INVESTIGATING THE BIOPHYSICAL PROPERTIES AND AGGREGATION BEHAVIOUR OF OPTINEURIN AND ITS MUTANTS

ANJALI DIXIT

KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

ANJALI DIXIT

KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

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I dedicate this thesis to my Parents

विद्या ददाति विनयं, विनयाद्याति पात्रताम्। पात्रत्वाद्धनमाप्नोति,

धनाद्धर्मं ततः सुखम्

CERTIFICATE

This is to certify that the thesis titled "**Investigating the biophysical properties and aggregation behaviour of Optineurin and its mutants** " being submitted by **Ms. Anjali Dixit** to the Kusuma School of Biological Science, Indian Institute of Technology Delhi, New Delhi for the award of the degree of '**Doctor of Philosophy**' is a record of the bonafide research work carried out by her, prepared under my supervision in conformity with the rules and regulations of 'Indian Institute of Technology Delhi'. The research report and the results presented in the thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.

Prof. Bishwajit Kundu

Professor

Kusuma School of Biological Sciences

Indian Institute of Technology Delhi New Delhi-110016, India

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ABSTRACT

Optineurin (OPTN) is a multifunctional, ubiquitously expressed cytoplasmic protein. The name 'Optineurin' has been derived from OPTIc NEURopathy INducing protein. The mutants of OPTN are linked with primary open-angle glaucoma (POAG), a pathological condition that causes irreversible blindness and amyotrophic lateral sclerosis (ALS). The presence of OPTN in ocular tissues like the iris, retina, cornea, trabecular meshwork, and lenses is intriguing. This is because mammalian ocular tissues, particularly eye lenses, often experience various stress conditions. Interestingly, the ocular protein OPTN also harbors heat shock elements in its promoter region. Sequence analysis of OPTN exhibits intrinsically disordered regions and nucleic acid binding domains. These properties hinted that OPTN might be endowed with sufficient thermodynamic stability and chaperoning activity. However, these attributes of OPTN have not yet been explored. In the first part of this thesis we have studied these properties in OPTN and its ALS-related mutant through thermal and chemical denaturation experiments and monitored the processes using CD, fluorimetry, differential scanning calorimetry, and dynamic light scattering. We found that upon heating, OPTN reversibly forms higher-order multimers. OPTN also displayed a chaperone-like function by reducing the thermal aggregation of bovine carbonic anhydrase. It regains its native secondary structure, RNA-binding property, and melting temperature (T_m) after refolding from a thermally as well as chemically denatured state.

Considerable progress has been made over a decade in understanding the physiological and pathological functions of OPTN. It is well reported that OPTN is an aggregation prone protein that is involved in neurodegenerative disease progression. OPTN tends to form aggregates under certain stress conditions such as oxidative stress. Moreover, OPTN is associated with the with the RNA binding proteins and stress granules. However Despite such advancements, there

remains a lacuna of knowledge in the fundamental biophysical and aggregation mechanisms of OPTN's involvement in disease progression. To understand the aggregation kinetics of OPTN and its ALS-related mutants E478G and Q398X, we explored their aggregation behaviour in the second part of this thesis. It is known that intrinsically disordered proteins form multivalent interactions which leads to polymerization which eventually undergo phase separation. Similarly, RNA binding protein, under stress conditions, form multivalent interactions with other proteins and RNA molecules to undergo LLPS in the form of stress granules. On sequence and structure analysis it was found that OPTN is intrinsically disordered glutamine rich protein, consisting of both zinc finger and leucine zipper domains, which are known to bind oligonucleotides. This inclined us to propose that OPTN may act as an RNA binding protein having a significant role in stress granule dynamics and phase separation. Therefore we studied its RNA-binding and phase separation properties as well.

From our study we found that OPTN is a protein with significant structural reversibility after denaturation. OPTN is a versatile protein that could mould into a conformational multimer. We found that it interacts with RNA in its native state and also it regains this functionality after refolding. Similar phenomenon was not observed with the mutants, which does not show complete reversibility. Reversible thermal transition of OPTN to multimeric form is of special interest as it displays chaperone-like activity, presumably to perform a myriad of cellular functions. The thermodynamic properties of OPTN presented here lays the foundation for further explorations and validation with additional experiments. Our study gives a reasonable correlation of OPTN’s existence in the ocular tissues, pertaining to its chaperoning potential. We have shown that prolonged stress makes the wild type OPTN and its mutants prone to aggregation and we have established aggregation kinetics *in-vitro*. We found that the wild type OPTN forms worm-like fibres while the mutants form amorphous aggregates. The initial study

of liquid-liquid phase separation and formation of gel like particle further throws light on the complexity of this protein and its progressive role in neurological disorders in the cell. Further studies and exploration of LLPS to solid structure or aggregation pathway will be helpful in designing potential therapeutics to inhibit or delay in OPTN- associated disease progression.

सार

ऑप्टिनेरिन एक बहुक्रियाशील, सर्वव्यापी रूप से व्यक्त साइटोप्लाज्मिक प्रोटीन है। 'ऑप्टीन्यूरिन' नाम ऑप्टिक न्यूरोपैथी उत्प्रेरण प्रोटीन से लिया गया है। ओपीटीएन के म्यूटेंट प्राथमिक ओपन-एंगल ग्लूकोमा से जुड़े हुए हैं, एक रोग संबंधी स्थिति जो अपरिवर्तनीय अंधापन और एमियोट्रोफिक लेटरल स्क्लेरोसिस का कारण बनती है। आईरिस, रेटिना, कॉर्निया, ट्रैब्युलर मेशवर्क और लेंस जैसे ऑक्यूलर टिश्यू में ओपीटीएन की उपस्थिति पेचीदा है। ऐसा इसलिए है क्योंकि स्तनधारी ओकुलर ऊतक, विशेष रूप से आंखों के लेंस, अक्सर विभिन्न तनाव स्थितियों का अनुभव करते हैं। दिलचस्प बात यह है कि ऑक्यूलर प्रोटीन ओपीटीएन भी अपने प्रवर्तक क्षेत्र में हीट शॉक तत्वों को परेशान करता है। ओपीटीएन का अनुक्रम विश्लेषण आंतरिक रूप से अव्यवस्थित क्षेत्रों और न्यूक्लिक एसिड बाइंडिंग डोमेन को प्रदर्शित करता है। इन गुणों ने संकेत दिया कि ओपीटीएन पर्याप्त थर्मोडायनामिक स्थिरता और चैपरिंग गतिविधि से संपन्न हो सकता है। हालाँकि, ओपीटीएन की इन विशेषताओं का अभी तक पता नहीं लगाया गया है। इस थीसिस के पहले भाग में हमने थर्मल और रासायनिक विकृतीकरण प्रयोगों के माध्यम से ओपीटीएन और इसके पेशीशोषी पार्श्व काठिन्य से संबंधित म्यूटेंट में इन गुणों का अध्ययन किया है और सीडी, फ्लोरीमेट्री, डिफरेंशियल स्कैनिंग कैलोरीमेट्री और डायनेमिक लाइट स्कैटरिंग का उपयोग करके प्रक्रियाओं की निगरानी की है। हमने पाया कि गर्म करने पर, ओपीटीएन उत्क्रमणीय रूप से उच्च-क्रम वाले मल्टीमर्स बनाता है। ओपीटीएन ने गोजातीय कार्बोनिक एनहाइड्रेज़ के थर्मल एकत्रीकरण को कम करके एक चैपरोन जैसा कार्य भी प्रदर्शित किया। यह अपनी मूल माध्यमिक संरचना, आरएनए-बाध्यकारी संपत्ति, और पिघलने के तापमान (टीएम) को एक ऊष्मीय और साथ ही रासायनिक रूप से विकृत अवस्था से पुन: प्राप्त करता है।

ओपीटीएन के शारीरिक और रोग संबंधी कार्यों को समझने में एक दशक में उल्लेखनीय प्रगति हुई है। यह अच्छी तरह से बताया गया है कि ओपीटीएन एक एकत्रीकरण प्रवण प्रोटीन है जो न्यूरोडीजेनेरेटिव रोग प्रगति में शामिल है। ओपीटीएन कुछ तनाव स्थितियों जैसे ऑक्सीडेटिव तनाव के तहत समुच्चय बनाता है। इसके अलावा, ओपीटीएन आर एन ए बाइंडिंग प्रोटीन और स्ट्रेस ग्रैन्यूल्स के साथ जुड़ा हुआ है। हालांकि इस तरह की प्रगति के बावजूद, रोग की प्रगति में ओपीटीएन की भागीदारी के मौलिक जैवभौतिकीय और एकत्रीकरण तंत्र में ज्ञान की कमी बनी हुई है। ओपीटीएन और इसके पेशीशोषी पार्श्व काठिन्य से संबंधित म्यूटेंट ई*478*जी और क्यू*398*एक्स के एकत्रीकरण कैनेटीक्स को समझने के लिए, हमने इस थीसिस के दूसरे भाग में उनके एकत्रीकरण व्यवहार का पता लगाया। यह ज्ञात है कि आंतरिक रूप से अव्यवस्थित प्रोटीन बहुस्तरीय अंतःक्रियाओं का निर्माण करते हैं जो पोलीमराइज़ेशन की ओर ले जाती हैं जो अंततः चरण पृथक्करण से गुजरती हैं। इसी तरह, आरएनए बाध्यकारी प्रोटीन, तनाव की स्थिति में, अन्य प्रोटीनों और आरएनए अणुओं के साथ एलएलपीएस से गुजरने के लिए तनाव कणिकाओं के रूप में बहुसंख्यक संपर्क बनाता है। अनुक्रम और संरचना विश्लेषण पर यह पाया गया कि ओपीटीएन आंतरिक रूप से अव्यवस्थित ग्लूटामाइन समृद्ध प्रोटीन है, जिसमें जिंक फिंगर और ल्यूसीन ज़िपर डोमेन दोनों शामिल हैं, जो ऑलिगोन्यूक्लियोटाइड्स को बांधने के लिए जाने जाते हैं। इसने हमें यह प्रस्ताव देने के लिए प्रेरित किया कि ओपीटीएन एक आर एन ए बाइंडिंग प्रोटीन के रूप में कार्य कर सकता है जिसकी तनाव ग्रेन्युल गतिकी और चरण पृथक्करण में महत्वपूर्ण भूमिका है। इसलिए हमने इसके आरएनए-बाइंडिंग और फेज सेपरेशन गुणों का भी अध्ययन किया।

हमारे अध्ययन से हमने पाया कि ओपीटीएन विकृतीकरण के बाद महत्वपूर्ण संरचनात्मक प्रतिवर्तीता वाला प्रोटीन है। ओपीटीएन एक बहुमुखी प्रोटीन है जो एक गठनात्मक मल्टीमर में ढाला जा सकता है। हमने पाया कि यह आरएनए के साथ अपनी मूल स्थिति में इंटरैक्ट करता है और साथ ही यह इस कार्यक्षमता को रिफॉल्ड

करने के बाद फिर से हासिल करता है। म्यूटेंट के साथ इसी तरह की घटना नहीं देखी गई, जो पूर्ण प्रतिवर्तीता नहीं दिखाती है। ओपीटीएन के मल्टीमेरिक रूप में प्रतिवर्ती थर्मल संक्रमण विशेष रुचि का है क्योंकि यह चैपरोन जैसी गतिविधि को प्रदर्शित करता है, संभवतः सेलुलर कार्यों के असंख्य प्रदर्शन करने के लिए। यहां प्रस्तुत ओपीटीएन के थर्मोडायनामिक गुण अतिरिक्त प्रयोगों के साथ आगे की खोज और सत्यापन की नींव रखते हैं। हमारा अध्ययन नेत्र संबंधी ऊतकों में ओपीटीएन के अस्तित्व का एक उचित सहसंबंध देता है, जो इसकी संरक्षिका क्षमता से संबंधित है। हमने दिखाया है कि लंबे समय तक तनाव जंगली प्रकार ओपीटीएन और इसके म्यूटेंट को एकत्रीकरण के लिए प्रवण बनाता है और हमने इन-विट्रो में एकत्रीकरण कैनेटीक्स स्थापित किया है। हमने पाया कि जंगली प्रकार ओपीटीएन कृमि जैसे तंतुओं का निर्माण करता है जबकि म्यूटेंट अनाकार समुच्चय बनाते हैं। तरल-तरल चरण पृथक्करण और जेल जैसे कण के गठन का प्रारंभिक अध्ययन आगे इस प्रोटीन की जटिलता और कोशिका में तंत्रिका संबंधी विकारों में इसकी प्रगतिशील भूमिका पर प्रकाश डालता है। ठोस संरचना या एकत्रीकरण मार्ग के लिए एलएलपीएस के आगे के अध्ययन और अन्वेषण, ओपीटीएन से जुड़े रोग की प्रगति को रोकने या देरी करने के लिए संभावित चिकित्सीय डिजाइन करने में सहायक होंगे।

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ABBREVEATIONS & SYMBOLS

ALS	Amyotrophic Lateral Sclerosis
FTD	Frontotemporal dementia
AD	Alzheimer's disease
RNA	Ribonucleic acid
RBP	RNA binding proteins
IDR	Intrinsically disordered regions
LLPS	Liquid-liquid phase separation
mRNP	Messenger ribonucleoprotein
TDP-43	TAR DNA-binding protein
SOD1	Superoxide dismutase 1
C9ORF72	Chromosome 9 open reading frame 72
FUS	Fused in sarcoma
SG	Stress Granules
OPTN	Optineurin
POAG	Primary open angle glaucoma
TNF-α	Tumor necrosis factor- α
UBD	Ubiquitin-binding domain
LZ	Leucine zipper
ZnF	Zinc finger
LC3	Microtubule-associated protein 1A/1B-light chain 3
LIR	LC3-interacting region
TANK	RAF Family Member Associated NFKB Activator
TBK-1	TANK binding kinase-1

RIP-1	serine/threonine kinase receptor-interacting protein 1
T	Temperature
K	kelvin
R	gas constant
3-D	three dimensional
nm	nanometres
mm	millimetres
μm	micrometres
mM	milli molar
AFM	atomic force microscopy
TEM	transmission electron microscopy
dsRBD	double-stranded RNA-binding domain
RRM	RNA recognition motif
K/R-rich	Lysine/Arginine-rich
PB	Processing bodies
TIA-1	T-Cell-Restricted Intracellular Antigen-1
TTP	Tristetraprolin
G3BP	GTPase-activating protein-binding protein 1
5′-UTR	5’ untranslated regions
NEMO	NF-kappa-B essential modulator
NRP	NEMO-related protein
CYLD	conserved cylindromatosis
MYO6	Myosin VI
TAX1BP1	Tax1-binding protein 1
Aβ	Amyloid β

UV	Ultra violet
PD	Paget Disease
CJD	Creutzfeldt-Jakob disease
RGC5	Retinal Ganglion cells 5
PC12	adrenal phaeochromocytoma
ROS	Reactive oxygen species
WT	wild type
MYOC	myocilin
NTG	normal tension glaucoma
LB	Luria-Bertani
OD	Optical Density
IPTG	isopropylthio-β-galactoside
SDS	Sodium dodecyl-sulfate
PAGE	polyacrylamide gel electrophoresis
Ni-NTA	nickel-nitrilotriacetic acid
kDa	kilo Daltons
°C	degree celcius
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
PMF	peptide mass fingerprinting
CAN	acetonitrile
DTT	dithiothreitol
µg	microgram
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid

SDM	site-directed mutagenesis
. µl	microlitre
Hrs	hours
CD	circular dichroism
Min	minutes
T_m	midpoint of melting temperature
ΔH	enthalpy
ΔS	entropy
ΔG	Gibbs free energy
GdnCl	guanidium hydrochloride
R_h	hydrodynamic radii
%	percent
PBS	phosphate buffer saline
ThT	Thioflavin T
Pa	pascal
kV	kilo volts
s	seconds
bp	base pairs
RU	response units
HMCs	high molecular weight complexes
CC	coiled coil
HSP	heat shock protein
BCA II	bovine carbonic anhydrase II