UNDERSTANDING STRUCTURAL AND MECHANISTIC IMPACT OF AMYOTROPHIC LATERAL SCLEROSIS PATIENT DERIVED MUTATIONS IN D-AMINO ACID OXIDASE

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by

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Dedicated to the Almighty Allah, and my beloved parents, whose unwavering love and support have been my guiding light throughout this journey

Certificate

This is to certify that the thesis titled "**Understanding structural and mechanistic impact of Amyotrophic lateral sclerosis patient derived mutations in D-amino acid oxidase**", submitted by Ms. **Shumayila Khan** to the Indian Institute of Technology Delhi for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by her, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology Delhi, India. The results prescribed in it have not been submitted in part or full to any other University for the award of any degree or diploma.

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Shumayila Khan

Abstract

The D-amino acid oxidase protein modulates neurotransmission by controlling the levels of D-serine, a co-agonist of N-methyl-D-aspartate receptors. Mutations in the DAO gene have been associated with Amyotrophic Lateral Sclerosis with some studies reporting certain pathogenic mechanisms of the R199W mutation. We characterized two novel mutations R38H and Q201R found in ALS patients and report certain novel findings related to R199W mutation. Structure of any DAO mutant identified in ALS patients has yet to be solved. We also report the first instance of crystal structure analysis of a patient-derived mutant of DAO, R38H, to gain insights into the structural implications of this mutation. The structure revealed significant perturbations and altered binding with the cofactor (FAD) and the inhibitor benzoate. These observations were further confirmed through biochemical assays. The Q201R variant exhibited an even more pronounced reduction in binding affinity towards these ligands. Furthermore, we evaluated the kinetic parameters of all variants with three substrates, which demonstrated a diminished oxidase activity and reduced substrate binding for all three mutations. Notably, the R38H variant exhibited oxidase activity levels comparable to WT, but only at very high substrate concentrations, while R199W and Q201R showed drastically diminished levels of activity. Additionally, our findings hint at substantial structural disruptions for both the R199W and Q201R variants. This is supported by the higher oligomeric state observed in the holoenzyme form of both mutants, as well as the thermal instability observed in the case of R199W. We hypothesize that the mutant enzymes may be rendered non-functional in a cellular context, resulting in the possibility of excitotoxicity at the NMDAR receptor. The research provides novel insights into structural and functional aspects of DAO mutations in ALS.

सार

डी-अमीनो एसिड ऑक्सीडेज प्रोटीन एन-मिथाइल-डी-एस्पार्टेट रिसेप्टर्स के सह-एगोनिस्ट डी-सेरीन के स्तर को नियंत्रित करके न्यूरोट्रांसमिशन को नियंत्रित करता है। डीएओ जीन में उत्परिवर्तन को एमियोट्रोफिक लेटरल स्क्लेरोसिस से जोड़ा गया है, कुछ अध्ययनों में आर199डब्लू उत्परिवर्तन के कुछ रोगजनक तंत्रों की रिपोर्ट की गई है। हमने ALS रोगियों में पाए जाने वाले दो नए उत्परिवर्तन R38H और Q201R की विशेषता बताई है और R199W उत्परिवर्तन से संबंधित कुछ नए निष्कर्षों की रिपोर्ट की है। एएलएस रोगियों में पहचाने गए किसी भी डीएओ उत्परिवर्ती की संरचना को अभी तक हल नहीं किया जा सका है। हम इस उत्परिवर्तन के संरचनात्मक निहितार्थों में अंतर्दृष्टि प्राप्त करने के लिए DAO, R38H के रोगी-व्युत्पन्न उत्परिवर्ती के क्रिस्टल संरचना विश्लेषण के पहले उदाहरण की भी रिपोर्ट करते हैं। संरचना में महत्वपूर्ण गड़बड़ी और सहकारक (एफएडी) और अवरोधक बेंजोएट के साथ परिवर्तित बंधन का पता चला। इन अवलोकनों की जैव रासायनिक जांच के माध्यम से और पुष्टि की गई। Q201R वैरिएंट ने इन लिगेंड्स के प्रति बाइंडिंग एफ़िनिटी में और भी अधिक स्पष्ट कमी प्रदर्शित की। इसके अलावा, हमने तीन सब्सट्रेट्स के साथ सभी वेरिएंट के गतिज मापदंडों का मूल्यांकन किया, जिसने कम ऑक्सीडेज गतिविधि और तीनों उत्परिवर्तन के लिए कम सब्सट्रेट बाइंडिंग का प्रदर्शन किया। विशेष रूप से, R38H वैरिएंट ने WT के बराबर ऑक्सीडेज गतिविधि स्तर प्रदर्शित किया, लेकिन केवल बहुत उच्च सब्सट्रेट सांद्रता पर, जबकि R199W और Q201R ने गतिविधि के स्तर को काफी कम दिखाया। इसके अतिरिक्त, हमारे निष्कर्ष R199W और Q201R दोनों वेरिएंट के लिए पर्याप्त संरचनात्मक व्यवधानों का संकेत देते हैं। यह दोनों म्यूटेंट के होलोनीजाइम रूप में देखी गई उच्च ऑलिगोमेरिक अवस्था के साथ-साथ R199W के मामले में देखी गई थर्मल अस्थिरता द्वारा समर्थित है। हम परिकल्पना करते हैं कि उत्परिवर्ती एंजाइमों को सेलुलर संदर्भ में गैर-कार्यात्मक प्रदान किया जा सकता है, जिसके परिणामस्वरूप एनएमडीएआर रिसेप्टर में एक्साइटोटॉक्सिसिटी की संभावना होती है। वर्तमान अध्ययन एएलएस में डीएओ उत्परिवर्तन के संरचनात्मक और कार्यात्मक पहलुओं में नवीन अंतर्दृष्टि प्रदान करता है।

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CHAPTER 3 Material and Methods

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Abbreviations

A_{280}	Absorbance of the protein-dye conjugate at 280 nm
ALS	Amyotrophic Lateral Sclerosis
bp	Base pair
cm	Centimetre
CD	Circular dichroism
°C	Degree Celsius
Da	Dalton
DAO	D- amino acid oxidase
dH2O	Distilled water
ddH2O	Double distilled water (Sterilised distilled water)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ε	Molar extinction coefficient of the solute
FAD	Flavin Adenine Dinucleotide
g	Gram
HCl	Hydrochloric acid
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
kb	Kilo base
k_{cat}	Turnover number
KCl	Potassium chloride
kDa	Kilo Dalton

K_d	Dissociation coefficient
K_m	Michaelis coefficient
l	Sample pathlength
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
mg	Milligram
min	Minute
ml/l	Millilitre per litre
µM	Micromolar
µmole	Micromole
µg	Microgram
mM	Millimolar
M	Molar
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometre
NMDA	N-methyl-D-aspartate
$(NH_4)_2SO_4$	Ammonium sulphate
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein Data Bank

PEG	Polyethyleneglycol
pI	Isoelectric point
Psi	Pounds per square inch
rpm	Revolutions per minute
[S]	Substrate concentration
$S_{0.5}$	Substrate concentration at 1/2 V_{max} (Hill model)
SDS	Sodium dodecyl sulphate
SOC	Super Optimal broth with Catabolite repression
U	µmole NADH/sec
T_m	Melting temperature
UV	Ultraviolet
V_{max}	Maximum reaction velocity
v/v	Volume to volume
WT	Wild Type
w/v	Weight to volume