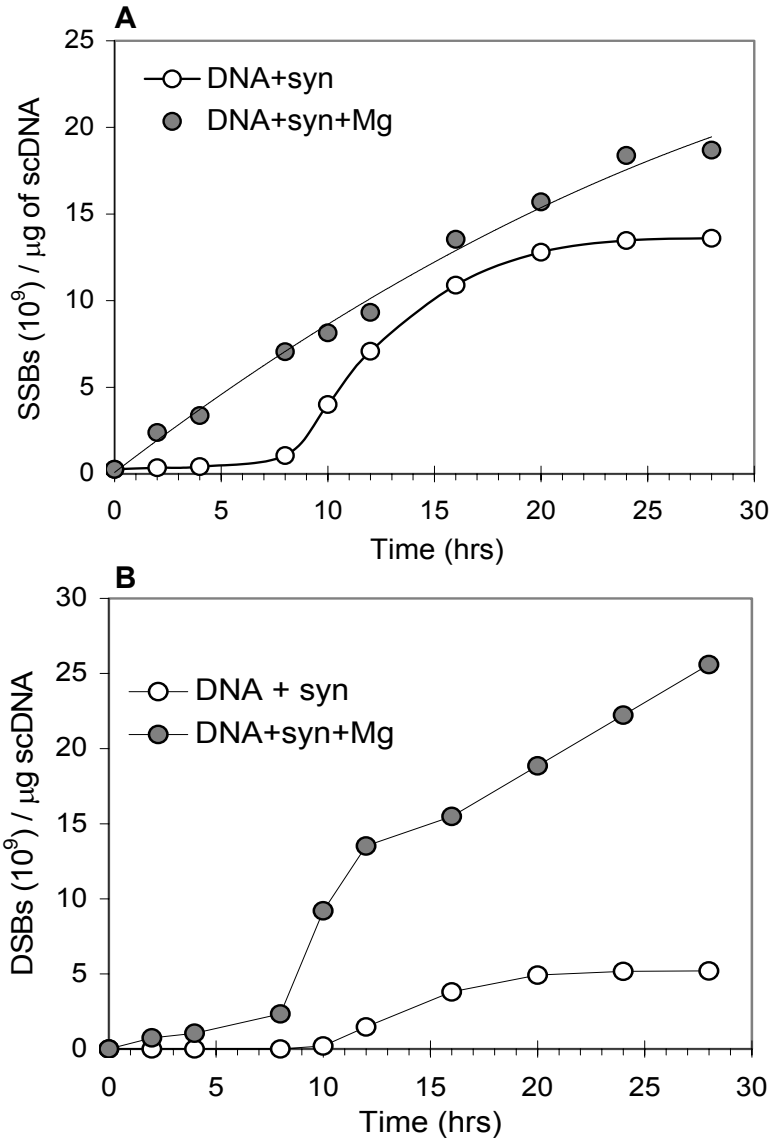



**Figure 3.13**

**Time kinetics of formation of SSBs and DSBs in scDNA by α-synuclein.** 1 μg of scDNA was treated with 10 μM α-synuclein for various time intervals in separate reactions at 37$^0$C and subjected for Nick translation assay.

**A. Estmation of SSBs by Nick Translation using DNA polymerase assay.** The kinetics for SSBs caused by α-synuclein in scDNA showed sigmoidal curve pattern with a lag phase of ~10 hrs. However in the presence of $MgCl_2$ a linear pattern was observed.

**B. Estimation of DSBs by terminal transferase assay.** The kinetics for DSBs formation in scDNA by α-synuclein showed sigmoidal curve pattern both in the absence and presence of $MgCl_2$. However, the DSBs formed were few times more in the presence of $Mg^{2+}$.

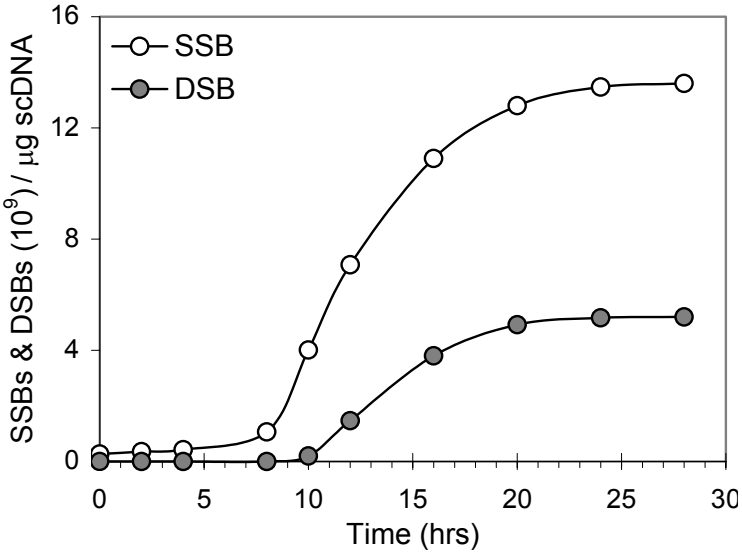



**Figure 3.14**

**Comparison of kinetics patterns for formation of SSBs and DSBs in scDNA by α-synuclein.** The graph showed that initially till ~8 hrs of incubation only SSBs were observed and DSBs started appearing after prolonged incubations (>~12 hrs). This indicates that α-synuclein damages the scDNA by the formation of SSBs and whatever DSBs observed after >~12 hrs of incubation may be due to accumulation of more number of SSBs at close vicinity in scDNA.

Fig. 3.15 shows the activity of α-synuclein on circular supercoiled and linear DNA 50 ng of each DNA was incubated with 10 µM α-synuclein at $37^0$C for ~12 hrs (overnight) and analyzed by running 1.2% agarose gel electrophoresis at 3V/cm. It was observed that the nicking activity was much less for linearised scDNA (Eco R1 treated) compared to scDNA.

These results showed that α-synuclein induced DNA nicking property is specific to single stranded DNA. The few strand breaks observed in case of linear DNA (Fig. 3.15 D) might be because of the single strand tails present at the ends after Eco R1 cleavage.

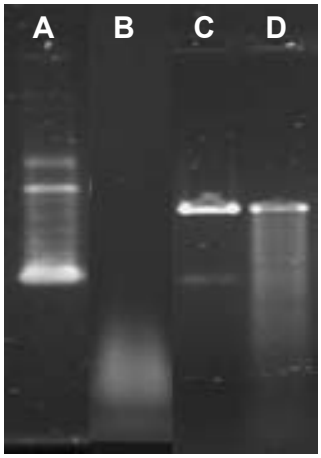



**Figure 3.15**

**Nicking activity of α-synuclein on linear double stranded linear DNA.** A, scDNA alone; B, scDNA + 20μM α-synuclein; C, Linearised scDNA (Eco R1 treated); D, Linearised DNA + 10 μM α-synuclein. The nicking activity was significantly less for double stranded linear DNA compared to scDNA.

However, scDNA is degraded by α-synuclein though it is double stranded. The scDNA owing to the superhelical twisting and torsion energy will unwind in some regions to form single stranded loops [Cherny et al, 1999]. It appears that α-synuclein binds to these single stranded regions in scDNA first and once the nicking is initiated further activity will be cooperative digesting the whole DNA. Similar mechanism was proposed by researchers on p53 protein binding to scDNA [Cherny et al, 1999].

Further, to confirm the above mechanism that α-synuclein activity is specific to single stranded regions of scDNA, the effect/influence of single stranded DNA (small single stranded fragments of calf-thymus DNA formed by heat denaturation followed by sonication) on α-synuclein induced scDNA digestion was studied. Single stranded DNA at 10:1 (pUC 18: ssDNA) concentrations

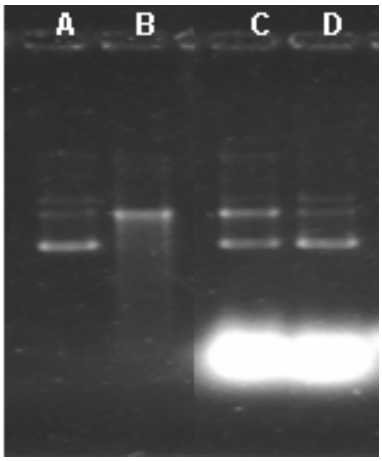



**Figure 3.16**

**Competitive inhibition of α-synuclein induced scDNA nicking by single stranded DNA.** A, supercoiled DNA alone; B, scDNA + 1 μM α-synuclein; C, scDNA + 1μM α-synuclein + 10:1 single stranded DNA; D, scDNA + 1 μM α-synuclein + 1:1 single stranded DNA. Heat denatured and sonicated calf-thymus DNA was used as single stranded DNA.

partially protected pUC 18 digestion (Fig. 3.16, lane C), while 1:1 concentrations, completely protected scDNA from α-synuclein induced DNA strand scission (Fig. 3.16, lane D).

## 3.3-9. α-synuclein interactions with single and double stranded circular DNA

The single strand DNA nicking specificity of α-synuclein was further studied by interacting α-synuclein with single strand circular (M13mp18 phage) and double stranded circular DNA (M13mp18 phage). M13 phage DNA is a single stranded circle, which is 7250 bases in length. Double stranded circular DNA arises as an

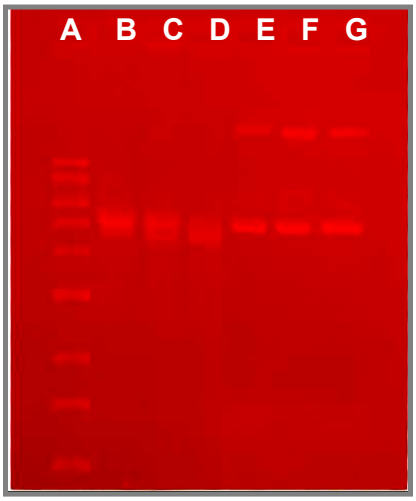



**Figure 3.17**

**Nicking activity of α-synuclein with single and double stranded circular DNA.** Agarose (1%) gel electrophoresis. A, 1kB molecular weight marker; B, single stranded circular (ssc) DNA; C, ssc DNA + 1 μM α-synuclein (wild-type); D, ssc DNA + 1 μM α-synuclein + 1 mM $MgCl_2$; E, double stranded circular (dsc) DNA; F, dsc DNA + 1 μM α-synuclein; G, dsc DNA + 1 μM α-synuclein +1 mM $MgCl_2$. α-synuclein nicked only single stranded circular DNA and double stranded circular DNA remains intact.

intermediate form during DNA replication. The interaction was carried out by incubating 1 μg of DNA with 10 μM α-synuclein at $37^0$C for ~12 hrs (overnight) and analyzed by 1 % agarose gel electrophoresis (Fig. 3.17). It was observed that α-synuclein nicks only single stranded circular DNA and the activity was enhanced by $MgCl_2$ as in the case of scDNA. However, double stranded circular DNA was not affected by α-synuclein. These results showed that α-synuclein induced nicking property is specific to single stranded DNA.

### 3.3-10. α-synuclein interactions with poly d(GC).d(GC) and poly d(AT).d(AT) nucleotide specific DNA

The nucleotide specificity of DNA nicking property of α-synuclein was studied by interacting α-synuclein with poly d(GC).d(GC) and poly d(AT).d(AT). The DNA-protein reaction mixtures were incubated at 37$^0$C for ~12 hrs and analyzed by electrophoresing with 1% agarose gel (Fig. 3.19). The results showed that α-synuclein could nick/damage both poly d(GC).d(GC) and poly d(AT).poly d(AT). Closer examination of the gel revealed that the nicking activity was slightly more with GC compared to AT specific DNA.

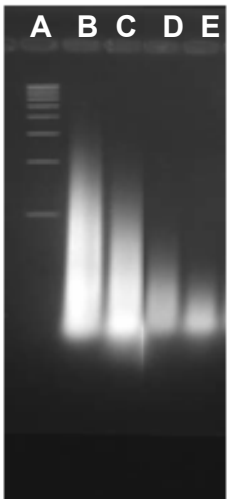



**Figure 3.19**

**Sequence specificity of α-synuclein induced DNA nicking activity with poly GC and poly AT.** 1% agarose gel, run at 4V/cm. A, 1 kB molecular weight marker; B, poly GC (0.5 μg); C, poly GC (0.5 μg) + α-synuclein (1 μM); D, poly AT (0.5 μg); E, poly AT (0.5 μg) + α-synuclein (1 μM). The DNA-α-synuclein reaction mixtures were incubated at 37$^0$C for ~12 hrs (overnight). The nicking activity was observed with both poly GC and poly AT, however the activity was more with GC specific DNA.

### 3.3-11. α-synuclein interactions with Genomic DNA

The nicking activity of α-synuclein was also examined with genomic DNA like Calf-Thymus DNA and Lamda phage DNA. For interaction, 0.5 μg of each DNA was incubated with 1 μM α-synuclein at 37$^0$C for ~12hrs (overnight) and analyzed by running 1% agarose gel electrophoresis at 4V/cm. The results showed that α-synuclein damaged both Calf-Thymus and Lamda DNA (Fig. 3.20).

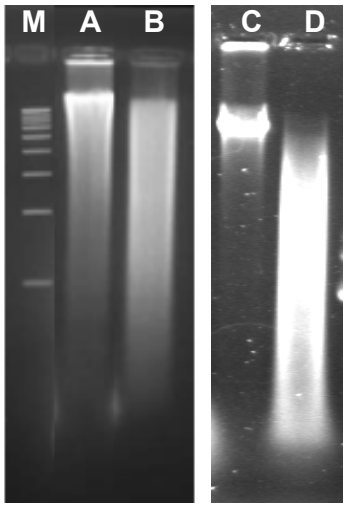



**Figure 3.20**

**Nicking activity of α-synuclein with genomic DNA.** M, 1kB molecular weight marker; A, Calf-Thymus DNA (0.5 μg); B, Calf-Thymus DNA (0.5 μg) + α-synuclein (1 μM); C, lamda DNA (0.5 μg); D, lamda DNA (0.5 μg) + α-synuclein (1 μM). The DNA-α-synuclein solutions were incubated at 37$^0$C for ~12 hrs (overnight) and electrophoresed on 1% agarose gel at 4V/cm. The gel was stained with ethidium bromide.

### 3.3-12. Familial mutant α-synuclein interactions with scDNA

The above results showed that α-synuclein (wild-type) in its native/normal sequence has DNA nicking property. Further, three

mutations have been identified in α-synuclein namely Ala30Pro (A30P), Ala53Thr (A53T) and Glu46Lys (E46K) in familial or inherited PD [Kruger et al, 1998; Polymeropoulos et al, 1997; Zarranz et al, 2004]. In this perspective, we examined the DNA nicking ability of two commonly occurring mutant α-synucleins, A30P and A53T. The mutants and wild type α-synuclein were interacted with scDNA similarly at 37$^0$C for ~12 hrs (overnight) and run on 1.2% agarose gel electrophoresis (Fig. 3.21). It was observed that all the three forms of α-synuclein (wild-type, mutants: A30P and A53T) showed similar kind of DNA nicking activity.

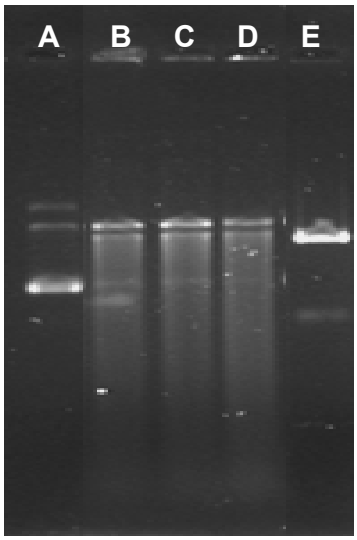



**Figure 3.21**

**DNA nicking activity of mutant forms of α-synuclein protein.** The two common familial mutants, Ala30Pro and Ala53Thr were interacted with scDNA and the activity was compared with wild-type α-synuclein. The samples were incubated at 37$^0$C for ~12 hrs and electrophoresed on 1.2% agarose gel and stained with sybre gold. A, scDNA (50 ng); B, scDNA + α-synuclein wild-type; C, scDNA + mutant Ala30P; D, scDNA + mutant Ala53Thr; E, linearised scDNA (Eco R1 treated scDNA). The protein concentrations used were 1 μM. All the three forms of the protein showed similar DNA nicking activity.

## 3.3-13. Role of α-synuclein folding/aggregation on its inherent DNA nicking activity

To examine the DNA nicking property of monomers, oligomers and aggregates of α-synuclein, the protein (50 μM α-synuclein) was incubated at 37$^0$C with continuous stirring using micro magnetic bars. The aggregation was monitored using Thioflavin T fluorescence assay (Fig. 3.23 A). An aliquot was withdrawn at various time intervals and incubated with scDNA at 37$^0$C for ~12 hrs. The strand breaks in DNA were studied using 1.2 % agarose gel electrophoresis and SSBs/ DSB were estimated (Fig. 3.22).

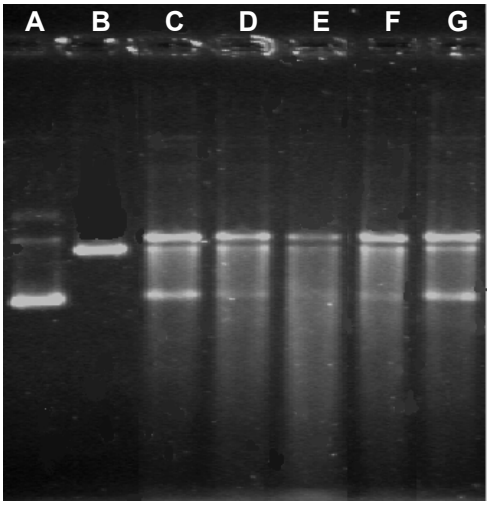



**Figure 3.22**

**Effect of Aggregation on DNA nicking activity of α-synuclein.** α-synuclein (50 μM) was incubated at 37$^0$C with constant stirring using micro-magnetic bars which makes α-synuclein into β-sheet and aggregates. Aliquots were withdrawn at C, 1; D, 5; E, 10; F, 25; G, 40 hrs time and studied for scDNA nicking by 1.2% agarose gel electrophoresis. A, supercoiled DNA alone; B, linearised supercoiled DNA as reference (Eco R1 treated supercoiled DNA); C-G, DNA (0.5 μg) + α-synuclein (1μM) incubated at 37$^0$C for 12 hrs. The oligomers or partially folded α-synuclein showed more DNA nicking activity than monomers and aggregates.

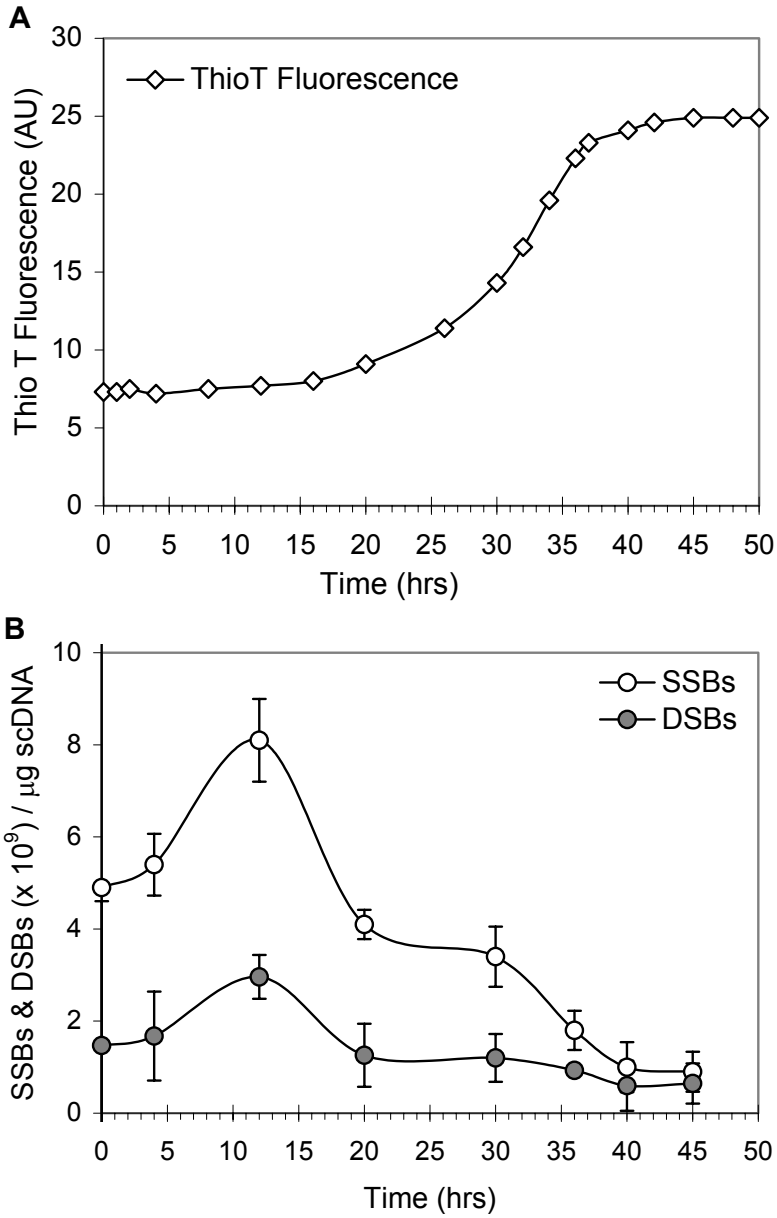



**Figure 3.23**

**Comparison of α-synuclein aggregation and formation of SSB and DSB. A, Thioflavin T fluorescence assay for α-synuclein aggregation.** 50 µM α-synuclein was incubated with constant stirring using micro-magnetic bars and aggregation was monitored using Thioflavin T fluorescence assay (Excitation, 446 nm and Emission, 482 nm).

**B, DNA nicking activity of α-synuclein aliquots withdrawn from the above incubated sample at various time intervals.** The SSBs and DSBs were measured by Nick Translation method using DNA polymerase I assay for SSBs and Terminal transferase assay for DSBs.

The results showed that the oligomers of α-synuclein has more DNA nicking activity than monomers and aggregates

Fig. 3.23A, represents the kinetics of fibrillization of α-synuclein monitored by Thio T fluorescence (Excitation, 446 nm and Emission, 482 nm). It was observed that the nicking activity of α-synuclein was maximum after 10 hrs of incubation where the protein was expected to be in oligomeric or partially folded form.

Further, the number of SSBs and DSBs were estimated in scDNA (0.5 μg) treated with aliquots of α-synuclein (1 μM) (Fig.3.23 B) withdrawn at various time intervals from aggregating α-synuclein solution. It was observed that both the SSBs and DSBs increase from 1 to ~12 hrs and start decreasing after ~12 hrs (Fig.3.23 B). The maximum DNA nicking activity was observed at 10-12 hrs of incubation (Fig. 3.22). At this time incubation, the Thio T fluorescence values began to increase indicating that α-synuclein must be in oligomeric form (Fig. 3.23 A). The least DNA nicking activity was shown by aggregates of α-synuclein compared to other intermediate forms. This is an interesting finding in view of the recent debate on the exact toxic form/species of α-synuclein.

## 3.3-14. Transmission Electron Microscopic evidence for α-synuclein-DNA complex formation

The physical association between α-synuclein fibrils and scDNA was studied using transmission electron microscopic (TEM) images. For EM experiments wild-type α-synuclein was incubated with stirring which led to the formation of aggregates as indicated by Thio T fluorescence. The aggregated α-synuclein was mixed with scDNA for 1 hr and observed under electron microscope (The sample preparation for TEM has been explained in Experimental procedure section). It was observed that almost all the scDNA molecules were in

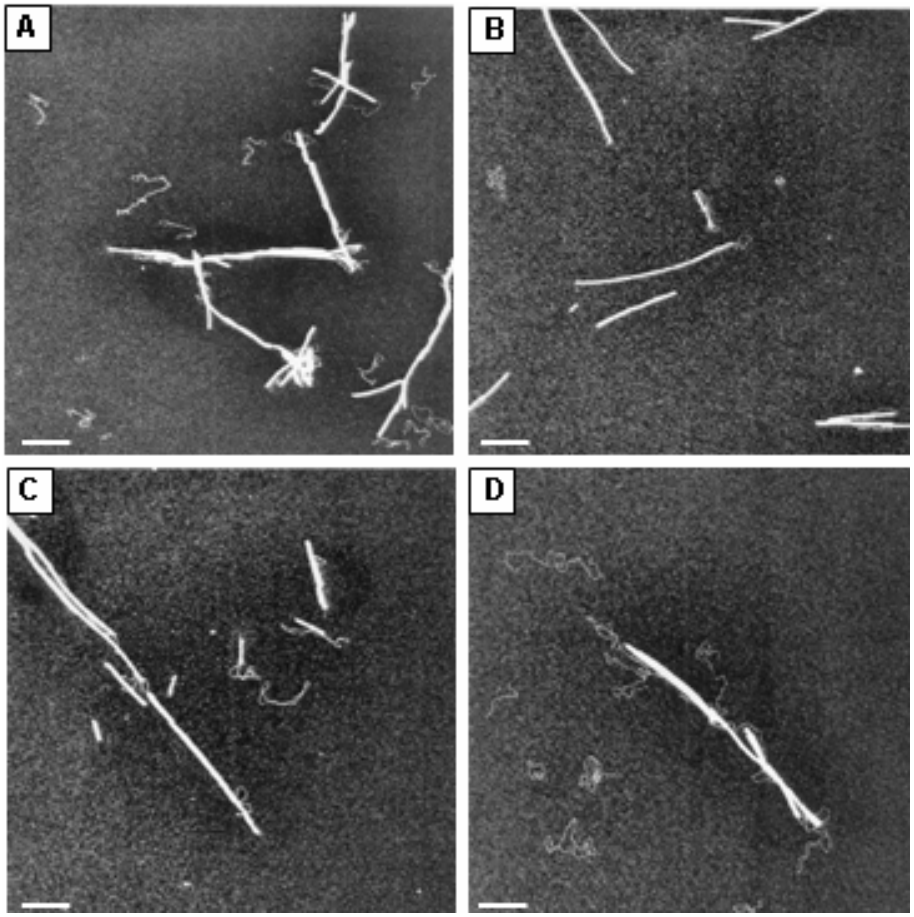



**Figure 3.24**

**Transmission Electron Microscopic (TEM) images evidencing association of scDNA with preformed α-synuclein fibrils.** TEM images of the samples were obtained after the incubation of scDNA with preformed fibrils assembled from the wild-type α-synuclein. For fibrillization α-synuclein solution was incubated at $37^0$C with constant stirring using micro-magnetic bars and aggregation monitored by Thio T fluorescence. For the DNA-α-synuclein association reaction, the preformed α-synuclein fibrils were mixed with scDNA and kept for 1 hr before taking the TEM images. Images were acquired in an angular dark-field mode. The scale bar represents 100 nm.

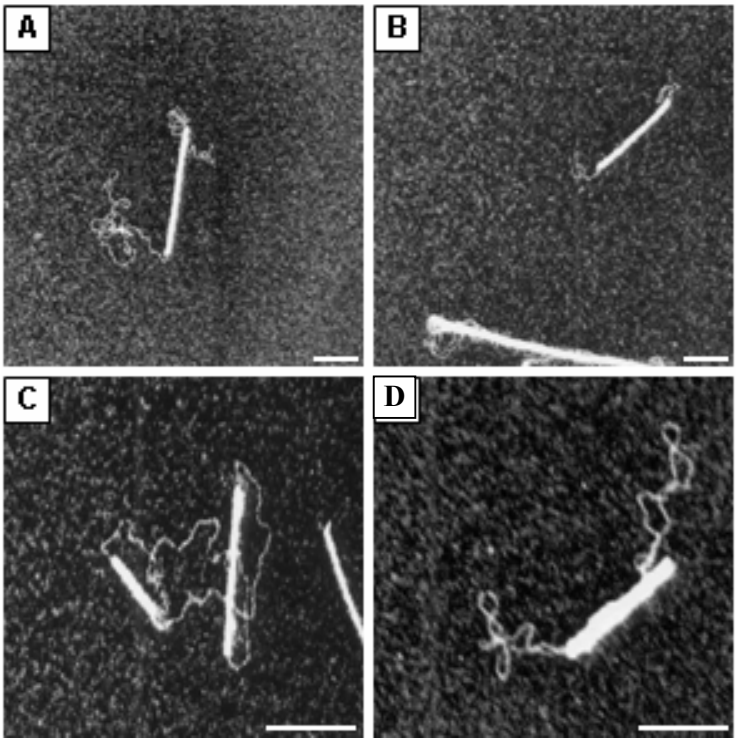



**Figure 3.25**

**TEM images of α-synuclein-scDNA complexes: indicating the binding of DNA to the ends of fibrils.** The formation of α-synuclein and association of α-synuclein and scDNA were carried out as explained in Fig 3.24. Scale bars represent 100 nm.

complexation with the α-synuclein fibrils, forming various types of complexes (Fig. 3.24). The DNA was visible as thin supercoiled threads in physical association with α-synuclein fibrils. Interestingly, the binding between α-synuclein fibrils and DNA appeared strong making it impossible to trace the entire DNA molecule due to its close association with the fibril body. Similar observations were recently made by Cherny et al, [2004]. Some DNA molecules were in open circular or linear forms (Fig. 3.24 D). This was because of DNA nicking of scDNA by α-synuclein leading to relaxation of supercoiling and linearization.

Further magnification of TEM images interestingly evidenced that the DNA molecules were bound to the ends of fibrils in most cases (Fig. 3.25). It also appeared that the DNA binds to α-synuclein fibrils through its own ends. There were hardly any DNA molecules crossing the fibrils.

## 3.4 Discussion

Our results demonstrate two new and novel properties of α-synuclein, a prime protein implicated in PD. **First**, we showed that α-synuclein binds to DNA and alter the conformation of DNA (Fig. 3.2). **Second**, α-synuclein was shown to have DNA nicking activity and it behaves like a nuclease enzyme. These are new evidences in literature, there were no such reports previously on α-synuclein binding to DNA.

The mechanism of α-synuclein induced DNA nicking activity was established using several approaches and using different DNA. It was concluded experimentally from our data that α-synuclein preferentially nicks only single stranded DNA and intact double stranded DNA was unaffected by α-synuclein. It was also observed that the nicking activity involves the formation of only SSBs. However, during long term incubations with α-synuclein DSBs were formed chopping the DNA into small pieces. This was probably due to accumulation of more number of SSBs in close vicinity which indirectly leads to the formation of DSBs. A schematic representation of nicking mechanism of α-synuclein with scDNA has been represented in Fig. 3.26.

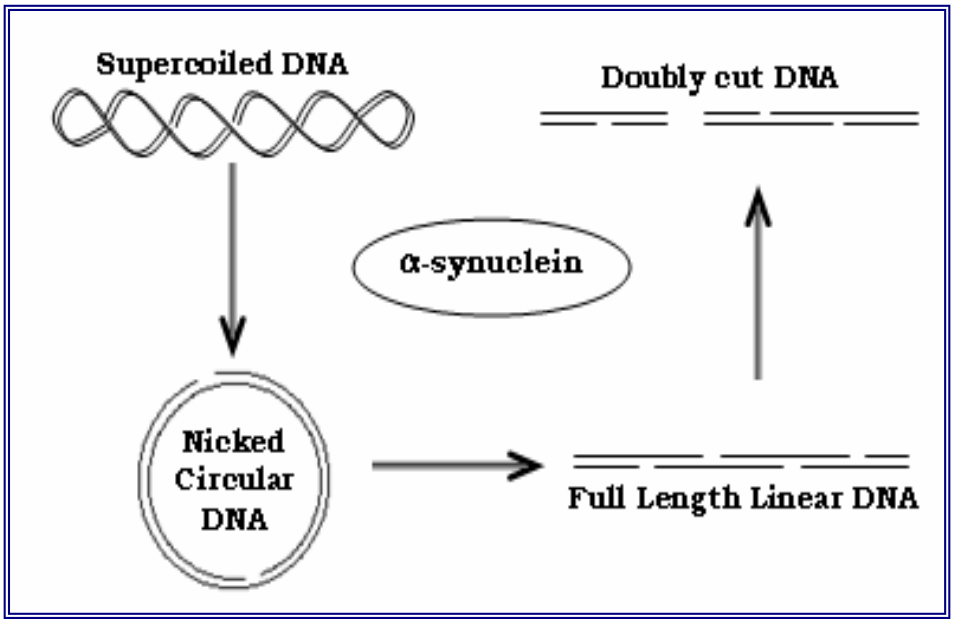



**Figure 3.26**

**Schematic Representation of the mechanism of DNA nicking activity of α-synuclein.** It is evident that α-synuclein relaxes the scDNA to open circular form by causing single strand nicks and on prolonged incubation lead to the formation of linear and further fragmentation of DNA due to accumulation of more number of SSBs in close vicinity

Further, the ability of known nuclease inhibitors, ATA and DEPC to abolish DNA nicking activity of α-synuclein reveals few interesting points on the importance of histidine in this property (Fig. 3.7). ATA is a powerful inhibitor of proteins whose biological function depends on the formation of complex with nucleic acid [Gonzalez et al, 1980]. DEPC is also very effective to inactivate nucleases. Both ATA and DEPC derivatize histidine (H) in the active site of nucleases and hence make the proteins unable to bind to DNA. Moreover, ATA is the only well documented compound capable of interacting with a broad variety of nucleic acid binding proteins [Gonzalez et al, 1980].

α-synuclein has one H in its sequence (out of 140 amino acids). This histidine is at 50th position from N-terminal end (Fig. 3.27). As ATA and DEPC could protect DNA from α-synuclein induced nicking, it is evident that the lone H residue at 50$^{th}$ position plays a crucial role in the nicking activity.

| | | | |
|---|---|---|---|
| MDVFMKGLSK$^{10}$ | AKEGVVAAAE $^{20}$ | KTKQGVAEAA$^{30}$ | GKTKEGVLYV$^{40}$ |
| GSKTKEGVV**H**$^{50}$ | GVATVAEKTK$^{60}$ | EQVTNVGGAV$^{70}$ | VTGVTAVADK$^{80}$ |
| TVEGAGSIAA$^{90}$ | ATGFVKKDQL$^{100}$ | GKNEEGAPQE$^{110}$ | GILEDMPVDP$^{120}$ |
| | DNEAYEMPSE$^{130}$ | EGYQDYEPEA$^{140}$ | |

**Figure 3.27**

**Amino acid sequence of α-synuclein.** The numerical superscripts denote number of amino acids after each 10 amino acids. The sequence is from N-terminal to C-terminal. There is one histidine (H) residue at 50$^{th}$ position (marked in red color). Our results showed that this H plays a crucial role in the DNA nicking property of α-synuclein. ATA and DEPC, two classical nuclease inhibitors derivatize H residue and hence abolish α-synuclein induced DNA nicking property.

A plausible scenario for DNA binding to α-synuclein could be as follows: It appears that initially on mixing with α-synuclein in solution, α-synuclein monomers interact electrostatically with DNA phosphate groups. DNA interacts possibly with the positively charged lysine side chains located predominantly in the N-terminal and partly in the central region of α-synuclein sequence. Because it is highly unlikely to bind to the C-terminal end of α-synuclein which is rich in negatively charged amino acid residues [Cherny et al, 2004]. These electrostatic interactions may lead to (i) formation of

non-sequence specific complex of α-synuclein with DNA, and (ii) increase in the local concentration of α-synuclein on DNA [Cherny et al, 2004]. However, our results on the role of H in DNA nicking property of α-synuclein indicates that once α-synuclein binds to DNA by electrostatic forces, there could be a conformational change in α-synuclein making the protein enzymatically bind to DNA.

Our results on differential nicking activity of monomers, partially folded intermediates or oligomers and aggregates of α-synuclein (Fig. 3.22 and 3.23) indicated that a conformational change or oligomerization in α-synuclein would enhance the nicking activity. It was also observed from kinetics study that α-synuclein induced DNA nicking activity follows a sigmoidal curve pattern with time (Fig. 3.13 and 3.14). It is likely that α-synuclein undergoes a conformational change on binding electrostatically to DNA as explained above. Hence, after a lag phase of about ~10 hrs and then a cooperative increase in nicking activity was observed. Moreover in presence of $Mg^{2+}$ a linear increase in nicking activity was observed possibly because of the ability of divalent $Mg^{2+}$ to aid in partial folding of α-synuclein thereby eliminating the initial lag phase in the kinetics. The conformational change from random coil to a folded or partially folded conformation may help α-synuclein exert enzymatic nuclease activity through H amino acid residue. Studies showed that α-synuclein has a propensity to form broken helices in the N-terminal region owing to the presence of 6-7 eleven amino acid repeats [Chandra et al, 2003]. It is possible that the initial folding in α-synuclein, forming broken helices in N-terminal end is essential for nicking activity. It is interesting to note that many of the classical nucleases have helix-coil-helix conformation, which makes them effectively bind to DNA.

It will be evocative to understand the potential implications of the above *in vitro* findings to neurodegenerative changes associated with PD. Though α-synuclein is primarily a presynaptic nerve terminal protein predominantly present in the membrane, several studies have evidenced the presence of α-synuclein in nuclei as well [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004]. However, the mode of appearance of α-synuclein into the neuronal nuclei and functions of α-synuclein in nuclei is still obscure. According to Maroteaux et al, [1988] the mode of localization of α-synuclein in the nucleus could involve a lateral diffusion along the endoplasmic reticulum and outer nuclear membrane [Torrisi et al. 1987] or more conventional transport through nuclear pores. They proposed that α-synuclein family proteins may be involved in coordinating nuclear and synaptic events. (The possible functions of α-synuclein have been represented in table 1.3). However, under PD conditions, the nuclear localization of α-synuclein could be enhanced due to non-specific transportation through oxidatively damaged nuclear membrane [Hegde and Rao, 2003]. Recent studies showed that α-synuclein is present in neuronal nuclear inclusions and neuritis in multiple system atrophy [Lin et al, 2004]. Further, Goers et al, [2003] provided evidence for the co-localization of α-synuclein with histones in the nuclei of nigral neurons from mice exposed to a toxic insult. The authors observed that histones stimulate α-synuclein fibrillation *in vitro* [Goers et al, 2003].

The DNA binding property of α-synuclein characterized in the present study may have a significant effect on nuclear-translocated α-synuclein functioning. In particular, α-synuclein may interact with

histone-free DNA segments and induce nicking.

These lucid evidences for the presence of α-synuclein in neuronal nuclei indicate that the DNA nicking activity of α-synuclein characterized in the present study may have very significant role to play in neuronal cell death in PD through DNA instability. Moreover, the results also raise the argument that whether the α-synuclein aggregation is pathological feature of the disease or it is adaptation to counter the toxicity of soluble form of α-synuclein (in terms of DNA damage).

We have previously shown that amyloid β peptides which form major proteinacious aggregates in AD also bind and alter the topology of scDNA [Hegde et al, 2004a]. The present study highlights the similar properties of α-synuclein and amyloid peptides and in turn demands a need to understand the parallel mechanisms underlying PD and AD, the two major neurodegenerative disorders.

✡ ✡ ✡ ✡ ✡

# CHAPTER 4

# *DNA induces folding in α-synuclein: Understanding the mechanism using chaperon property of osmolytes*

## 4.1 Introduction

α-synuclein is a small (14 kDa), soluble, intracellular, highly conserved protein, of unknown function and it is abundant in various regions of the brain [Maroteaux et al, 1988; Jakes et al, 1994; Iwai et al, 1995; George, 2002]. α-synuclein is characterized by the presence of acidic stretches within the C-terminal region and a repetitive motif, KTKEGV, in the first 93 residues [George et al, 1995; George, 2002]. Such a periodicity is characteristic of the amphipathic helices of apolipoproteins [Segrest et al, 1990; Segrest et al, 1992].

Structurally, purified α-synuclein is a natively unfolded protein [Hegde and Rao, 2003; Weinreb et al, 1996; Uversky et al, 2001a; Eliezer et al, 2001]. This lack of folding has been shown to correlate with the specific combinations of low overall hydrophobicity and large net charge [Uversky et al, 2000; Uversky, 2002a; Uversky, 2002b]. Few studies have highlighted the importance on being unfolded/disordered and it has been proposed that the increased intrinsic plasticity represents an important pre-requisite for effective molecular recognition [Plaxco et al, 1997; Dunker et al, 2001; Uversky, 2003].

Deposition of aggregated forms of α-synuclein in neuronal or glial cytoplasm is pathological hallmark of PD and other neurological disorders like dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and multiple system atrophy [Lucking and Brice, 2000; Trojanowsky and Lee, 1998]. *In vitro* α-synuclein readily assembles into fibrils, with morphologies and staining characteristics similar to those of fibrils extracted from PD affected brain [Uversky et al, 2001a; Crowther et al, 1998; El-Agnaf et al, 1998; Wood et al, 1999; Giasson et al, 1999; Narhi et al, 1999; Serpel et al, 2000;

Conway et al, 2000a; Li et al, 2001; Hoyer et al, 2002; Munishkina et al, 2003]. The kinetics of fibrillation occurs via a nucleation dependent mechanism [Conway et al, 2000b; Wood et al, 1999], with the critical primary stage being formation of a *partially folded intermediate* [Uversky et al, 2001a].

α-synuclein folding and fibrillation have been found to be promoted on binding to long chain fatty acids [Perrin et al, 2001] and also upon its interaction with lipid droplets [Cole et al, 2002]. It was also shown that membrane interactions induce a large conformational change from random coil to α-helix in α-synuclein and these interactions may be physiologically important [Jo et al, 2000]. On the basis of these observations, it has been assumed that α-synuclein may exist in two structurally different isoforms *in vivo*: a helix-rich, membrane-bound form and a disordered, cytosolic form, with the membrane-bound α-synuclein generating nuclei that seed the aggregation of the more abundant cytosolic form [Lee et al, 2002; Munishkina et al, 2003].

In the presence of several divalent and trivalent metal ions [Uversky et al, 2001b] or several common pesticides [Uversky et al, 2001d; Manning-Bog et al, 2002] α-synuclein has been shown to adopt a partially folded conformation, which is critical for fibrillation. These studies indicate that electrostatic interactions with α-synuclein resulting in neutralization of net negative charge would favor folding of the protein. Recently it was shown that double stranded DNA promotes aggregation of α-synuclein [Cherny et al, 2004]. In the previous chapter, our results indicated that the DNA binding may induce folding in α-synuclein which makes the protein bind to DNA enzymatically and have DNA nicking activity. In this perspective, the present study has been carried out to understand

the ability of different DNA to induce conformational changes in α-synuclein.

Further, it was shown that the binding of α-synuclein to synthetic and natural membranes is accompanied by a dramatic increase in α-helical content [Perrin et al, 2000; Jo et al, 2000; Volles et al, 2001; Jo et al, 2004]. It was also observed that naturally occurring osmolytes like trimethylamine-N-oxide (TMAO) causes natively unfolded α-synuclein to fold in a manner described for this protein at high temperatures, and low pH [Uversky et al, 2001c]. These results indicate that the osmophobic effect must be added to the previously described factors that induce the transformation of natively unfolded α-synuclein in to partially folded intermediate [Uversky et al, 2001c].

In a quest to understand the DNA induced conformational transition in α-synuclein, we have used chaperon properties of five osmolytes viz. glycerol, betaine, taurine, TMAO, sarcosine for their ability to induce folding in α-synuclein, as a model system.

## 4.2 Experimental procedures

### 4.2-1. Materials

Supercoiled DNA pUC 18 plasmid, Cesium chloride purified, 90% supercoiled structure), lamda DNA, Calf thymus DNA, single stranded circular (ssc)DNA (MP3), double stranded circular (dsc)DNA (MP3), Tris and HEPES buffers were purchased from Bangalore Genei, India. Poly d(GC).d(GC) and poly d(AT).d(AT), Sarcosine, TMAO, Taurine, Betaine, glycerol and Copper grids (300 mesh size) for electron microscopy, were purchased from Sigma Chemical Company USA. Uranyl acetate was purchased from B.D.H. laboratory

chemicals division. The stock solutions of 7M Sarcosine, 5M each of Betaine and TMAO and 0.8M Taurine were prepared in distilled water depending on the solubility of respective osmolytes.

### 4.2-2. α-synuclein expression and purification

α-synuclein was expressed in BL-21 cells using pT7-7 plasmid and purified by anion-exchange chromatography as explained in the methodology section of Chapter 3. α-synuclein was also purchased from rPeptides, USA.

### 4.2-3. Circular Dichroism Studies

The CD spectra (190–260 nm) were recorded for α-synuclein in the presence / absence of increasing concentrations of various DNA (supercoiled DNA, lamda DNA, single and double strand circular DNA etc) in 0.01 M HEPES buffer (pH 7.4) on a JASCO J-700 Spectropolarimeter. The cell length was of 1 mm width and 1 mm length. Each spectrum was the average of four repetitions. The CD contributions from DNA alone was corrected in the α-synuclein–DNA complex spectra. 10 μM α-synuclein was used for each measurement.

The effect of osmolytes, Sarcosine, TMAO, Taurine, Betaine and Glycerol on α-synuclein secondary conformation were also determined by CD. The protein was diluted to a final concentration of 2 μM in HEPES buffer, pH 7.0. The protein solutions were combined with $1x10^{-6}$ M to 1 M Sarcosine, TMAO, Taurine and Betaine and 1-50% by volume of Glycerol. Spectra were collected after 15 min of equilibrium period at room temperature. Spectra were acquired on a JASCO J-700 spectropolarimeter in a 0.1-cm path length cell over a wavelength range of 200-260nm and 190 to 260nm wherever

possible, with a 1.0-nm bandwidth, 0.1-nm resolution, 4-s response time and 20 nm/min scan rate. All spectra were corrected by substraction from buffer and respective osmolyte.

The deconvolution or secondary structure analysis of CD spectra of α-synuclein was carried out using *j-fit* software.

### 4.2-4. Intrinsic Fluorescence

Tyrosine intrinsic fluorescence spectra were collected on a HITACHI 2000 spectrofluorimeter in semimicro quartz cuvette with a 1-cm excitation light path. The light source was a 150-watt xenon lamp. For tyrosine intrinsic fluorescence α-synuclein containing solution was excited at 275 nm and emission monitored in the range from 290 to 350 nm. The maximum emission was observed at 306 nm. α-synuclein concentrations for intrinsic fluorescence measurements were kept at 2 μM. α-synuclein contains four tyrosine residues and there are no tryptophan. Hence the tyrosine fluorescence has been used to monitor folding of α-synuclein [Uversky et al, 2001b]. In the present investigation, the tyrosine intrinsic fluorescence was used to understand the osmolyte induced folding in α-synuclein.

### 4.2-5. Acrylamide fluorescence quenching

Acrylamide quenching studies of the intrinsic fluorescence of α-synuclein were performed by adding aliquots from a stock solution of the quencher (acrylamide) into a cuvette containing the protein (α-synuclein) solution. Fluorescence intensities were corrected for dilution effects. Fluorescence quenching data were analyzed using the general form of the **Stern-Volmer equation,** taking in to account not only the dynamic but also static quenching

[Eftink and Ghiron, 1981] as shown in the equation below:

$$I_o/I = (1+K_{sv}[Q])e^{V[Q]}$$

Where, $I_o$ and I are the fluoroscence intensities in the absence and presence of quencher; $K_{sv}$ is the dynamic quenching constant; V is a static quenching constant; and [Q] is the quencher concentration.

### 4.2-6. ANS (1-Anilinonaphthaleine-8-sulphonic acid) binding

ANS is frequently used to demonstrate the presence of partially folded conformations of proteins, characterized by the presence of solvent-exposed hydrophobic clusters. This is because ANS binds to solvent exposed hydrophobic clusters, resulting in a considerable increase in the ANS fluorescence intensity and in a pronounced blue shift of the fluorescence emission maximum.

We measured the change of ANS fluorescence to monitor the gain of structure in α-synuclein in terms of solvent exposed hydrophobic clusters upon binding with the osmolytes: Sarcosine, TMAO, Betaine, Taurine and Glycerol. A stock solution of 1 mM ANS was prepared in distilled water. The [ANS]/[α-synuclein] ratio in all experiments was kept equal to 5. Fluorescence measurements were performed at $25^0$C in 1 mL semi-micro quartz cuvette. Emission spectra were recorded from 400 to 600nm with excitation at 350nm. The protein concentration was 2 μM.

### 4.2-7. Trypsin cleavage and Tricine-SDS-PAGE

Proteolytic cleavage of proteins has been used as a probe of protein conformation and stability [Nadig et al, 1996; Fontana et al, 1997]. Trypsin was used at a final concentration of 0.1 mg/mL. The

effect of the presence of osmolytes on tryptic digestion was studied by 15% Tricine-SDS-PAGE.

### 4.2-8. Thioflavin T fluorescence for fibril formation assay

***Preparation of α-synuclein incubations for aggregation studies:*** α-synuclein at 50 μM concentration was dissolved/ diluted in 10 mM Tris-HCl, pH 7.4 and incubated at $37^0$C with agitation/stirring using micro-magnetic bars (Teflon coated) in glass vials.

***Thio T Fluorescence:*** A 100 μM aqueous solution of Thio T was prepared and filtered through 0.2-μm polyether sulfone filter. At various time intervals, aliquots of the α-synuclein incubations were diluted to 10 μM in 50 mM glycine-NaOH buffer, pH 9.0. Assay solutions contained 10 μM Thio T and α-synuclein at a concentration of 5 μM in 50 mM glycine-NaOH buffer, pH 9.0.

### 4.2-9. Transmission Electron Microscopy of α-synuclein aggregates

α-synuclein aggregates were observed under JEOL 1010 TEM. A drop (5μL) of α-synuclein solution was placed on the carbon-coated copper grid and was allowed to dry in air for 30 min. A second drop was applied after blotting the first drop with filter paper. This process was repeated 4-6 times after which the grids were negative stained by adding a drop of 1% uranyl acetate (pH 5.1) on the grid and blotted with a filter paper after 10 s. The grids were completely dried so as to avoid moisture for TEM examination [Thomas and Bloomfield, 1985]. Detailed methodology for TEM has been given in Chapter 3.

## 4.3 RESULTS

### 4.3-1. scDNA induces ordered conformation in α-synuclein

Circular dichroism spectroscopy (CD) was used to determine the effects of DNA binding on the secondary structure of α-synuclein. It was observed that scDNA caused a *biphasic* conformational transition in α-synuclein. Natively, α-synuclein is in random coil conformation. On immediate mixing of the DNA and α-synuclein at room temperature a partial folding was induced in α-synuclein (Fig. 4.1) while α-helix conformation was formed on long term incubation at $4^0$C (Fig. 4.2).

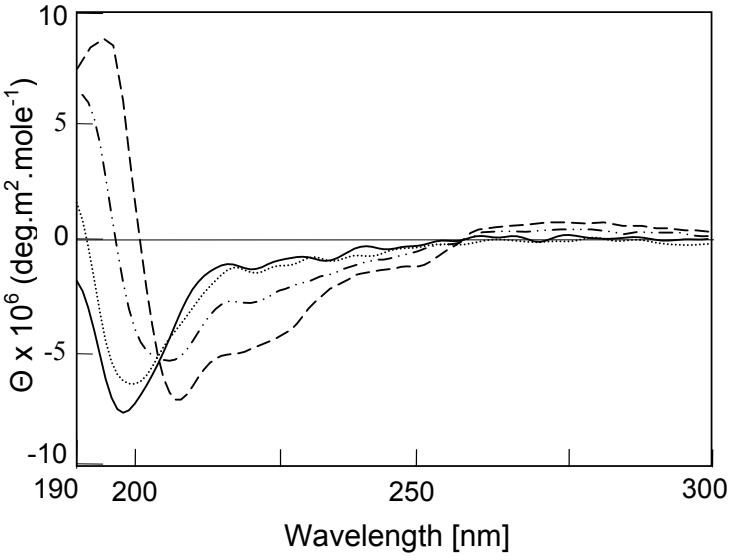



**Figure 4.1**

**scDNA induced partially folded conformation in α-synuclein.** The CD spectra of 5 μM α-synuclein was measured in the absence or presence of scDNA (1-10μg) and allowed to interact for 15 min. (–), α-synuclein; (.....), α-synuclein+1μg scDNA; (·—·), α-synuclein+5μg scDNA; (---), α-synuclein+ 10μg scDNA.

The CD spectrum of native α-synuclein was characterized by a strong negative CD band in the 195 to 200-nm region, indicative of a disordered or random coil conformation (Fig. 4.1, solid line). On interaction of α-synuclein with scDNA (for 15 min at room temperature) the CD spectrum of α-synuclein revealed a partially folded conformation with a subtle shift toward *α-helical* conformation.

On incubation of α-synuclein with scDNA for long time (~one month) at $4^0$C, the CD spectrum of α-synuclein clearly showed a typical α-helical secondary structure with characteristic minima at 208 and 222 nm (Fig. 4.2). The CD contribution from scDNA was very small and it was substracted from the complex spectra.

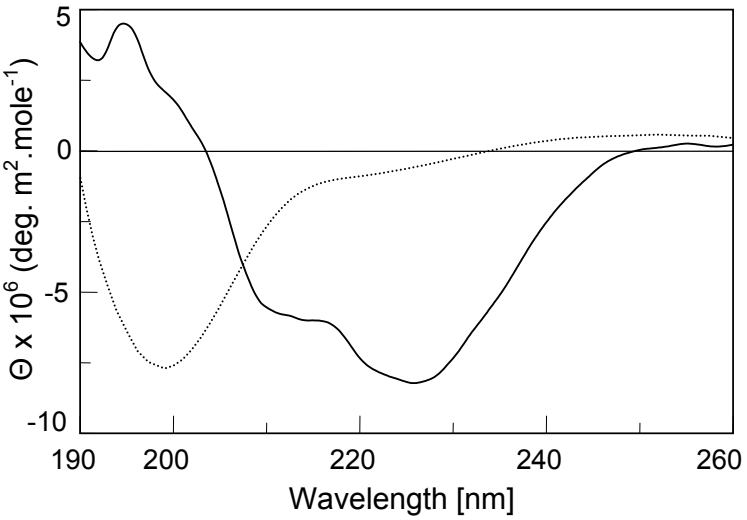



**Figure 4.2**

**scDNA induced α-helix in α-synuclein.** (...), α-synuclein (5μM) in 1 mM HEPES, pH 7.4; (–), α-synuclein +scDNA (10μg) incubated for ~30 days at $4^0$C. It was observed that scDNA induced α-helical conformation in α-synuclein.

### 4.3-2. Single strand circular (ssc)DNA induced α-helix in α-synuclein

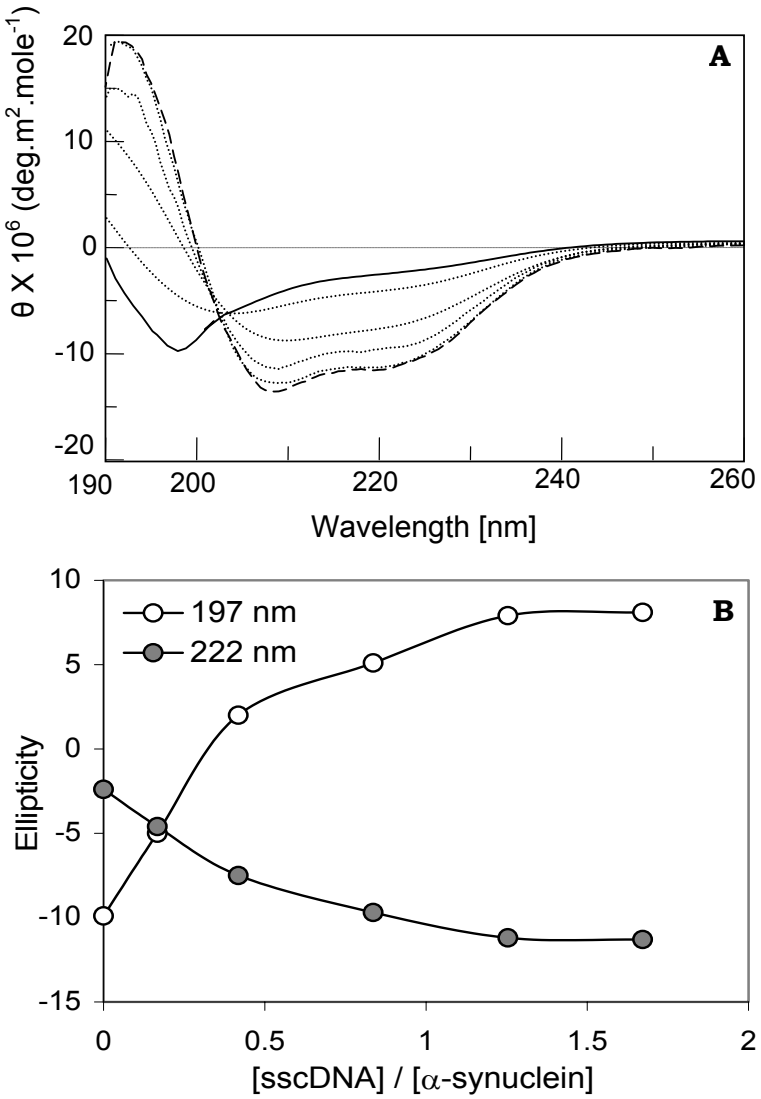



**Figure 4.3**

**Single strand circular (ssc) DNA-induced conformational changes (coil to helix) in α-synuclein.** The measurements were carried out at 25$^0$C.

**A, Far-UV CD spectra of α-synuclein as a function of sscDNA concentration.** (–), α-synuclein alone (10 μM); dotted lines (...), α-synuclein + sscDNA (1, 2.5, 5, 7.5 μM); (--), α-synuclein + sscDNA (10μM).

**B. Dependence of Fractional CD change with ratio of Concentration of sscDNA and α-synuclein.** Open circles-$[\theta]_{197}$ and closed circles-$[\theta]_{222}$.

The effect of sscDNA (non-supercoiled) binding to α-synuclein was observed by CD spectroscopy. The results revealed that sscDNA converts random coiled α-synuclein to a typical α-helix (Fig. 4.3). The percentage secondary structure content was calculated for the CD signal of α-synuclein-sscDNA complex using *j-fit* soft ware (Table 4.1). It was observed that on binding to sscDNA (1:1, synuclein-sscDNA molar ratio) the helix content increases from ~5% to 64%, while the random coil decreases from ~95% to 33%.

**Table 4.1**

**The percent secondary structural content of α-synuclein on binding to various concentrations of sscDNA.** Deconvolution of CD spectra of α-synuclein in the presence of DNA was carried out using *J-fit* software.

| sscDNA in μg | [sscDNA] / [α-synuclein] | coil | β-sheet | Helix |
|---|---|---|---|---|
| 0 | 0 | 94.60 | 0.06 | 4.80 |
| 1 | 0.17 | 76.89 | 2.97 | 12.73 |
| 2.5 | 0.42 | 54.24 | 2.02 | 44.74 |
| 5.0 | 0.84 | 38.52 | 3.46 | 58.01 |
| 7.5 | 1.25 | 33.45 | 4.20 | 62.10 |
| 10 | 1.67 | 33.10 | 3.15 | 63.75 |

We also studied the effect of double strand circular (dsc) DNA binding on the CD spectra of α-synuclein. The dscDNA-α-synuclein was incubated in 0.01M HEPES, pH 7.4, and incubated for 15min at room temperature. However, no conformational change was induced by dscDNA (10 μg) on α-synuclein (Fig. 4.4). In the previous chapter (Chapter 3) we have shown that α-synuclein induced DNA nicking activity is specific to sscDNA and the dscDNA was unaffected by α-

synuclein. The above results are in agreement with the previous results that α-synuclein has specificity for single stranded DNA.

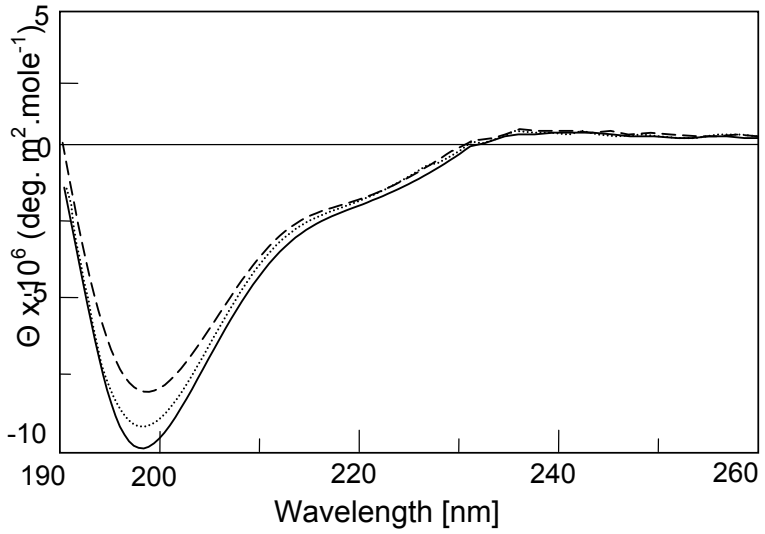



**Figure 4.4**

**CD spectra of dscDNA binding to α-synuclein.** (–), α-synuclein (10μg); (…), α-synuclein+ dscDNA (5μg); (---), α-synuclein + dscDNA (10μg). the dscDNA showed only binding to α-synuclein but no conformational folding was induced unlike sscDNA.

## 4.3-3. GC* and AT* specificity of DNA induced conformational transition of α-synuclein

In order to understand the GC and AT nucleotide sequence specificity of DNA binding to α-synuclein, we studied the interaction of α-synuclein with poly d(AT).(AT) and poly d(GC).d(GC). The study provided interesting insight on sequence specific binding affinity of DNA to α-synuclein. In case of poly d(GC).d(GC) a partially folded conformation was formed in natively random coiled α-synuclein (Fig.

4.5A), where as no such conformational transition was observed in case of poly d(AT).d(AT) binding to α-synuclein (Fig.4.5B). Both the DNA was allowed to interact with α-synuclein at room temperature for 15 min in 0.01 M HEPES buffer, pH 7.4.

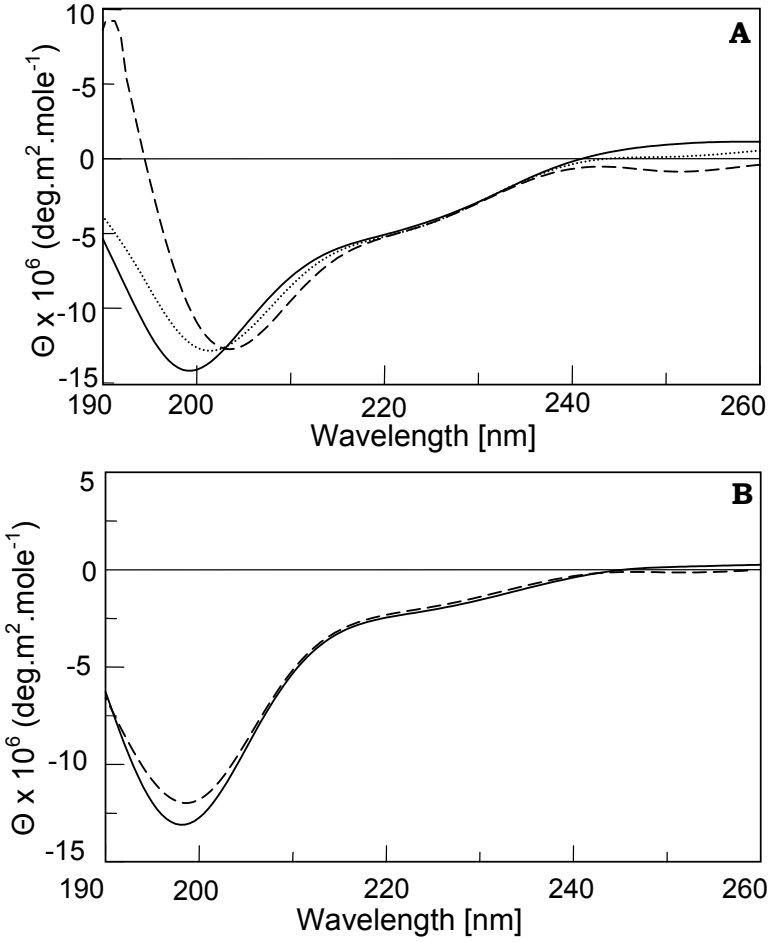



**Figure 4.5**

**The interaction of poly d(GC).d(GC) and poly d(AT).d(AT) with α-synuclein.** The DNA-α-synuclein solutions were allowed to interact for 15 min at room temperature.

**A, CD spectra of α-synuclein-poly d(GC).d(GC) complex.** (–), α-synuclein, 10 μM; (···), α-synuclein + 5μg poly d(GC).d(GC); (---), α-synuclein + 10μg poly d(GC).d(GC).

**B, CD spectra of α-synuclein-poly d(AT).d(AT) complex.** (–), α-synuclein, 10 μM; (---), α-synuclein + 10 μg poly d(AT).d(AT).

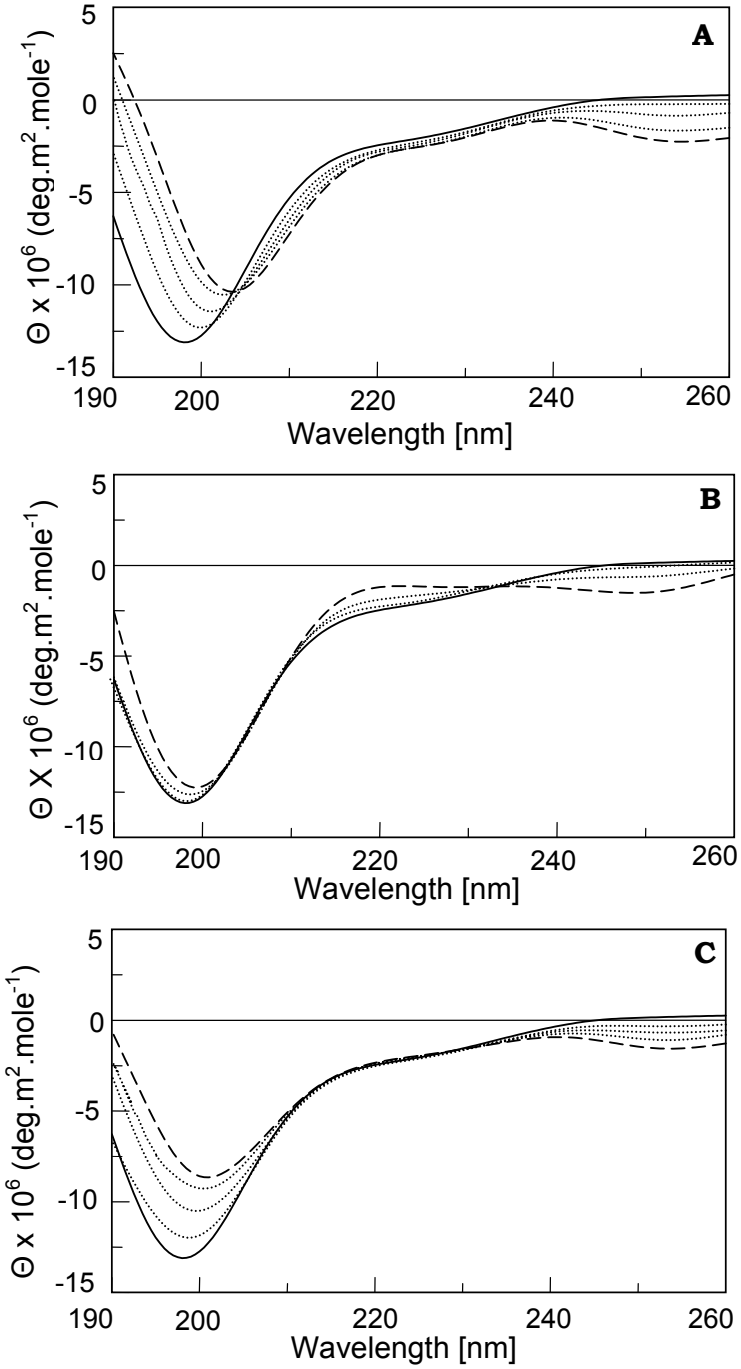



**Figure 4.6**

**Interactions of α-synuclein with GC, AT oligonucleotides.** A, CD spectra of d(GCGCGCGC) interactions with α-synuclein; B, CD spectra of d(ATATATAT) interactions with α-synuclein; C, CD spectra of d(GCATGCAT) interactions with α-synuclein. (—), α-synuclein alone, 10μM; (....), Complex spectra of α-synuclein with different concentrations (1.0, 2.5, 5.0, 7.5 μM for d(GCGCGCGC) and d(GCATGCAT) and 5 μM for d(ATATATAT). (---), α-synuclein + 10μM DNA oligonucleotide.

Further, the ability of GC and AT specific 8-mer oligonucleotides to induce conformational transition in α-synuclein was also studied using CD. It was observed that d(GCGCGCGC) induced a partial folding in α-synuclein (Fig. 4.6A), while d(ATATATAT) showed only binding but did not bring about any such conformational change (Fig. 4.6B). Interestingly, d(GCATGCAT) also induced a partial folding in α-synuclein (Fig. 4.6C). Closer examination of the CD data indicated that the folding induced by d(GCGCGCGC) was more in magnitude compared to d(GCATGCAT).

### 4.3-4. Interaction of Genomic DNA with α-synuclein

The effect of binding of lamda and Calf-thymus DNA on α-synuclein conformations was studied by CD experiments. It was observed that both these genomic DNA caused the formation of a partially folded structure in α-synuclein. However, the amount of folding induced by lamda DNA was more when compared to calf-thymus DNA (Fig. 4.7).

To understand the differential ability of calf-thymus and lamda DNA in inducing conformational transition in α-synuclein, the GC and AT nucleotide content of these DNA was correlated with their ability to induce folding. The GC content of calf-thymus DNA is ~70%, while for lamda DNA it is ~42%. We have shown previously that GC* specific DNA is more efficient in inducing conformational transition in α-synuclein compared to AT rich DNA.

The above CD studies showed that DNA binding, results in a conformational transition in α-synuclein. sscDNA and scDNA induced α-helix conformation, while genomic DNA and GC* specific DNA formed partially folded conformation, indicating a specificity for

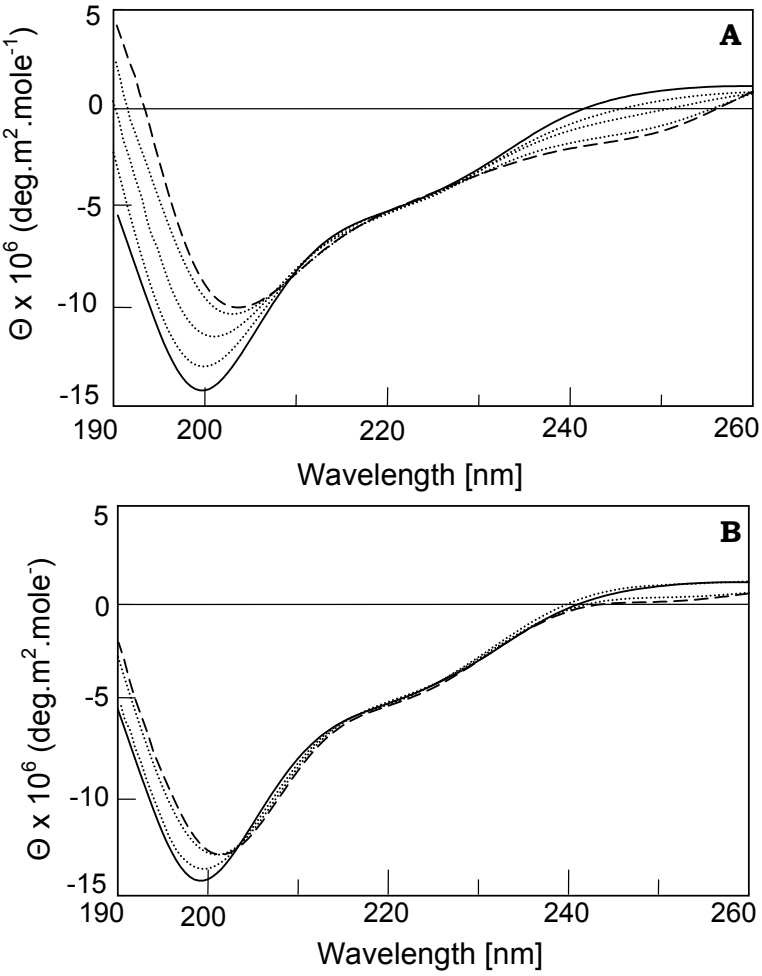



**Figure 4.7**

**Interaction α-synuclein with lamda DNA and Calf-thymus DNA.** Both lamda and Calf-thymus DNA induced partial folding in α-synuclein, however, the amount of ordered conformation induced by lamda DNA was higher compared to Calf-thymus DNA. The DNA-synuclein complexes are allowed to interact in 0.01 M HEPES, pH 7.4, for 15 min at room temperature and CD spectra were measured.

**A, Lamda DNA induces partial folding in α-synuclein.** (—)**,** α-synuclein, 10 μM; (….), α-synuclein + lamda DNA (1.0, 2.5 and 5.0μg respectively); (---), α-synuclein + 10μg lamda DNA.

**B, CD spectra of Calf-thymus DNA -α-synuclein interaction.** (—)**,** α-synuclein, 10 μM; (….), α-synuclein + Calf-thymus DNA (1.0 and 5.0μg respectively); (---), α-synuclein + 10 μg Calf-thymus DNA.

single stranded DNA and GC sequence in inducing folding in α-synuclein.

It appears that the DNA binding to α-synuclein is mediated through electrostatic interaction between negatively charged phosphate groups of DNA and the epsilon amino group of lysine aminoacids in α-synuclein. The DNA molecule is richly negatively charged on its surface as it is laced with phosphate groups, where as α-synuclein has 15 basic lysine residues which are mostly clustered in the N-terminal of its sequence. The neutralization of basic charge on epsilon amino group side chain of lysine residues will reduce the repulsion between the like charges in the N-terminal end of α-synuclein and this appears to be the driving force in inducing DNA mediated folding in the protein. Studies have shown that the N-terminal half of α-synuclein sequence has a very high propensity to form ordered conformation [Chandra et al, 2003].

To understand the mechanism of DNA binding and DNA induced folding in α-synuclein, we studied the effect of naturally occurring osmolytes on α-synuclein folding dynamics.

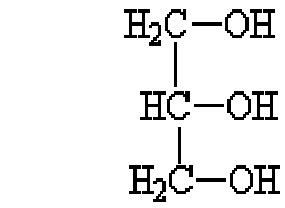
### 4.3-5. Role of Naturally occurring Osmolytes in inducing partial folding in α-synuclein

Osmolytes are molecules used in nature to protect organisms against stresses at high osmotic pressure. These compounds have also been found to stabilize the native state of proteins relative to the unfolded state [Ratnaparkhi and Varadarajan, 2001]. The mechanism of osmolytes triggered stabilization of protein has been studied in some detail [Arakawa and Timasheff, 1985; Pino and

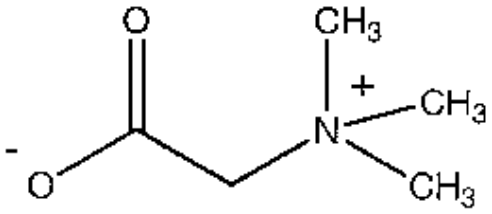
Sanchez-Ruiz, 1995; Liu and Bolen, 1995] and it is believed to result primarily from an unfavorable free energy of interaction between the osmolytes and the unfolded state of the protein [Xie and Timashef, 1997]. Proteins retain activity in the presence of osmolytes suggesting that the native state structure and dynamics are not greatly perturbed.

The main classes of osmolytes are sugars, methyl ammonium derivatives, polyhydric alcohols, and amino acids and their derivatives [Yancy et al, 1982; Ratnaparkhi and Varadarajan, 2001]. Molar concentrations of all the above classes of molecules have been shown to stabilize proteins.

The observation that α-synuclein and other amyloid peptides are generated within intracellular compartments, including endoplasmic reticulum, which is the quality control site for protein folding, has prompted us to investigate the role of organic chaperones in α-synuclein folding pathway [Yang et al, 1999]. These molecules have been shown to induce protein folding through preferential hydration of exposed polypeptide backbone and side chains of partially unfolded structures [Burg, 1995; Wang and Bolen, 1997]. Hydration effects are equally important in protein polymerization or aggregation where osmolytes are excluded through increased protein-protein interactions [Sackett, 1997]. We have used five osmolytes namely glycerol, Betaine, Taurine, Sarcosine, TMAO to understand the initiation of α-synuclein folding from random coil – to – β-sheet conformation resulting in aggregation and also to understand the DNA induced folding of α-synuclein.



**Glycerol**



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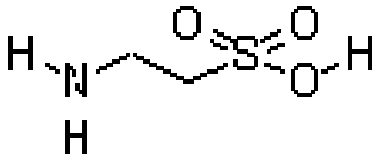
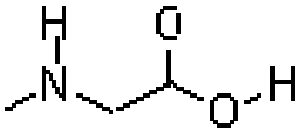
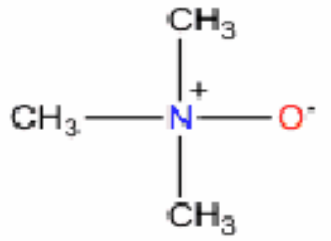


**Taurine**



**Sarcosine**



**TMAO**

**Figure 4.8**
**Chemical structure of naturally occurring osmolytes used in the present study.**

### 4.3-6. CD Spectroscopic studies on the osmolyte induced conformational changes in α-synuclein

The effect of Osmolytes, Glycerol, Betaine, Taurine, Sarcosine, and TMAO, (all from Sigma) on α-synuclein secondary conformation were determined by CD. The protein was diluted to a final concentration of 5 μM in HEPES, pH 7.4. The protein solutions were combined with $1x10^{-6}$ M to 1.0 M Betaine, Taurine, Sarcosine, and TMAO and 1.0-50% by volume of Glycerol. Spectra were collected after 15min of equilibrium period at room temperature.

The α-Synuclein dissolved in HEPES, pH 7.4 exhibited a random coil conformation indicative of an un-ordered secondary structure. The self-aggregation of α-synuclein involving conformational changes from random coil to beta sheet and subsequent fibril formation takes from 25-30 days, depending on the incubation conditions. In contrast, adjusting the protein solution to 25-50% v/v Glycerol, and 0.1-1.0 M Betaine and TMAO resulted in an immediate partial folding of the protein towards an ordered structure (Fig.4.9). However, Taurine and Sarcosine did not affect the CD signal of α-synuclein.

### 4.3-7. α-Synuclein Folding analysis using Intrinsic Fluorescence Measurements

The effect of osmolytes Glycerol, Betaine, TMAO, Taurine, and Sarcosine, on α-synuclein conformation were also studied using Tyrosine intrinsic fluorescence of α-synuclein. Tyrosine fluorescence was excited at 280nm and emission monitored in the range from 290 to 450nm. Protein concentrations for intrinsic fluorescence were kept at 2 μM at pH 7.4, in 0.01M HEPES buffer.

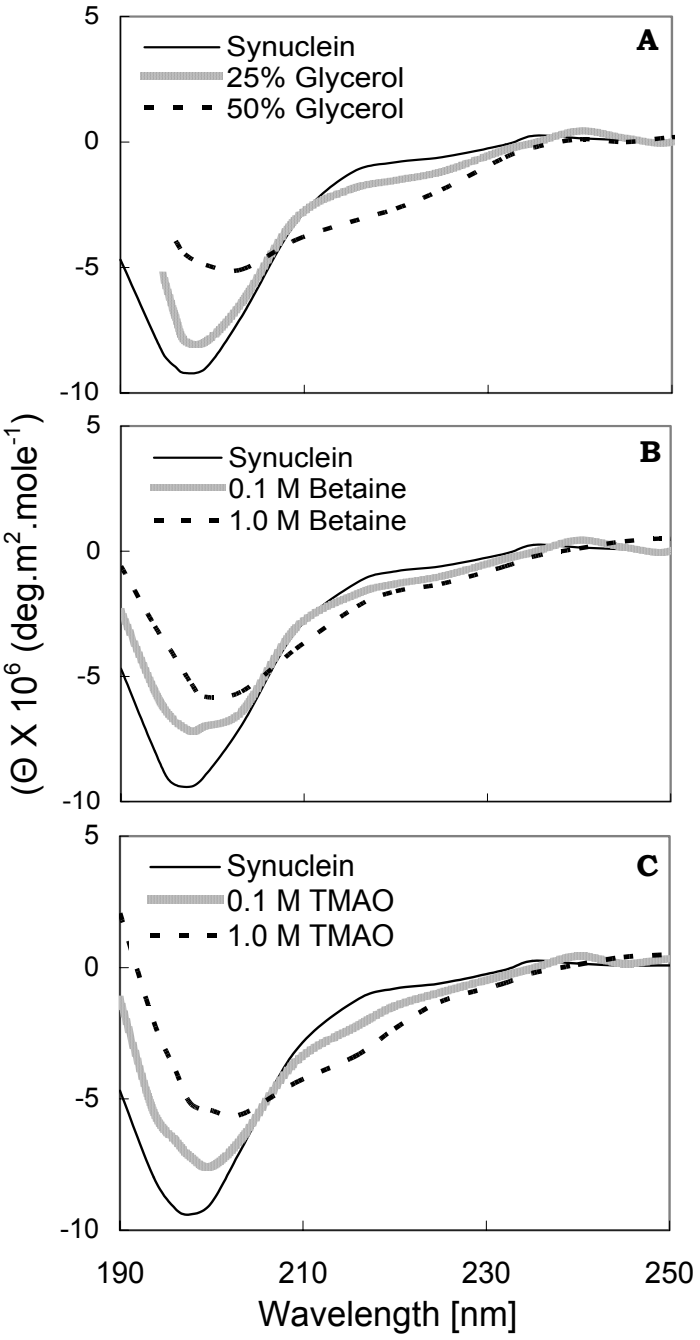



**Figure 4.9**

**CD spectra of α-synuclein in presence of osmolytes: A, glycerol; B, Betaine; and C, TMAO.** (—), α-synuclein, 10μM; (....), α-synuclein + 25% glycerol or 0.1M Betaine/ TMAO; and (---), α-synuclein + 50% glycerol or 1.0M Betaine/ TMAO. The results demonstrate immediate conversion of α-synuclein conformation from random coil to partially folded conformation by glycerol, betaine and TMAO.

The effects of different osmolytes on the intrinsic tyrosine fluorescence of α-synuclein varied significantly. Among the 5 osmolytes studied, 4 osmolytes (Betaine, Taurine and TMAO) quench the protein fluorescence emission, whereas glycerol enhanced the tyrosine fluorescence (Fig. 4.10). It is important to note that there are no tryptophan residues in α-synuclein. The fluorescence emission spectra also show that the osmolytes differ both in the amplitude of the induced spectral changes and in the efficiency in inducing protein folding.

Interestingly, the pattern of fluorescence changes with Betaine, Taurine, TMAO and glycerol was exponential while Sarcosine decreased the tyrosine fluorescence of α-synuclein in a linear manner against the concentration of osmolyte.

To confirm that the osmolyte induced fluorescence change in α-synuclein is associated with changes in the protein conformation or folding and not just direct fluorescence quenching of tyrosine, we investigated the effect of these osmolytes on the fluorescence of free L-tyrosine. It was found that all the osmolytes studied have much less pronounced effect on the fluorescence of free L-tyrosine compared to the intrinsic fluorescence of α-synuclein. In fact, the fluorescence intensity of L-tyrosine was not affected at all in the presence of Taurine, which decreased the intrinsic fluorescence of α-synuclein quite significantly. The figures shown below indicate that Sarcosine, Betaine, Glycerol and TMAO quenched L-tyrosine fluorescence to a much smaller extent than the intrinsic fluorescence of α-synuclein. It is also interesting to note that the quenching of free tyrosine fluorescence by osmolytes shows linear relationship, indicating that the process involves only collisional or dynamic quenching.

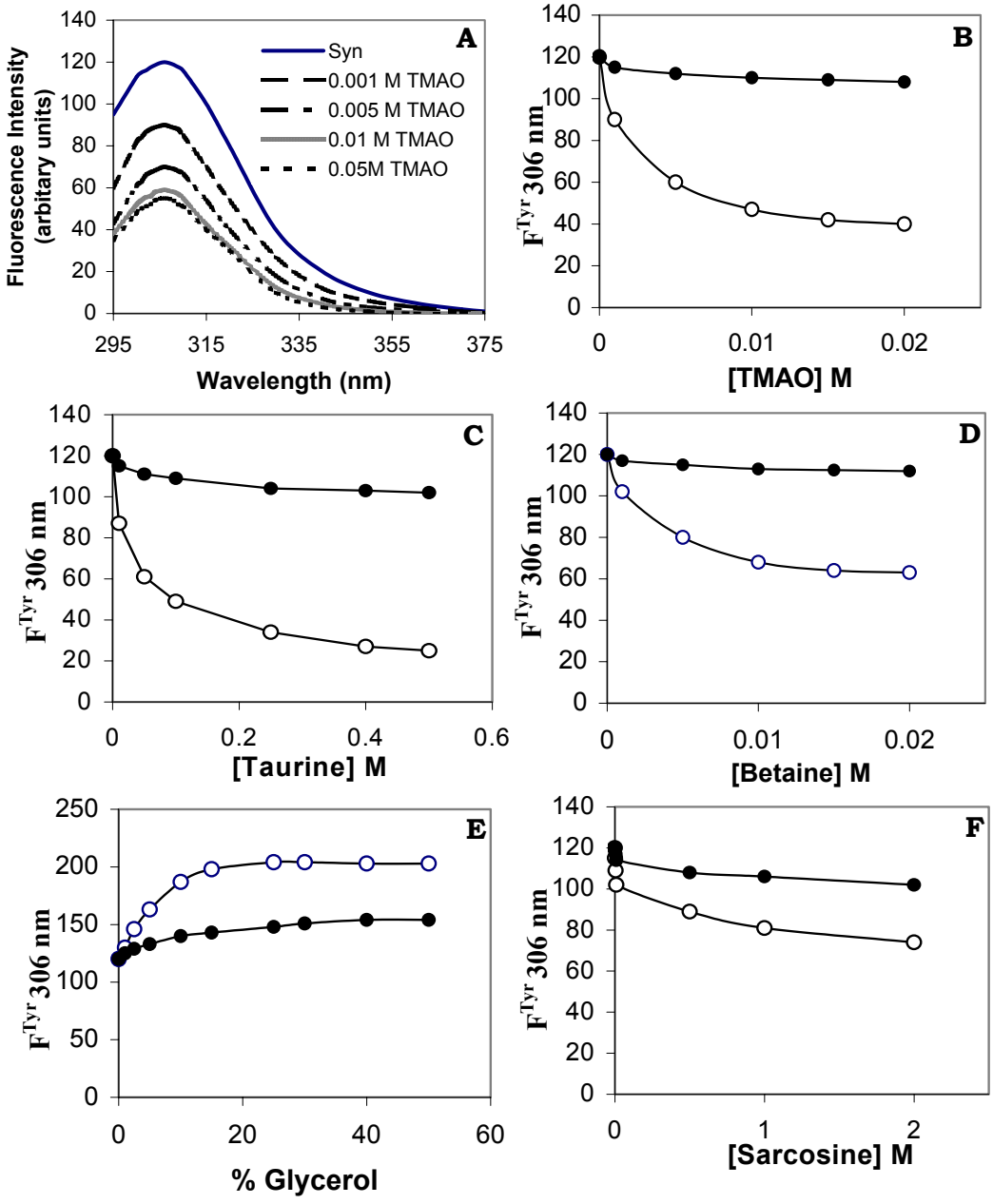



**Figure 4.10**

**The effect of osmolytes on α-synuclein intrinsic fluorescence.** (•), free L-tyrosine; (o), α-synuclein. A, Effect of increasing concentrations of TMAO on tyrosine intrinsic fluorescence of α-synuclein, excitation at 280nm, emission at 306 nm (295-375 nm). B to F, effect of TMAO, Taurine, Betaine, Glycerol and Sarcosine on α-synuclein intrinsic fluorescence and their comparison to free L-tyrosine. α-synuclein concentrations were kept at 2.0 μM.

However, major deviations from linear dependence are observed in the case of intrinsic fluorescence of α-synuclein. These observations indicate that the osmolytes under study induce structural reorganization of α-synuclein in the form of ordered folding.

### 4.3-8. ANS binding

Changes in ANS fluorescence are characteristic of the interaction of this dye with solvent – exposed hydrophobic surfaces of partially folded proteins [Semisotnov et al, 1991]. Fig. 4.11 represents the ANS fluorescence of α-synuclein in the absence/ presence of osmolytes. The results revealed that among the osmolytes used, Glycerol, TMAO, Betaine and Taurine enhanced the ANS fluorescence in the same order (Fig. 4.11).

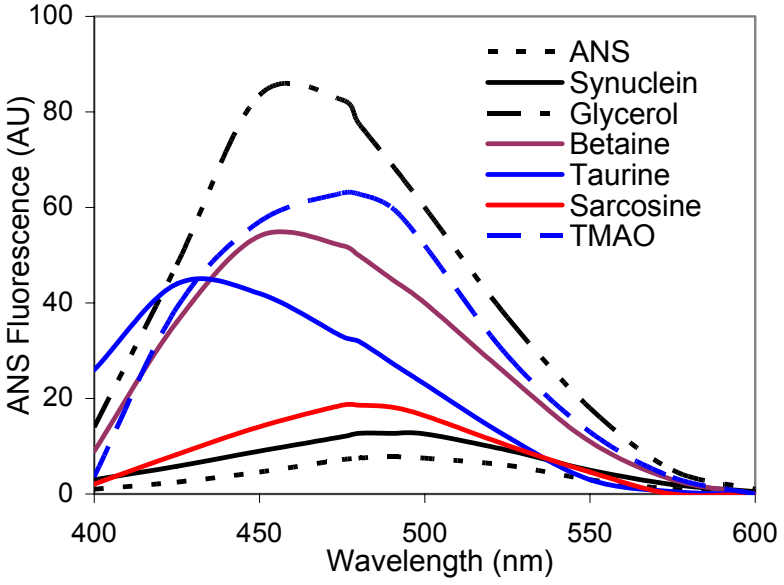



**Figure 4.11**

**Effect of osmolytes on ANS fluorescence of α-synuclein.** ANS Fluorescence spectra measured for free dye (*dotted line*) and in the presence of 5 μM α-synuclein and 50% v/v glycerol, 0.5 M Taurine, 1 M each of TMAO, Betaine and Sarcosine.

Interestingly, all these four osmolytes induced a blue shift (~30 to ~65nm) of ANS $\lambda_{max}$.

This means that in the presence of glycerol, TMAO, betaine and taurine, natively unfolded α-synuclein is converted into a partially folded conformation with solvent exposed hydrophobic patches. Glycerol, taurine and betaine did not affect the ANS fluorescence emission in the absence of protein. Sarcosine did not show any change in ANS $\lambda_{max}$ even in the absence of α-synuclein, indicating no conformational change in α-synuclein.

### 4.3-9. Acrylamide mediated fluorescence quenching studies

Further evidence for the osmolytes induced changes in the environment of tyrosine residues in α-synuclein has been studied by acrylamide induced tyrosine fluorescence quenching. Information on the relative solvent exposure of tyrosine residues (there are no tryptophans in α-synuclein) can be obtained from analysis of the effect of quencher molecules such as acrylamide [Eftink and Ghiron, 1981]. Fig. 4.12 represents the ***Stern-Volmer plots*** for α-synuclein and α-synuclein-osmolyte complexes. The upward curvature indicates the presence of both dynamic and static quenching [Eftink and Ghiron, 1981]. The results showed that in the absence of osmolytes, the Ksv for α-synuclein is smaller than the value for free L-tyrosine. This indicates that the tyrosine residues of natively unfolded α-synuclein are partially protected from quencher molecules. In the presence of osmolytes the Ksv value decreases and in turn, the degree of protection increases, in the following order: α-synuclein → α-synuclein + glycerol; → α-synuclein + taurine; → α-synuclein + sarcosine; → α-synuclein + TMAO; → α-synuclein + betaine.

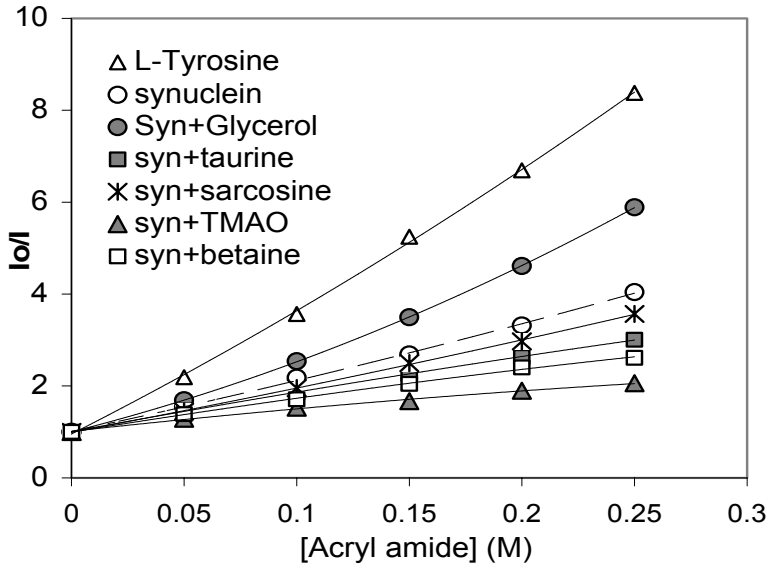



**Figure 4.12**

**Stern-Volmer plots for Acrylamide mediated tyrosine fluorescence quenching of α-synuclein in presence of osmolytes.** Synuclein (○), Free L-tyrosine (Δ), Glycerol (●), taurine (■), Betaine (ж), TMAO (▲), sarcosine (ĺ). The conditions were as described under 'experimental procedures'.

## 4.3-10. Effect of osmolytes on Tryptic digestion resistance of α-synuclein

α-synuclein appears to be sensitive to trypsin digestion in its native conformation as it contains 15 lysine residues in its sequence. We have used proteolytic (tryptic) cleavage of α-synuclein as a probe of protein conformation by looking at the sensitivity of α-synuclein in the presence/ absence of osmolytes. It was observed that a partial protection to tryptic action was shown by α-synuclein in the presence of osmolytes when compared to α-synuclein alone (Fig. 4.13). The restricted trypsin digestion of α-synuclein in the presence of osmolytes, along with CD and fluorescence data confirms that α-synuclein adopts a folded conformation in the presence of osmolytes.

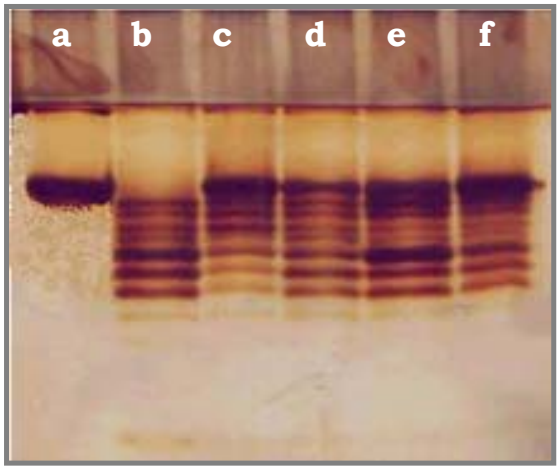



**Figure 4.13**

**Effect of osmolytes on tryptic cleavage of α-synuclein.** a, α-synuclein alone (4 μg); b to f, α-synuclein subjected to limited trypsin treatment (Detailed methodology given in 'experimental procedures') in the absence (b) and presence of 50 % v/v glycerol (c); 1M TMAO (d); 1M Betaine (e) and 0.5 M Taurine (f). The samples were electrophoresed on tricine-SDS-polyacrylamide gel and photographed after silver staining. α-synuclein showed restricted tryptic digestion in the presence of osmolytes.

## 4.3-11. Effect of presence of DNA and osmolytes on α-synuclein fibril formation

Previous studies have shown that the transformation of α-synuclein into a partially folded conformation (induced by pH or temperature or metal ions) is strongly correlated with the enhanced formation of α-synuclein fibrils [Uversky et al, 2001a; Uversky et al, 2001b]. In this context, we analyzed the aggregation propensity of α-synuclein in the presence of osmolytes which induce partially folded conformation and also in the presence of DNA which induce α-helix. In agreement with the results of Uversky et al, [2001a; 2001b], Fig. 4.14 showed that osmolytes which induced partial folding in

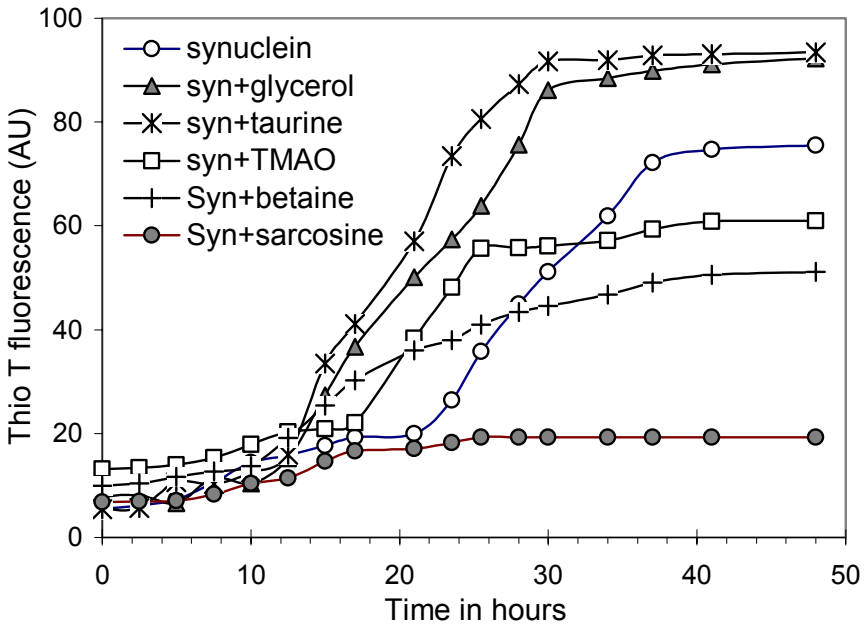



**Figure 4.14**

**Effect of osmolytes on fibrillation of α-synuclein.** The kinetics of α-synuclein was monitored by Thio T fluorescence. The symbols represent Thio T fluorescence intensities determined experimentally. The α-synuclein concentrations were 50 μM in 0.01 M Tris Cl buffer, pH 7.4.

α-synuclein resulted in a very substantial acceleration of the kinetics of aggregation. A shorter lag time and a larger rate of fibril formation were observed in presence of osmolytes when compared to α-synuclein alone.

However, sscDNA which formed α-helix conformation in α-synuclein (Fig. 4.3), delayed the aggregation by nearly ~25 hrs (Fig. 4.15). For α-synuclein alone, a lag time of ~18-20 hrs was observed before the aggregation started as indicated by the increase in the Thio T fluorescence emission which it reached a limiting value after 37 hrs. In the presence of sscDNA (0.13 μM), the kinetics of fibrillation was much slower, reflected in the increase in lag time for

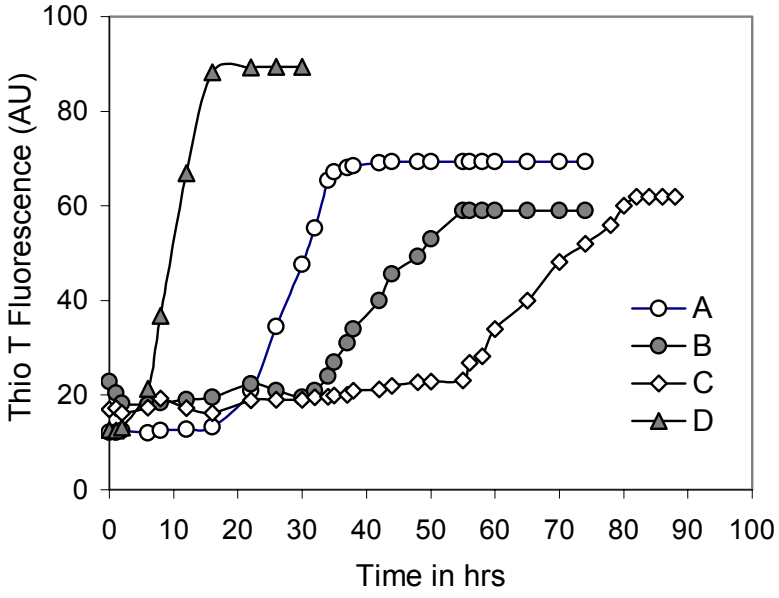



**Figure 4.15**

**Modulation of α-synuclein aggregation by DNA**. Variation of flourescence emission intensity of Thio T at 482 nm for α-synuclein incubated in the absence (A) and presence of pUC 18 scDNA (B); single stranded circular DNA (C); d(GCGCGCGC) (D). The DNA: α-synuclein molar ratio was 0.13 μM : 50 μM. The excitation wavelength was 450 nm. A significant increase in the lag time of aggregation of ~10 hrs was observed for synuclein in the presence of DNA.

the Thio T intensity signal. Also the time it took for reaching the limiting value (complete fibril formation was much greater compared to synuclein alone). This indicates that sscDNA inhibits synuclein fibril formation to a considerable extent. The observations were confirmed repeating the experiment three times, where similar trend was observed. scDNA also delayed the α-synuclein aggregation by ~10 hrs. However, the results obtained with scDNA were not very consistent, the lag-time varying between 20-30hrs. The d(GCGCGCGC) DNA which induced a partially folded intermediate conformation in α-synuclein (Fig. 4.6), caused significant enhancement in α-synuclein aggregation kinetics (Fig. 4.15).

Similar observations were made by Uversky et al, [2001c], where they showed that TMAO induces a partial folding and acceleration of fibrillization in α-synuclein at low concentrations, where as, at high concentrations causes the formation of α-helix conformation and inhibits aggregation to a considerable extent. Our results are in agreement with Uversky et al, [2001c]. Hence, it appears that a partially folded intermediate conformation is a very critical step in α-synuclein aggregation pathway.

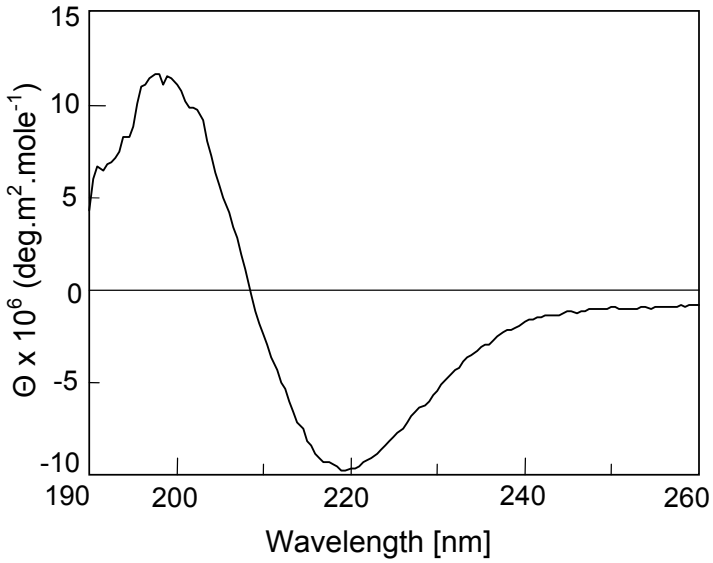



**Figure 4.16**
**CD spectra of aggregated α-synuclein indicating β-sheet secondary conformation.**

The α-synuclein aggregation is associated with a conformational transition from random coil to β-sheet secondary structure. Hence, we confirmed the formation of β-sheet conformation in α-synuclein in the presence of osmolytes/ DNA once the Thio T fluorescence reached a limiting value. The CD spectra of

representative aggregated α-synuclein showing β-sheet secondary structure is represented in Fig. 4.16.

### 4.3-12. TEM study of α-synuclein aggregates

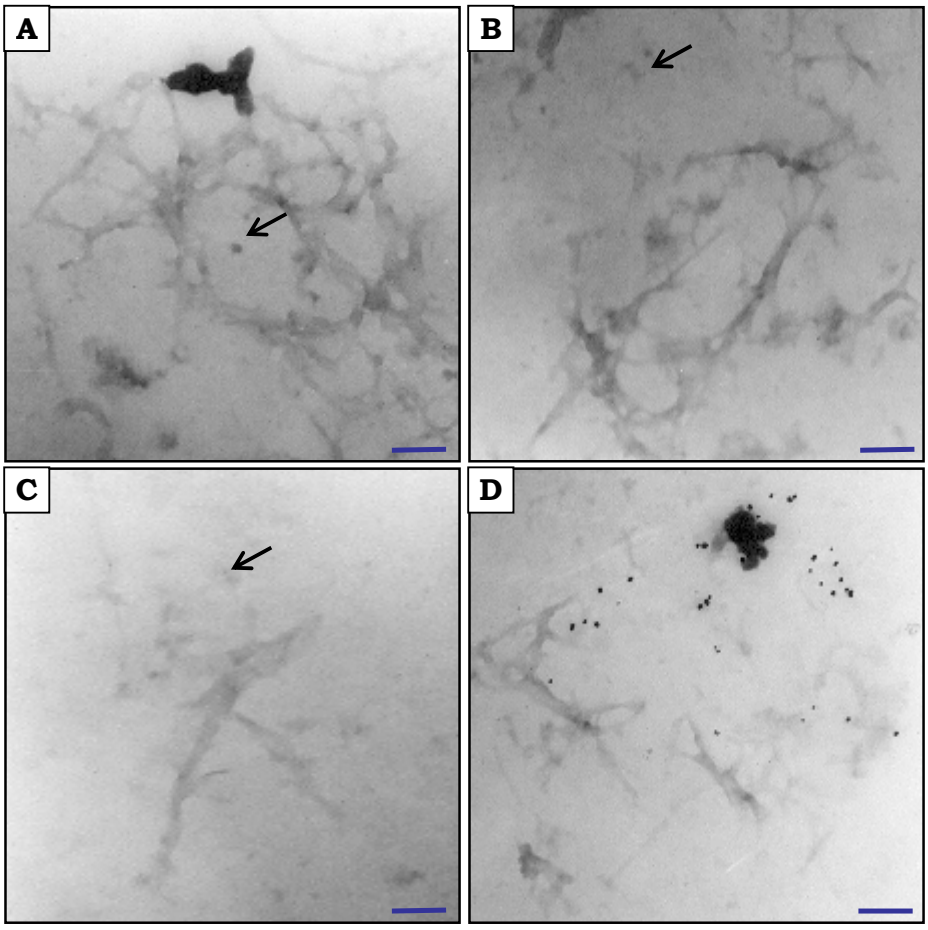



**Figure 4.17**

**Negatively stained transmission electron micrographs of fibrils formed from α-synuclein.** The protein samples were applied on carbon-coated Formver grids, and allowed to dry in air. After staining for 2 min with 2% uranyl acetate, samples were observed under a JEOL 1010 transmission electron microscope. Magnification: scale bar, 100 nm. A-D represent fibrils observed at different regions in the EM stage.

The structures of α-synuclein aggregates formed upon incubation at $37^0$C with stirring using micro-magnetic bars were examined by TEM. The aggregates were seen as long, often forming large networks, and distributed rather uniformly on the surface of the carbon film (Fig. 4.17). Some small aggregates of variable size (more or like amorphous) were also seen in the image (Fig. 4.17, see arrow), however, the aggregates were predominantly fibrillar. The structure of α-synuclein aggregates/ fibrils were qualitatively similar in the presence or absence of DNA (See Chapter 3, Fig. 3. 24 and 3.25) or osmolytes. This could be because, the images were taken when the fibrillization was complete as indicated by the maximum Thio T fluorescence (Fig. 4.14 and 4.15).

## 4.4 DISCUSSION

α-Synuclein is a natively unfolded protein with little or no ordered structure under physiological conditions. At neutral pH, it is calculated to have 24 negative charges (15 of which are localized in the last third of the protein sequence), leading to a strong electrostatic repulsion, which hinders the folding of α-synuclein [Uversky et al, 2001b]. As previously reported [Weinreb et al, 1996; and Uversky et al, 2001a], α-synuclein at neutral pH has a far-UV-CD spectrum typical of an unfolded polypeptide chain, and reflecting the lack of ordered secondary conformation under these conditions. This raises the question of how an essentially disordered protein is transformed into highly organized fibrils in Parkinson's brain?. In the search for an answer to this question, we have investigated the effects of DNA and osmolyte binding on the conformational properties of α-synuclein. Aggregation or self-association is a characteristic property of a partially folded (denatured) proteins and most

aggregating protein systems probably involve a transient partially folded intermediate as the key precursor of fibrillation [Wetzel, 1996; Fink, 1995]. It has also been shown that in some cases the self-association induces additional structure and stability in the partially folded intermediates.

The natively unfolded character of α-synuclein arises from its low intrinsic hydrophobicity and high net charge at neutral pH (pI 4.7) [Uversky et al, 2000]. Thus the conditions that decrease the net charge and that increase the hydrophobicity would be expected to result in partial folding. Osmolytes provide such conditions by affecting the hydration properties at the protein surface [Liu and Bolen, 1995; Santoro et al, 1992]. In the present study, the shape and intensity of the far-UV-CD and intrinsic fluorescence spectra change significantly with an increase in the osmolyte concentration, indicating that the conformation of natively unfolded α-synuclein is significantly affected by interaction with the osmolytes, which induce secondary conformation in synuclein. This process is accompanied by an increase in $[\theta]_{220}$ (negative CD intensity) and change in the intrinsic fluorescence at 305nm. These changes are attributed to the osmolyte-induced changes in the environment of tyrosine residues, possibly due to their solvent accessibility because of partial folding of the protein.

It has been shown previously that [Uversky et al, 2001a] either an increase in temperature or a decrease in pH level rapidly transformed natively unfolded α-synuclein into a partially folded conformation. Moreover, the same study showed a tight correlation between the increase of this intermediate and the enhanced formation of α-synuclein fibrils. Interestingly, in the present study, the magnitude of the osmolyte mediated structural perturbations, as

monitored by CD, is comparable with that induced by low pH or high temperature [Uversky et al, 2001a]. Thus, natively unfolded α-synuclein adopts a partially folded conformation even at neutral pH in the presence of osmolytes. This partially folded conformation is characterized by an increased amount of ordered secondary structure and by the appearance of solvent accessible hydrophobic clusters. In agreement with Uversky et al, [2001a], our results showed that the osmolytes-induced partial folding in α-synuclein is associated with enhanced assembly of α-synuclein into fibrils.

Munishkina et al, [2003] observed that organic solvents (simple alcohols, trifluoroethanol (TFE) etc) cause natively unfolded α-synuclein to fold in a multiphasic manner, the mechanism of such folding differing for different solvents. However, all the solvents lead to the formation of a previously described partially folded intermediate [Uversky et al, 2001a; Uversky et al, 2001b; Uversky et al, 2001c; Manning-Bog et al, 2002]. The subsequent fate of the partially folded intermediate was dependent on the nature of the solvent: simple alcohols and moderate concentrations of TFE induced β-structure-enriched oligomers, where as high concentrations of fluoro alcohols gave rise to α-helical conformation. It was further shown that under conditions where this partially folded intermediate is populated, α-synuclein fibrillation is significantly faster, indicating that the partially folded intermediate is a critical species in the α-synuclein fibrillation pathway [Munishkina et al, 2003]. Our results on DNA and osmolytes induced conformations in α-synuclein is in agreement with Munishkina et al, [2003] as far as the fibrillation propensity of partially folded intermediate is concerned.

Importantly, the strong correlation observed between the degree of protein folding and its efficiency of fibril formation suggests

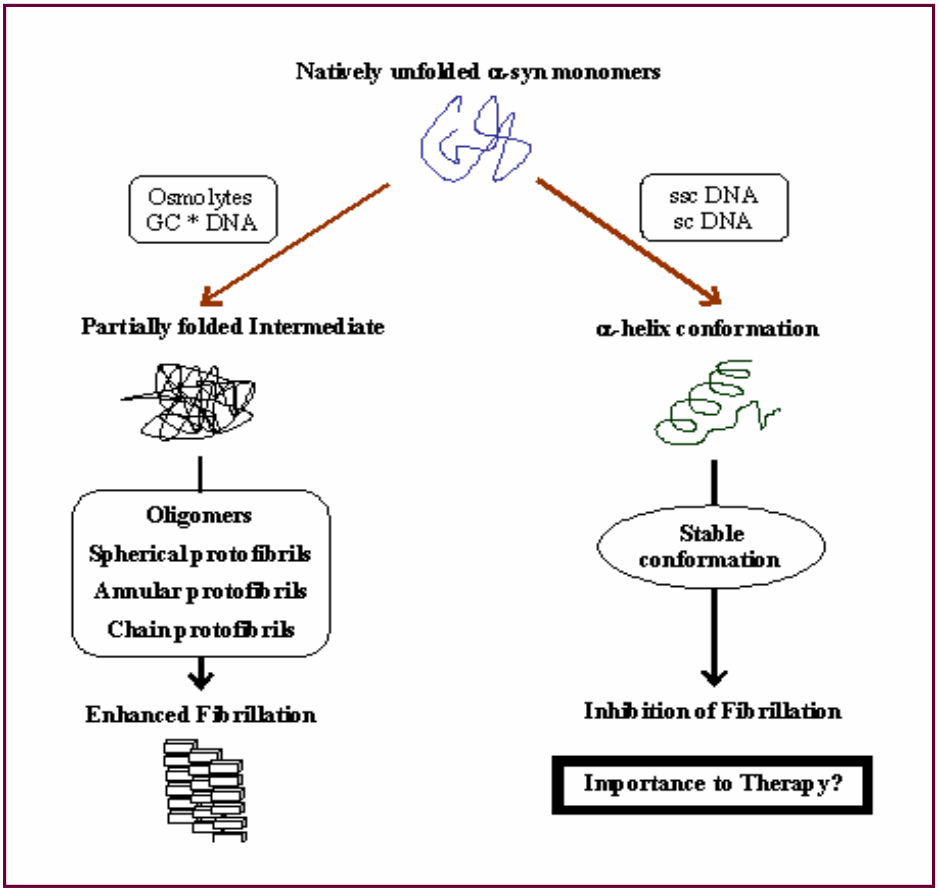



**Figure 4.18**

**Schematic representation of DNA and osmolytes induced conformational changes and fibrillation of α-synuclein.** Legends: sscDNA, single strand circular DNA; scDNA, supercoiled DNA.

that this intermediate partially folded form can be a precursor of fibrils [Uversky et al, 2001a].This is because, in contrast to an unfolded protein, a partially folded intermediate is anticipated to have contiguous hydrophobic patches on its surface (also indicated by ANS binding studies), which are likely to foster self-association and hence potentially fibrillation. Since hydrophobic interactions

increase with increase in osmolyte concentration, the simplest explanation for the osmolyte-induced formation of aggregation competent intermediate (confirmed by Thio T binding) is due to increased hydrophobic interactions.

There are two interesting features of the α-synuclein partially folded intermediate: It has some β-structure, which is the major type of secondary structure in α-synuclein fibrils, and it is relatively unfolded (i.e. more similar to random-coil conformation than a native tightly folded globular conformation). Khurana et al, [2001] have recently observed that a partially folded intermediate of IgG light chains that form amyloid fibrils is relatively unfolded and they have also predicted that it could be a common feature in amyloid fibril formation that the critical monomeric partially folded intermediate is relatively unfolded.

The question arises as to the physiological significance of the observation of a partially folded intermediate of α-synuclein formed with osmolytes. Clearly, these particular conditions will not be found in the dopaminergic cells of potential PD patients. However, the existence of such an intermediate on the pathway to fibrils means that *in vivo* conditions that lead to population of the intermediate will lead to increased risk of the disease. Thus, intracellular factors that lead to a shift in the equilibrium position between the natively unfolded state and the partially folded intermediate will increase the likelihood of α-synuclein fibril formation. Such factors could include relatively non-polar molecules that would preferentially bind to the partially folded intermediate.

The ability of DNA and osmolytes in inducing conformational transition in α-synuclein, indicates that two factors are critical in

modulating α-synuclein folding: (i) Electrostatic interaction as in the case of DNA, and (ii) Hydrophobic interactions as in the case of osmolytes.

The physiological significance of DNA induced α-synuclein conformation and modulation of its assembly/ fibrillation is unclear at the present time. However, several studies have shown the presence of α-synuclein into neuronal nuclei [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanji et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004]. Recently it was shown that α-synuclein is present in neuronal nuclear inclusions and neuritis in multiple system atrophy [Lin et al, 2004]. Goers et al, [2003] provided evidence for the co-localization of α-synuclein with histones in the nuclei of nigral neurons from mice exposed to a toxic insult. It was further shown that the stoichiometry of the histone: α-synuclein complex was 2:1 and histones stimulate α-synuclein fibrillation *in vitro* [Goers et al, 2003]. A more recent study on interaction of double stranded DNA with α-synuclein and its ability to enhance the fibrillation of α-synuclein revealed that both the components of chromatin, histone and DNA may have a significant effect on nuclear translocated α-synuclein functioning [Cherny et al, 2004]. The authors proposed that α-synuclein may interact with histone-free, transcriptionally active DNA segments and hence may lead to a decreased transcriptional activity of some genes responding to environmental stimuli. These results along with our present study reveal that the interactions of α-synuclein with DNA and histones may function to regulate gene expressions.

The possible mechanisms of double stranded DNA promoting α-synuclein fibrillation has been proposed recently by Cherny et al,

[2004]. The authors also observed that neuronal nuclear inclusions potentially account for a significant fraction of the total amount of α-synuclein in a cell. Hence, minute variations in local α-synuclein concentrations or the presence of factors enhancing its fibrillation, e.g. DNA or histones, may stimulate significantly the aggregation of α-synuclein into fibrillar structures. It was further proposed that effective mechanisms preventing unnecessary or occasional conversion of a soluble α-synuclein into insoluble isoforms must exist in both cytoplasm and nucleus [Cherny et al, 2004]. However, in the present study, we provide a comprehensive picture of DNA binding effect on α-synuclein fibrillation using different DNAs such as double and single stranded DNA, AT and GC sequence specific DNA of different sizes etc and showed that only those DNA which induce a partial folding in α-synuclein (GC* rich DNA) promote its aggregation, while, sscDNA forms α-helix conformation in α-synuclein and also inhibit aggregation to a considerable extent (Fig. 4.18). Hence, we feel that extrapolation of *in vitro* results on DNA binding property of α-synuclein to *in vivo* system in PD has to be more cautiously done.

However, we cannot ignore the significance of DNA binding property of α-synuclein as a non-specific event. It is because, amyloid β peptides (Aβ), tau and prion proteins implicated in AD and prion diseases have also been shown to have DNA binding property [Ahn et al, 2000; Jang and Surh, 2002; Kampers et al, 1996; Nandi and Leclerc, 1999; Nandi, 1998; Cordeiro et al, 2001; Gabus et al, 2001a; 2001b]. Nucleic acids was shown to induce structural changes in prion peptides by forming stable complexes, which catalyzed further polymerization [Nandi et al, 2002; Cordeiro et al, 2001]. Association of Aβ(1-40) and Aβ(25-35) with double stranded DNA was detected [Ahn et al, 2000]. Aβ(25-35) was shown to cause

formation of open circular DNA from scDNA in presence of ferrous ions [Jang and Surh, 2002]. We have recently observed binding of Aβ(1-42) and Aβ(1-16) peptides with scDNA and their ability to convert scDNA into open circular form [Hegde et al, 2004a; Hegde et al, 2004b].

From the above evidences, it appears that the DNA binding is a unifying property of amyloidogenic peptides (α-synuclein, Aβ, tau, prions) implicated in various neurodegenerative disorders. Hence, we feel that further multiple systematic approaches including *in vivo* studies using animal models and cell cultures are required to understand the implications of these *in vitro* evidences on DNA binding properties of α-synuclein and other amyloidogenic peptides. Further, we feel that the property of sscDNA in inducing α-helical conformation in α-synuclein and inhibiting the fibrillation may be of significance in engineering DNA-chip based therapeutic approaches to PD and other amyloid disorders.

# CHAPTER 5

# *Serum trace elemental levels and the complexity of inter-elemental relationships in patients with PD*

## 5.1 Introduction

Trace elements in optimum biological levels are required for a number of metabolic and physiological processes in human body. Elements like Na, K, Mg, Ca and P serve as structural components of tissues, as constituents of the body fluids and therefore, are essential for the functioning of cells [Mertz, 1981]. Imbalance in the optimum levels of these elements along with other trace metals such as Fe, Cu, Zn, Al etc. may adversely affect the biological activity and are associated with many neurological diseases [Strong et al, 1994; Lai et al, 2002; Richardson, 2004; Zecca et al, 2004a; 2004b; Gotz et al, 2004].

The pathogenesis of neuronal degeneration in the pars compacta of substantia nigra (SN) in patients with PD is still not clearly known. Several studies have suggested the presence of oxidative stress in SN of PD patients [Jimenez and Luquin, 1996; Fahn and Cohen, 1992; Marsden, 1990; Gotz et al, 1990; Riederer et al, 1989; Marttila, 1988; Saggu et al, 1989]. Increase in Fe and other paramagnetic trace metals in SN could hypothetically elicit the oxidative process [Zecca et al, 2004a; Zecca et al, 2004b].

Limited data is available concerning the levels of metals in serum during pathological conditions of PD. Studies suggest that levels of transition metals like Zn, Cu and Fe in serum do not play role as risk factor indicators for PD [Jimenez et al, 1998]. Moreover, most of the available information is limited to few selected elements [Jimenez et al, 1992; Valdivia et al, 1994] and there is no study, which examines inter-elemental relationships with regard to severity of PD. The aim of this study is to assess the serum levels of 12 elements (Na, K, Fe, Al, Cu, Zn, Ca, Mg, Mn, Si, P and S) in patients with early and severe PD compared with a control population and

also to understand the possible relevance of inter-elemental relationships to the progression of PD.

## 5.2 Patients and Methods

### 5.2-1. Patients

We assessed the serum levels of 12 elements in early and severe PD patients in comparison with controls. Blood samples were collected from 52 patients with PD (27 early and 25 severe), recruited from among the outpatients attended in the Neurology Departments of two urban hospitals (Sri Venkateswara Institute of Medical Science, and J.S.S. Medical Hospital, India). The PD patient group was graded into early PD and severe PD according to clinical severity. All the patients met the commonly accepted diagnostic criteria for PD [Weiner et al, 1989] and were evaluated by the Unified Parkinson's Disease Rating Scale (UPDRS) [Fahn et al, 1987] and the Hoehn and Yahr staging [Hoehn and Yahr, 1967]. The first stage of the Hoehn and Yahr staging of PD was considered as Early PD while the latter stages of Hoehn and Yahr staging were graded into Severe PD. Among the 27 early PD cases 14 were untreated, the other 13 were treated with antiparkinsonian drugs alone or in combination including levodopa (11 cases), anticholinergics (2 cases), while among 25 severe PD patients, 6 were untreated, the other 19 were treated with antiparkinsonian drugs alone or in combination including levodopa (18 cases), anticholinergics (3 cases). The main clinical features of the PD patients and controls are summarized in Table 5.1. The cases related to occupational exposures for Fe and Mn were eliminated in the present study.

The control group comprised 25 “healthy” voluntary persons having no significant medical illness or medications for atleast

3-months duration at the time of blood collection.

The following exclusion criteria were applied to both the PD patients and controls [Fell and Burns, 1981; Triplett, 1985]: (a) Ethanol intake higher than 80g/day in the last 6 months. (b) Previous history of chronic hepatopathy or diseases causing mal-absorption. (c) Previous history of severe systemic disease. (d) Atypical dietary habits (diets constituted exclusively by one type of food stuff, such as vegetables, fruits, meat, or others, special diets because of religious reasons, etc). (e) Previous blood transfusions, anemia and polycytemia. (f) Intake of supplements of Fe, Cu, Al, Zn or chelating agents. (g) Therapy with chlorotiazides, ACTH or steroids. (h) Acute infectious disorders, traumatisms or surgery in the last 6 months. (i) haemolytic anemia.

**Table 5.1**
**Clinical features of Control and PD-patient groups.**
Data of quantitative variables are expressed as mean ± standard deviation

| **Features** | **Early PD** (n=27) | **Severe PD** (n=25) | **Control** (n= 25) |
|---|---|---|---|
| Age (years) | 57.15±5.2 | 59.30±3.9 | 55.4±6.4 |
| Sex | 13 F / 14 M | 11 F / 14 M | 12 F / 13 M |
| Duration of PD (years) | 2.68±0.86 | 7.0±1.4 | |
| Hoehn and Yahr stage | 1st stage | 2nd stage onwards | |
| Scores of Unified PD Rating Scale | | | |
| Total (items 1-31) | 23.1±11.4 | 57.6±15.6 | |
| ADL subscale (items 5-17) | 9±5.2 | 22.3±7.3 | |
| Motor subscale (items 18-31) | 17.8±5.8 | 29.7±13.6 | |

### 5.2-2. Ethical issue

Ethical approval for collecting blood samples from patients with PD and control humans were obtained from research ethical committee of J.S.S. Medical College and Hospital and Sri Venkateswara Institute of Medical Science, India. A written consent was obtained from the patients/carers prior to the collection of blood samples.

### 5.2-3. Precaution to avoid cross contamination during sample collection and storage

A 10-mL volume of venous blood sample was collected using canula to avoid iron contamination from each PD patient/control and serum was separated by centrifugation. Blood collection and serum separation was carried out in dust free environments. The serum was frozen at $-20^0$ C and protected from exposure to light until analysis. All the tubes used were polypropylene in nature and no glass material was used to prevent Al and Si contamination. All the precautions were taken to eliminate metal contamination during blood collection and storage in accordance with NCCLS criteria [1997].

### 5.2-4. Instrumentation and Elemental analysis

*Estimation and quality assurance test:* Elemental analysis was carried out using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) either by sequential or simultaneous mode depending on the elements to be analyzed. All dilutions were made with ultra pure milli Q water (18-mega ohms resistance) in dust free environment. The optimization of ICP-AES was evaluated by line selection and detection limits for each element. The validation of the

analysis was tested by analyzing serum matrix match multi-element synthetic standard and certified standard reference material (Bovine liver, 1577a) obtained from National Bureau of Standards, USA [Rajan et al, 1998]. The lines were selected for each element in such a way that interference from other elements was minimum. The wavelength used and detection limit of the elements are summarized below in Table 5.2.

**Table 5.2**

Inductively coupled plasma atomic emission spectrometry: wavelenghts and detection limits

| Element | Wavelength | Detection limit* | |
|---|---|---|---|
| | (nm) | μg/ml | μmole/ |
| Na | 588.995 | 0.03 | 0.00130 |
| K | 766.49 | 0.06 | 0.00153 |
| S | 182.98 | 0.05 | 0.00156 |
| P | 213.618 | 0.05 | 0.00162 |
| Ca | 393.366 | 0.002 | 0.00005 |
| Mg | 279.806 | 0.001 | 0.00004 |
| Cu | 224.7 | 0.002 | 0.00003 |
| Zn | 213.856 | 0.002 | 0.00003 |
| Fe | 259.94 | 0.005 | 0.00009 |
| Al | 396.152 | 0.002 | 0.00007 |
| Mn | 257.61 | 0.001 | 0.00002 |
| Si | 251.611 | 0.08 | 0.00285 |

*Detection limit (μ g/ml) for each element was calculated by running a multi-element standard solution containing 500 ng/ml of each of the above-cited elements.

## 5.2-5. Data analysis

The concentration of elements was expressed as micromoles/mL with mean, range of values and standard deviation. The mole percentage (Elemental concentration in mole% = Elemental concentrations (μmole/mL) x 100/Total elemental concentration

(μmole/ml) of analyzed elements in each sample) was calculated for the analyzed elements and the relative distribution based on the mole percentage was computed. Mole percentage calculations are essential to understand the relative distribution of each element in relation to other elements in biological matrix and this will also help to normalize the data of different samples to arrive at a clear inter-element relationships. Element to element ratios and correlations were calculated based on mole percentage to arrive at a possible elemental inter-relationships (direct and inverse) between control and PD serum. All statistical calculations such as inter-relationship and correlation coefficients, *t*-tests were carried out using Microsoft Excel 2000 and 'graph pad prism' software.

## 5.3 RESULTS

### 5.3-1. Elemental concentration

Elemental concentration (μmole/ml) for control, early PD and severe PD serum are given in Table 5.3. The data are presented in μmole concentration in order to calculate mole ratio of elements and also to determine inter-elemental correlation. The percent change of elements in early and severe PD compared to control group and also between early & severe PD have been presented in table 5.4. The results clearly showed that serum levels of K, Mg, Cu and P were higher ($p < 0.01$) in both early and severe PD compared to control. S and Al were significantly low ($p < 0.05$) in both early and severe PD, while Fe and Zn were decreased significantly ($p < 0.01$) in only severe PD compared to control, which may reflect the severity of PD. Interestingly, in early PD serum the concentrations of P, Cu, K and Ca were higher than control and severe PD. However, there was only

**Table 5.3**

Concentration of trace elements in micromole /mL ± Standard deviation in control, Early and Severe PD serum. Values represented in parenthesis indicate range. Reference values of metals (micromole/ml) in control human serum from 'Handbook on metals in clinical and analytical chemistry [1994] and Muniz et al, 2001. (Values with asterisk mark are from reference Muniz et al, 2001) as given in the second column for comparison

| **Element** | **Reference values For control** | **Control** | **Early PD** | **Severe PD** |
|---|---|---|---|---|
| Na | 139.2 * (134.8-147.8) | 135.4±4.1 (126.5-141.3) | 142.6±11.4 (125.0-164.5) | 142.9±9.8 (127.2-168.5) |
| K | (4.09-4.48) * | 3.54±0.6 (2.4-4.9) | 4.46±0.4 (3.3-5.4) | 3.79±0.4 (2.8-4.7) |
| S | Not Available | 36.6±3.7 (31.1-44.5) | 32.0±5.0 (25.3-45.6) | 31.1±3.3 (26.5-38.0) |
| P | 3.56 | 3.2±0.4 (2.3-4.0) | 4.12±0.9 (2.7-6.8) | 3.65±0.5 (2.8-4.5) |
| Ca | 2.35 (2.2-2.6) | 2.2±0.2 (1.8-2.5) | 2.41±0.3 (1.8-3.0) | 2.23±0.2 (21.8-2.69) |
| Mg | (0.6-1.07) | 0.9±0.09 (0.78-1.1) | 1.05±0.1 (0.82-1.3) | 1.05±0.08 (0.86-1.19) |
| Cu | 0.017 | 0.014±0.003 (0.009-0.019) | 0.022±0.008 (0.007-0.035) | 0.02±0.006 (0.011-0.035) |
| Zn | 0.013 | 0.009±0.001 (0.006-0.01) | 0.008±0.002 (0.006-0.012) | 0.007±0.001 (0.005-0.009) |
| Fe | (0.012-0.030) | 0.023±0.009 (0.016-0.047) | 0.02±0.004 (0.01-0.028) | 0.017±0.007 (0.004-0.035) |
| Al | < 0.0002 | 0.00059±0.00004 (0.0004-0.00068) | 0.00045±0.00005 (0.00034-0.0005) | 0.0004±0.0001 (0.0003-0.0005) |
| Mn | Not Available | <DL (DL:0.001) | 0.001±0.0002 (0.0005-0.034) | 0.001±0.0002 (0.0004-0.045) |
| Si | Not Available | <DL (DL: 0.08) | 0.032±0.01 (0.002-0.06) | 0.009±0.002 (0.004-0.014) |
| Total | | 181.8±6.3 (170.3-195.5) | 186.7±15.9 (162.7-218.4) | 184.8±10.5 (168.1-205.9) |

marginal increase in the total concentration (μmole/ml) of elements among early and severe PD serum, compared to control. There was no correlation of these values with age at onset, sex and antiparkinsonian therapy. These findings are in agreement with previous studies [Jimenez et al, 1998; Valdivia et al, 1992].

**Table 5.4**

Percentages changes of elements from control to early PD and severe PD and early PD to severe PD. The +ve and –ve signs indicate increasing and decreasing trends; Statistical significance, a: $p<0.01$; b: $p<0.05$.

| Metals | Control to Early PD | Control to Severe PD | Early to Severe PD |
|---|---|---|---|
| Na | 5.3[a] | 5.6[a] | 0.2 |
| K | 26.0[a] | 7.2[b] | -15.0[a] |
| S | -12.6[a] | -15.1[a] | - 2.8[b] |
| P | 30.0[b] | 15.4[a] | -11.4[b] |
| Ca | 8.7[b] | 0.6 | - 7.5[b] |
| Mg | 16.2[a] | 16.2[a] | 0.0 |
| Cu | 63.2[a] | 62.1[a] | - 9.1[b] |
| Zn | - 8.4[a] | -19.4[b] | -12.5[b] |
| Fe | - 13.9[b] | -29.5[a] | -15.0[a] |
| Al | - 23.7[a] | -37.3[a] | -17.8[a] |

The data generated in the present study on elemental concentrations in control human subjects were compared with the reference values from the 'Handbook on metals in clinical and analytical chemistry' [1994] and Muniz et al, [2001] (Table 5.3). The values matched for most of the elements except K, Al and Zn where larger variations are observed. The normal values for Al in serum

sample is a debatable question. Though it is agreed that Al level should be less than 0.005 μg/mL of control human serum, in many reports, value was shown to be around 0.010-0.020 μg/mL. Al level in our investigation for control human serum was 0.016 μg/mL.

### 5.3-2. Relative Mole Percentage

To elucidate the inter-elemental relationships among the data sets in control, early PD and severe PD, the concentration levels (μmole/ml) were normalized by calculating mole percentage of each element in a sample. The relative distribution is represented in Bar chart (Fig.5.1). The mole percentage data indicated that levels of Al, Fe and S levels were high in control serum compared to early and severe PD. Na and K increased in both the PD groups than control. Among the divalent elements, mole percentage of Mg, Cu were high, while Zn was low in early and severe PD serum compared to control. However, no significant change was observed for Ca.

### 5.3-3. Inter Elemental Correlations and Mole percent ratio

The inter-elemental relationships among the analyzed elements for control, early PD and severe PD showed a distinct pattern of direct and inverse correlations for selective elements. The correlation co-efficients and the statistical confidence levels at which the correlations were drawn are given in Table 5.5. Na showed inverse correlation ($r \geq -0.95$) with S in all the groups. This correlation was also established in our previous studies in cerebrospinal fluid of Alzheimer's patients [Rao et al, 1999b]. Na showed direct correlation with K and Al in control while they showed a tendency towards inverse correlation in both early and severe PD serum. K with S, Zn

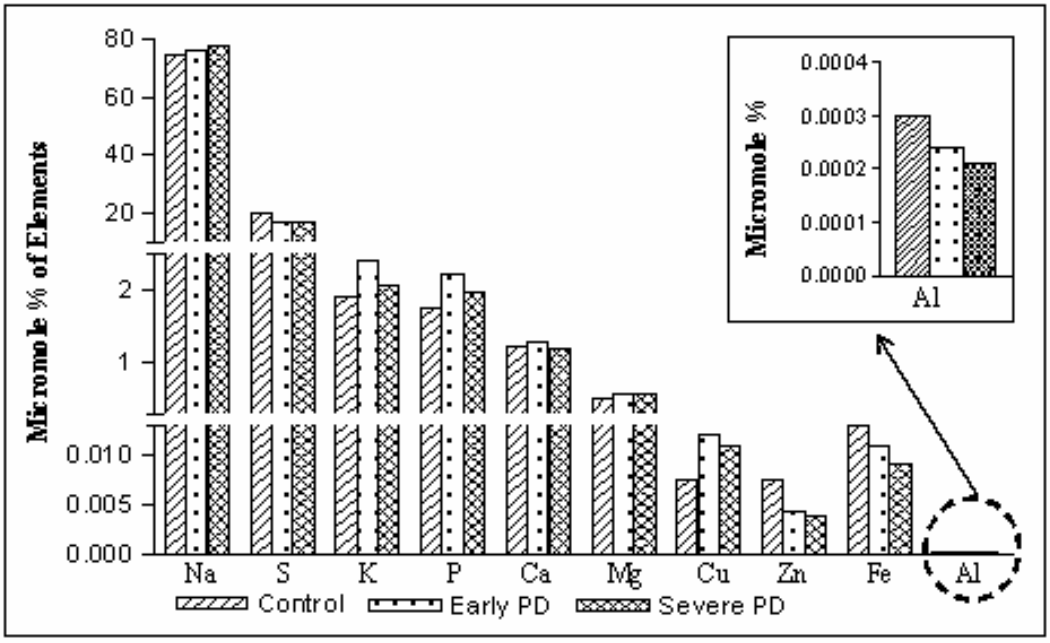



**Figure 5.1**

Comparison of relative mole percentage of elements in control, early and severe PD human serum samples. Inset: Al levels in PD and control serum magnified.

and Ca, and S with Al, and P with Fe showed inverse correlation in control and direct correlation in early and severe PD. S and Fe showed no correlation in PD while they were in direct relation in control. K to Zn and P to Fe correlations were inverse in control but they showed a tendency towards direct correlation in both types of PD serum.

The data were further analyzed in terms of element-to-element mole percentage ratio in control, early and severe PD serum. The ratios were calculated in order to understand the inter-relationship of elements in biological systems [Rajan et al, 1998; Rao et al, 1999a;

1999b]. The ratios of Na/K, Na/Cu, Fe/Cu, S/Mg, and S/Cu were significantly decreased in early and severe PD serum than control while the ratios of K/Al, S/Al, Mg/Al, P/Al, K/Fe, K/Zn and Cu/Zn showed an increasing trend in PD serum compared to control. However, only moderate change was observed in other ratios.

**Table 5.5**

Comparison of inter elemental relations between normal, early PD and severe PD Serum. Confidence levels: * >99.9%, ** >95%. At 99.9% and 95% confidence levels, the expected correlation co-efficient for sample size of 25 are 0.613 and 0.396 respectively. A positive and negative signs indicate direct and inverse correlations respectively.

| Correlation between elements | | Correlation co-efficient | | |
|---|---|---|---|---|
| 1 | 2 | Control (n=25) | Early PD (n=27) | Severe PD (n=25) |
| Na | S | -0.99* | -0.95* | -0.95* |
| Na | Ca | -0.46** | 0.00 | -0.59** |
| Na | K | 0.47** | -0.32 | -0.22 |
| Na | Al | 0.49** | -0.10 | -0.05 |
| K | S | -0.51** | 0.11 | 0.36 |
| K | Zn | -0.42** | 0.42** | 0.48** |
| K | Ca | -0.29 | 0.49** | 0.36 |
| S | Al | -0.49** | 0.09 | 0.05 |
| S | Fe | 0.40** | 0.03 | 0.12 |
| Cu | Zn | -0.09 | 0.29 | 0.49** |
| P | Fe | -0.58** | -0.12 | 0.39 |

## 5.3-4. Charge distribution

The relative percent charge distribution in control, early PD and severe PD were computed (Fig. 5.2). Triply charged ions (Al and Fe) decreased significantly in early and severe PD serum compared to

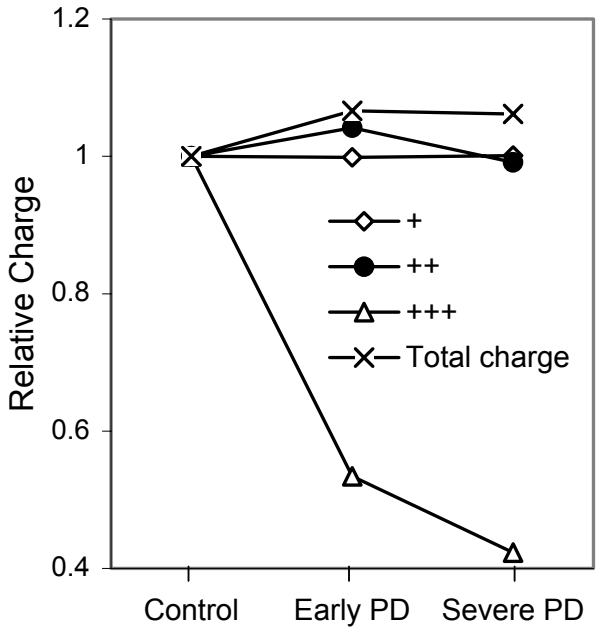



**Figure 5.2**
Charge Distribution in control, early PD and severe PD: Graph shows relative percent charge distribution with respect to control.

control while, no significant change was observed in case of monovalent ions (Na and K). However, doubly charged ions (Mg, Ca, Cu, and Zn) were marginally high in early PD compared to both control and severe PD serum. Among the divalent ions, the increase of Mg and Cu were probably neutralized by the decrease of Zn and as a result only marginal change was observed in doubly charged ions as a whole in PD serum. The total charge was high in early and severe PD than control indicating an increased charge burden in PD.

## 5.4 Discussion

In the last decade there has been increasing interest for the possible role of metals in the pathogenesis of PD [Zecca et al, 2004a; Zecca et al, 2004b; Jimenez et al, 1998; Basun et al, 1991; Dexter et

al, 1991; Smith et al, 1989]. Substantial information is available on trace metals distribution in brains of control [Rao et al, 1999a; Marksbery et al, 1984] and neurodegenerative diseases like, Parkinson's, Alzheimer's and Huntington's disease, [Rao et al, 1999a; Rao et al, 1999b; Dexter et al, 1991; Zecca et al, 1994; Zecca et al, 2004a]. Previous investigations [Dexter et al, 1989a] showed an increase in Fe and Zn concentrations in the SN, lateral putamen and caudate nucleus in PD brain. However, limited data is available on few selective elements in serum of PD affected patients. Moreover, there has been a controversy regarding the serum Zn and Cu levels in PD serum. Abbot et al, and Pan et al, [Abbot et al, 1992; Pan et al, 1997] reported decreased serum Zn concentrations, while, Jimenez et al, [1992] reported no significant change in Zn in PD serum compared to control. Our results showed decreased Zn levels in both early and severe PD serum, which is in agreement with Abbot et al, and Pan et al, [Abbot et al, 1992; Pan et al, 1997]. Serum Cu concentrations were normal in the previous studies [Jimenez et al, 1992; Campanella et al, 1973; Pall et al, 1987] with the exception of decreased copper levels in a single study [Pan et al, 1997]. In contrast, we observed increased Cu in both early and severe PD serum (>45-65%) compared to control. Further, Jimenez-Jimenez et al, [1998] reported that the serum levels of Fe, and Mn did not differ significantly between PD-patient and control groups. However, other studies [Abbot et al, 1992; Logroscino et al, 1997] reported decreased serum Fe levels. In the present study, significant decrease in Fe and moderate increase in Mn concentrations were observed in PD compared to control serum. Interestingly, Fe showed a gradual decreasing trend with the severity of PD. Fe decreased by ~14% and ~30% in early and severe PD respectively compared to control. However, there are no previous studies on serum levels of other elements like Na, K, S, P, Ca, Mg, Si, Al etc. In the present study

mole percentage ratios and the correlation patterns of elements indicated that there is a definite imbalance in the element-to-element inter-relationship pattern in serum of PD affected individuals (Table 5.5).

There has been a controversy regarding metal levels in PD serum and also possible role of metals as risk factors for PD. It is not clear whether alteration in metal homeostasis is a cause or consequence of disease pathology. So far, there is no detailed or comprehensive database on metal homeostasis and inter-relationships. The available reports only indicated the changes in levels of one or two individual elements, but fall short to correlate the element-to-element inter-relationship pattern with the disease progression. In this perspective, the present study provides a comprehensive database on concentrations of as many as 12 elements (majority of essential and few important toxic elements) in PD serum in comparison with control groups.

There is limited information concerning the correlation of elemental homeostasis in the brain, CSF, serum and other vital organs. Pall et al, [1987] and Pan et al, [1997] reported increased Cu levels in the CSF of patients with PD. The former report further suggested that this metal might be raised in the brain too. However, several studies suggested [Smith et al, 1989; Uitti et al, 1989] decreased levels of Cu and increased levels of Fe and Zn in the SN, lateral putamen and caudate nucleus in PD brain. The authors related this increase of Zn to an attempt of protection against oxidative stress arising from Fe increase. An inverse relationship between Fe and Cu (in liver) [Symes et al, 1969] and a direct relationship between Fe to S and Zn has been established [Basun et al, 1991]. Thus the increased Zn levels and decreased Cu levels in

PD brain may be compatible with the increase in Fe. CSF Zn levels were found to be decreased in a study conducted by Jimenez et al, [1998]. However, it is not clear, whether the source for increased Fe and Zn in brain comes from serum or CSF. Also, there is need to understand the primary factor that triggers the elemental imbalance in the body and its consequences. Trace metals play an important role in neuronal function. The levels of trace metals in serum may reflect brain levels with reference to essential metals, while this relation does not hold good for non-essential or toxic metals. All the essential metals cross blood brain barrier (BBB) by selective uptake mechanism. However, toxic metals cross BBB by displacing essential metals in carrier proteins like transferrin.

How or why a specific increase in the total Fe content of SN should occur in PD is not understood [Zecca et al, 2004a; Zecca et al, 2004b]. According to Lenders et al, [Leenders et al, 1993], Fe uptake into the brain across the BBB is significantly higher in PD patients than age-matched controls (PET study). They suggested that this increase in Fe in brain could be related to an increased transferrin receptor formation in PD. Fe is transported from blood to brain by a carrier protein transferrin. Further, Fe and transferrin are transported through the BBB by a transferrin receptor mediated transcytosis [Bank et al, 1989; Morris et al, 1992; Pardridge et al, 1991]. It has been argued that the increased Fe levels with the severity of neuropathological changes in PD are presumably due to increased transport through the BBB [Gotz et al, 2004]. Hence, the two likely pathways for increased Fe and Al uptake in dopaminergic neurons of SN may be due to the increase in transferrin receptor protein in PD brain and the non-specific pathological influx from other regions of the brain [Smith et al, 1989]. Al is also known to be co-transported with Fe-transferrin complex in neurological disorders

[Dexter et al, 1989a]. In normal brain, Fe and Al compete for transport across blood-brain barrier [Rao et al, 1999a] while in Alzheimer's disease, Fe and Al co-transport [Dexter et al, 1989a]. This differential mechanism is however still puzzling.

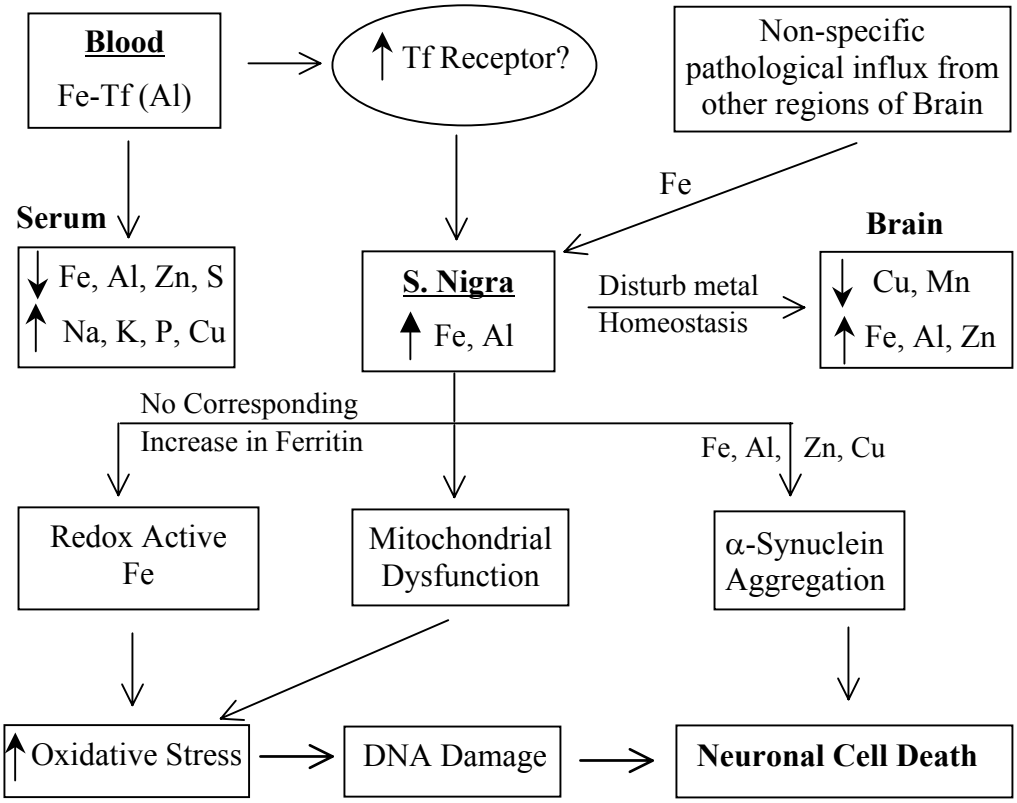
Fe within the brain exists in many complex forms not all of which are capable of catalyzing oxidative stress. The majority of Fe is bound and inactivated by association with ferritin under normal physiological conditions. Glial iron is mainly stored as ferric iron in ferritin, while neuronal iron is predominantly bound to Neuromelanin [Gotz et al, 2004]. The biosynthesis of ferritin is controlled by Fe availability [White and Munro, 1988]. The potential toxicity of increased Fe load in SN in PD therefore is determined by the extent to which it is deactivated by binding to ferritin and other moieties. However, in PD the increased total Fe level in SN was not associated with a compensatory increase in ferritin; instead the brain ferritin immunoreactivity was decreased [Dexter et al, 1990]. Hence the increased Fe load in PD may exceed the storage capacity of available ferritin, leading to excess reactive Fe, driving free radical generation [Smith et al, 1989]. This concept is supported by the increase in basal lipid peroxidation found in SN in PD [Dexter et al, 1987]. Hence, iron overload and imbalance in other redox metal levels may induce progressive degeneration of nigrostriatal neurons by facilitating the formation of reactive biological intermediates, including reactive oxygen species, and the formation of cytotoxic protein aggregates [Gotz et al, 2004; Uversky et al, 2001a; 2001b; 2001c].

With all the above facts in mind, we developed a hypothetical model explaining the possible relevance of metal homeostatic imbalance in brain and serum in modulating neurochemical events

leading to neuronal degeneration in PD (Fig. 5.3). We proposed two pathways. Pathway I explains two likely reasons for increased Fe and Al in PD brain: (a) the increase in transferrin receptor protein in PD brain and, (b) the non-specific pathological influx from other regions of the brain [Dexter et al, 1991].

We advocate that irrespective of metals being primary risk factors or consequences of disease mechanism, a moderate change in a single metal ion will upset the whole elemental homeostasis pool resulting in the significant imbalance in elemental levels in the body system (serum, CSF and brain). The effect of increase or decrease of a single metal is not restricted to the presenting metal alone, it will affect the total elemental and charge distribution pattern in the system.

An inverse relationship between Fe and Cu (in liver) [Symes et al, 1969] and a direct relationship between Fe to S and Zn have been established [Rajan et al, 1998]. Thus the increased Zn levels and decreased Cu levels in PD brain may be compatible with the increase in Fe. In PD, the increased total Fe level in substantia nigra was not associated with a compensatory increase in ferritin [White and Munro, 1988]. Hence the increased Fe load in PD may exceed the storage capacity of available ferritin, this leads to excess redox active Ferrous form of Fe. This in turn drive free radical generation [Dexter et al, 1991]. This concept is supported by the increase in basal lipid peroxidation found in SN in PD [Dexter et al, 1990]. Al also promotes Fe mediated oxidative stress by inhibiting Catalase activity and also by causing mitochondrial dysfunction [Anitha et al, 2002b; Gupta et al, 2005]. The oxidative stress finally leads to DNA damage, which may be one of the pathways leading to cell death in PD. In Pathway II, the increased levels of Fe, Al and Zn may likely to promote




Legend: Tf-Transferrin; The upward and downward arrows indicate increasing and decreasing trends

**Figure 5.3**
The possible biochemical events leading to metal homeostatic imbalance in brain and serum in Parkinson's disease and the role of metals in neuronal degeneration.

α-synuclein aggregation [Uversky et al, 2001b] leading to Lewy body formation. α-Synuclein is the major constituent of insoluble protein aggregates which is the hallmark pathological event in PD. α-Synuclein aggregation induced by changes in metals may be another mechanism contributing to degeneration of dopaminergic neurons in PD. The above factors give an overview of the complexities associated

with metal toxicity and the possible metals triggered neurochemical processes leading to neuronal degeneration.

Our results on comparative assessment of trace elements in PD serum and control subjects showed that a definite disturbance in metal homeostasis and inter-elemental relationships (direct and inverse) occurs in serum during the PD progression.

✡ ✡ ✡ ✡ ✡

# *General Summary and Conclusions*

Parkinson's disease (PD) is a progressive, neurodegenerative disorder that is characterized by the severe motor symptoms, including uncontrollable *tremor*, postural imbalance, slowness of movement and rigidity. The main pathological *hallmark* of PD is a pronounced loss of dopamine-producing neurons in the *substantia nigra* (SN), which results in a drastic decrease in dopamine in the striatum, to which these neurons project.

The etiology of PD has not been fully understood. Unproven hypotheses have included environmental toxins including metals, pesticides etc and genetic factors (See **Chapter 1**). A variety of mechanisms that are believed to cause accelerated cell death have also been suggested, including oxidative stress, excitotoxicity and mitochondrial dysfunction.

Recent studies showed that oxidative stress, DNA damage, chromatin condensation, and altered expression of genes [Enochs et al, 1994; Berg et al, 2004; Migliore et al, 2001; Jellinger 2000; Hirsch et al, 1999; Cohen, 1986; Youdim et al, 1989; Olanow, 1992; Dexter et al, 1992] are also associated with neurodegeneration in PD like Alzheimer's disease (AD). One of the consequences of redox imbalance is apoptosis and/or necrosis (programmed vs passive cell death) which are associated with neurodegeneration in PD [Burke and Kholodilov, 1998; Kingsbury et al, 1998; Ziv and Meland 1998; Tatton et al, 1998; Jenner and Olanov, 1998; Ziv et al, 1997; Robbins, 1987]. Studies have also shown that the levels of the nucleoside, 8-hydroxy -2'-deoxyguanosine (8-OHdG), a product of free radical attack on DNA were generally increased and differentially distributed in PD brains with highest levels in caudate, putamen, SN and cerebral cortex [Alam et al, 1997]. Further, our laboratory

evidenced that the genomic DNA undergoes a helicity change in AD from B-form to left handed Z-DNA form [Anitha et al, 2002a]. These observations suggest that topological changes in the genetic material may be involved in the pathogenesis of PD also. So far no studies have been reported on the DNA conformation in PD affected brain cells.

In this perspective, we studied the stability, integrity and topology of DNA isolated from 5 clinically and neuropathologically confirmed PD cases and 6 age-matched controls. Genomic DNA was isolated from eight regions in the human brain, namely frontal, temporal and occipital cortex, hippocampus, caudate/ putamen, thalamus, cerebellum and midbrain collected at post-mortem from cases of PD and controls and were analyzed for single and double strand breaks in DNA, and their conformations and topology (See **Chapter 2**). The results showed that DNA from midbrain in PD accumulated significant number of strand breaks than age-matched controls. Caudate nucleus/ putamen, thalamus and hippocampus also showed more DNA fragmentation compared to control brains. Circular dichroism studies showed that DNA conformation was altered with imprecise base stacking in midbrain, caudate nucleus/ putamen, thalamus and hippocampus in PD. However, DNA from frontal, temporal and occipital cortex, cerebellum was not affected significantly in PD group as compared to controls. This study provides a comprehensive database on stability, damage and conformations of DNA in different regions in brains of PD patients.

The above results suggested that the structural integrity and topology of genomic DNA is altered in many regions of PD brain. The changes include DNA damage in the form of single strand and double strand breaks and weakening of the base stacking as

indicated by the CD, Tm, EtBr binding and DNAse I treatment experiments. Interestingly, the changes observed appear to be region specific in the brain. The DNA isolated from frontal, temporal occipital cortex and cerebellum were largely unaffected while midbrain, caudate/ putamen, hippocampus and thalamus showed more strand breaks. It is however, evocative to understand the implications of the above findings in relevance to PD pathogenesis.

The pathological hallmark of PD is the formation of insoluble protein aggregates known as *Lewy bodies*. The major constituent of these fibrillar structures is **α-synuclein**, a 140 amino acid protein with a basic amino terminal and an acidic carboxy terminal. There is little information available about the neurobiology of α-synuclein under normal and neurodegenerative conditions. In amyloid plaques of AD and other neurodegenerative disorders α-synuclein has cross beta conformation while it adopts α-helical conformation in presence of phospholipids and lipid membranes [George, 2002; Jensen et al, 1998; Davidson et al, 1998; Jenco et al, 1998; McLean et al, 2000; Perrin et al, 1999]. The formation of α-helix is suggested as a protective mechanism against formation of beta sheet and aggregated structures [Uversky et al, 2001c; Munishkina et al, 2003]. The major efforts of drug development are focused on the prevention or delaying of the protein aggregation and formation of plaques and recently emphasis has been given to stabilizing the *non-toxic form of the protein*.

Recent observations showed that α-synuclein is localized in the chromatin region of nuclei in the brain [Maroteaux et al, 1988; Gomez-Tortosa et al, 2000; McLean et al, 2000; Tanzi et al, 2002; Leng et al, 2001; Sangchot et al, 2002; Goers et al, 2003; Lin et al, 2004]. Moreover, the presence of the majority of the lysine residues

in the N-terminal region of α-synuclein suggests a possible DNA binding role for α-synuclein. An understanding of the DNA binding could be exploited for developing specific gene chip based drugs that bind to the protein and prevent aggregation.

We demonstrated in the present study, two new and novel properties of α-synuclein. **First**, we showed that α-synuclein binds to DNA and alter the conformation of DNA. **Second**, α-synuclein was shown to have DNA nicking activity and it behaves like a endonuclease enzyme. These are new evidences in literature, there were no such reports previously on α-synuclein binding to DNA (See **Chapter 3**). It was also observed that the nicking activity involves the formation of only SSBs. However, during long term incubations with α-synuclein DSBs were formed chopping the DNA into small pieces indicating that α-synuclein preferentially nicks only single stranded DNA. Further, the ability of known nuclease inhibitors, Aurintricarboxylic acid (ATA) and Diethyl pyrocarbonate (DEPC) to abolish DNA nicking activity of α-synuclein reveals histidine aminoacid residue at 50$^{th}$ position in α-synuclein sequence plays a crucial role in the nicking activity. We further showed that conformational change or oligomerisation in α-synuclein would enhance the nicking activity. This indicates that the oligomers of α-synuclein are more toxic in terms of DNA nicking than monomers and aggregates. This work was awarded ***International Alzheimer's Fellowship*** and was presented in the ***9$^{th}$ International Meeting on Alzheimer's disease and Related Disorders*** held in Philadelphia, USA in July, 2004.

We discussed the potential implications of the above *in vitro* findings to neurodegenerative changes associated with PD (Chapter

3). We proposed that the DNA binding property of α-synuclein characterized in the present study may have a significant effect on nuclear-translocated α-synuclein functioning. In particular, α-synuclein may interact with histone-free DNA segments and induce nicking.

Structurally, purified α-synuclein is a natively unfolded protein [Hegde and Rao, 2003; Weinreb et al, 1996; Uversky et al, 2001a; Eliezer et al, 2001]. This lack of folding has been shown to correlate with the specific combinations of low overall hydrophobicity and large net charge [Uversky et al, 2000; Uversky, 2002a; Uversky, 2002b]. *In vitro* α-synuclein readily assembles in to fibrils, with morphologies and staining characteristics similar to those of fibrils extracted from PD affected brain [Uversky et al, 2001a; Crowther et al, 1998; El-Agnaf et al, 1998; Wood et al, 1999; Giasson et al, 1999; Narhi et al, 1999; Serpell et al, 2000; Conway et al, 2000; Li et al, 2001; Hoyer et al, 2002; Munishkina et al, 2003]. it has been assumed that α-synuclein may exist in two structurally different isoforms *in vivo*: a helix-rich, membrane-bound form and a disordered, cytosolic form, with the membrane-bound α-synuclein generating nuclei that seed the aggregation of the more abundant cytosolic form [Lee et al, 2002; Munishkina et al, 2003].

In **chapter 4**, our results indicated that the DNA binding may induce folding in α-synuclein which makes the protein bind to DNA enzymatically and have DNA nicking activity. In this perspective, we tried to understand the ability of different DNA to induce conformational changes in α-synuclein. We have used the effect of chaperonic properties five osmolytes viz. glycerol, betaine, taurine,

TMAO, sarcosine for their ability to induce folding in α-synuclein, as a model system to understand the DNA induced conformational changes in α-synuclein.

We provided a comprehensive picture of DNA binding effect on α-synuclein fibrillation using different DNAs such as double and single stranded DNA, AT and GC sequence specific DNA, of different sizes, genomic DNA etc and showed that only those DNA which induce a partial folding in α-synuclein (GC* rich DNA) promote its aggregation, while, sscDNA forms α-helix conformation in α-synuclein and also inhibit aggregation to a considerable extent. It was also observed that among the osmolytes used in the present study glycerol, TMAO, betaine and taurine induced partial folding in α-synuclein and enhanced the fibrillation kinetics.

The ability of DNA and osmolytes in inducing conformational transition in α-synuclein, indicates that two factors are critical in modulating α-synuclein folding: (i) Electrostatic interaction as in the case of DNA, and (ii) Hydrophobic interactions as in the case of osmolytes. We feel that the property of sscDNA in inducing α-helical conformation in α-synuclein and inhibiting the fibrillation may be of significance in engineering DNA-chip based therapeutic approaches to PD and other amyloid disorders.

Further, from the fact that other amyloidogenic peptides like Aβ, tau and prions also have DNA binding property [Hegde et al, 2004a; Hegde et al, 2004b; Ahn et al, 2000; Jang and Surh, 2002; Kampers et al, 1996; Nandi and Leclerc, 1999; Nandi, 1998; Cordeiro et al, 2001; Gabus et al, 2001a; Gabus et al, 2001b] it appears that the DNA binding is a unifying property of amyloidogenic peptides implicated in various neurodegenerative disorders.

Metals are well established to be risk factors for PD, though the significance of metals in the aetiopathogenesis of PD is still unknown [Zecca et al, 2003; Zecca et al, 2004; Dexter et al, 1991]. Several studies have shown decreased copper, increased zinc and iron in the *substansia nigra* and increased copper concentrations in the cerebrospinal fluid of PD affected patients [Dexter et al, 1991]. Though much work has been done on metal homeostasis in PD brain, limited data is available on trace elemental levels and their inter-relationships in serum of Parkinson's patients [Jimenez et al, 1998]**.** Moreover, most of the available information is limited to few selected elements [Jimenez et al, 1992; Valdivia et al, 1994] and there is no study, which examines inter-elemental relationships with regard to severity of PD. The aim of this study was to assess the serum levels of 12 elements (Na, K, Fe, Al, Cu, Zn, Ca, Mg, Mn, Si, P and S) in patients with early and severe PD compared with a control population and also to understand the possible relevance of inter-elemental relationships to the progression of PD.

The results showed a definite pattern of variation among certain elements in early and severe PD compared to control [Hegde et al, 2004c]. In both early and severe PD serum, Al and S ($p<0.05$) were decreased compared to control. Fe ($p<0.01$) and Zn ($p<0.05$) were significantly low in severe PD, while K, Mg, Cu ($p<0.01$) and P ($p<0.05$) were high in early and severe PD compared to control. The data revealed a clear imbalance in the elemental interrelationship in both early and severe PD serum compared to control as shown by the direct and inverse correlations. These results suggested that a definite disturbance in the elemental homeostasis occurs during the progression of PD. There has been a controversy regarding metal levels in PD serum and also possible role of metals as risk factors for PD. It is not clear whether alteration in metal homeostasis is a cause

or consequence of disease pathology. So far, there is no detailed or comprehensive database on metal homeostasis and inter-relationships. The available reports only indicated the changes in levels of one or two individual elements, but fall short to correlate the element-to-element inter-relationship pattern with the disease progression. In this perspective, the present study provided a comprehensive database on concentrations of as many as 12 elements (majority of essential and few important toxic elements) in PD serum in comparison with control groups (See **Chapter 5**).

We further advocated from the above results that irrespective of metals being primary risk factors or consequences of disease mechanism, a moderate change in a single metal ion will upset the whole elemental homeostasis pool resulting in the significant imbalance in elemental levels in the body system (serum, CSF and brain). The effect of increase or decrease of a single metal is not restricted to the presenting metal alone, it will affect the total elemental and charge distribution pattern in the system.

## Significance of the work:

1. The study provided a new data on DNA stability, integrity and topology of genomic DNA isolated from eight different brain regions from PD affected human brain samples (*Chapter* 2).

2. We evidenced two new and novel properties of α-synuclein: i) DNA binding and ii) DNA nicking. We showed that α-synuclein behaves as an endonuclease. These have been very intriguing observations which exposed a new toxic role of α-synuclein. The observations led to a new debate on whether α-synuclein

induced DNA damage has any role to play in DNA fragmentation observed in PD brain and hence open new avenues of research in this direction (*Chapter* 3).

3. We showed that single stranded circular DNA induces a stable, aggregation resistant, α-helical conformation in natively random-coil α-synuclein. This observation is very important in investigating the possibility of using DNA-chip based therapy for PD. We also showed that GC* rich DNA forms a partially folded intermediate conformation in α-synuclein and this conformation has high aggregation propensity. Using chaperonic properties of α-synuclein as model system we showed that increasing hydrophobicity and decreasing the net charge are critical in α-synuclein conformational transition and fibrillation (*Chapter* 4).

4. We generated a comprehensive database on the levels of 12 elements (both essential and toxic) in serum samples of PD affected patients and showed that there is a definite disturbance in inter-element homeostasis pattern in PD serum (*Chapter* 5).

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# *Publications Of The Candidate*

## *Papers in scientific journals*

1. **Muralidhar L. Hegde** and Jagannatha Rao KS (2003) Challenges and complexities of α-synuclein toxicity: New postulates in unfolding the mystery associated with Parkinson's disease. ***Arch Biochem Biophys***, 418: 169-178

2. **Muralidhar L. Hegde,** Anitha S, Latha KS, Mustak MS, Reuven Stein, Rivka Ravid, Jagannatha Rao KS (2004) First evidence for helical transitions in supercoiled DNA by Amyloid β peptide (1-42) and Aluminium: A new insight in understanding Alzheimer's disease. ***J Mol Neurosci,*** 22: 19-31

3. **Muralidhar L. Hegde,** Shanmugavelu P, Vengamma B, Sathyanarayana Rao TS, Menon RB, Rao RV and Jagannatha Rao KS (2004) Serum trace elemental levels and the complexity of inter-element relationships in patients with Parkinson's disease. ***J Trace Elem Med Biol,*** 18*:* 163-171

4. **Muralidhar L. Hegde**, Anitha S and Jagannatha Rao KS, (2004) Are monomer-oligomer-aggregates of amyloidogenic peptides toxic species in neurodegeneration? A new experimental evidence. ***Neurobiol Aging*****,** (Abstract) 25, S2: 170

*5.* Shanmugavelu P, Sathyanarayana Rao TS, **Muralidhar L. Hegde**, Mustak MS, Menon RB, Rao RV and Jagannatha Rao KS (2004) Altered trace elemental homeostasis in neurodegenerative (Alzheimer's and Parkinson's) and neuropsychiatric (bipolar) disorder. *In.* Neurodegenerative diseases: Alzheimer's disease (P21-1), ***J Neurochem,*** (Abstract) 88 (Suppl. 1): 49

6. Gupta VB, Anitha S, **Muralidhar L. Hegde**, Gurroto R, Zecca L, Rivka Ravid, Reuven Stein, Shankar KS, Shanmugavelu P and Jagannatha Rao KS, (2005) Aluminium in Alzheimer's disease: Are we still at a Cross-Road. ***Cell Mol Life Sci,*** 62: 143-158

7. Pande MBS, Nagabhushan P, **Muralidhar L. Hegde,** Sathyanarayana Rao TS, Jagannatha Rao KS, (2005) A novel Computational support for Diagnostic assessment of Neurological Disorder (Parkinson's Disease) through a new Dissimilarity Analysis. ***Comput Biol Med***, 35: 475-493

*8.* **Muralidhar L. Hegde**, Gupta VB, Anitha M, Harikrishna T, Shankar SK, Uday Muthane, Subba Rao K and Jagannatha Rao KS (2005) Studies on Genomic DNA topology and stability in brain regions of Parkinson's disease. *Revised and submitted to* ***Neurobiol Aging***.

## *Patent*

**Muralidhar L. Hegde** and Jagannatha Rao KS (2005) Patent filed to ***CSIR, India*** on Superhelicity based DNA chip' inhibited α-synuclein aggregation and favored ordered conformation: Therapeutic approach to Neurological disorders.

## *Paper / Poster presentations in symposia*

1. **Muralidhar L. Hegde** and K.S. Jagannatha Rao (2005) DNA induces folding in α-synuclein: understanding the mechanism using chaperon property of osmolytes. First award winning poster presented in meeting on 'Novel proteins in human nutrition and health organized by Solae-CFTRI, India on 22nd March, 2005. Awarded **First Prize** for the poster.

2. **Muralidhar L. Hegde**, Anitha S and Jagannatha Rao KS (2004) "Are monomer-oligomer-aggregates of amyloidogenic peptides toxic species in neurodegeneration? A new experimental evidence" poster presentation at the 9th International Conference on Alzheimer's Disease and Related Disorders in Philadelphia, Pennsylvania, **USA**, July 17-22, 2004. Awarded '**International Alzheimer's fellowship'** to attend the meeting by Alzheimer's Association, USA

3. **Muralidhar L. Hegde** and Jagannatha Rao KS (2003) "α-synuclein Nicks DNA: A new challenge in understanding Parkinson's disease" 10th Annual Conference of Andhra Pradesh Neuro Scientists Association (APNSA-2003), Tirupati, **India**, 26th –27th July 2003.