

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

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

May 2024

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LATERAL SCLEROSIS PATIENT DERIVED
MUTATIONS IN D-AMINO ACID OXIDASE**

by

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Submitted

In fulfillment of the requirements of the degree of Doctor of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

May 2024

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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Acknowledgments

I would like to express my heartfelt gratitude to several individuals and entities who have played pivotal roles in the successful completion of my thesis. First and foremost, I would like to express my heartfelt appreciation to my supervisor, Prof. James Gomes, for his unwavering support throughout my PhD, and for giving me the freedom to pursue my research interests. Prof. Gomes has been a pillar of encouragement throughout my thesis journey, and I am truly grateful for his mentorship.

I extend my sincere appreciation to the members of my SRC: Prof. Aditya Mittal, Prof. Vivekanandan Perumal, and Prof. Shashank Deep. Their constructive feedback and continuous monitoring of my progress played a pivotal role in shaping my research. Their supportive approach made this academic endeavor a truly enriching experience.

I am deeply appreciative of the invaluable learning experiences I have gained during my journey, and I would like to extend my heartfelt gratitude to Dr. Ravindra Makde, Dr. Biplab Ghosh, and Dr. Ashwani Kumar for their generous and indispensable support in the field of crystallography and diffraction. Their extensive knowledge and technical expertise were instrumental in facilitating the successful execution of my crystallographic experiments. I would like to offer a special commendation to Dr. Ashwani Kumar for his exceptional proficiency in structure solving, a pivotal contribution that led to the elucidation of the mutant protein structure and the understanding of its significance.

I would like to express my sincere appreciation for my friend Saurabh Upadhyay, whose companionship has consistently provided me with strength during my academic journey. Both of us started our respective PhD journeys together and Saurabh has made significant contributions to my research work. His exceptional planning skills played a pivotal role in

assisting me with my work, and I was truly fortunate to have such a dedicated friend and research companion through this often daunting journey.

I would also like to thank all the dedicated members of our lab, with a special mention of Dr. Upma Dave, who has been like a big sister to me. Her constant support and guidance during challenging times were invaluable, and I am deeply appreciative of her presence in my academic journey as well as of her contributions to my work.

I am grateful to all the students, and staff of KSBS, IITD, for their tremendous support and camaraderie. I wish to express my appreciation to the entire faculty at KSBS for their generosity in providing unrestricted access to their laboratories, and access to essential facilities during my research journey. The collaborative environment at our institution has been instrumental in my work.

I am profoundly indebted to my parents, whose support and sacrifices have been the cornerstone of my academic pursuits. Their boundless love, encouragement, and belief in my abilities have been a constant source of motivation. Everything I have achieved is a testament to their dedication and guidance, and I am forever grateful for the opportunities they have provided me. In addition, my sister holds a special place in my heart, and her unwavering support during the challenging moments of my journey has been a beacon of light. Her empathy, understanding, and also professional help in my work have been instrumental in my growth, and I cherish the deep bond we share.

Lastly, but most importantly, I express my profound gratitude to Almighty Allah for granting me the strength, wisdom, and perseverance to complete this thesis successfully. It is through His blessings that I have reached this milestone in my academic journey.

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 उत्परिवर्तन 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 उत्परिवर्तन 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 सबस्ट्रेट्स के साथ सभी वैरिएंट के गतिज मापदंडों का मूल्यांकन किया, जिसने कम ऑक्सीडेज गतिविधि और तीनों उत्परिवर्तन के लिए कम सबस्ट्रेट बाइंडिंग का प्रदर्शन किया। विशेष रूप से, R38H वैरिएंट ने WT के बराबर ऑक्सीडेज गतिविधि स्तर प्रदर्शित किया, लेकिन केवल बहुत उच्च सबस्ट्रेट सांद्रता पर, जबकि R199W और Q201R ने गतिविधि के स्तर को काफी कम दिखाया। इसके अतिरिक्त, हमारे निष्कर्ष R199W और Q201R दोनों वैरिएंट 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 उत्परिवर्तन 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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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
dH ₂ O	Distilled water
ddH ₂ O	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
$\mu mole$	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	$\mu\text{mole NADH/sec}$
T_m	Melting temperature
UV	Ultraviolet
$V_{\max}$	Maximum reaction velocity
v/v	Volume to volume
WT	Wild Type
w/v	Weight to volume