

## **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.