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Brief Article

Structural Discovery of Small Molecule Binding Sites in Cu−Zn Human Superoxide Dismutase Familial Amyotrophic Lateral Sclerosis Mutants Provides Insights for Lead Optimization†

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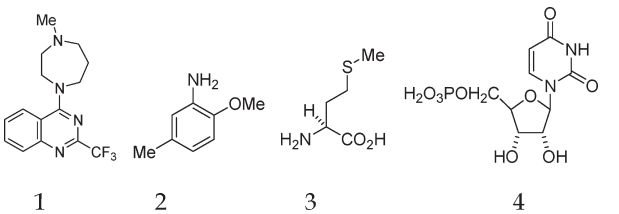
Dominant inheritance of point mutations in CuZn superoxide dismutase (SOD1) is the best characterized subset of familial amyotrophic lateral sclerosis (FALS) and accounts for some 20% of the known familial cases. We report the discovery and visualization via cocrystallography of two ligand-binding pockets in human SOD1 and its pathogenic mutants that have opened up the real possibility of undertaking lead compound discovery using a fragment-based approach for therapeutic purposes for SOD1 associated motor neuron disease.

Introduction

The neurodegenerative disease amyotrophic lateral sclerosis (ALS[a]), also known as motor neuron disease (MND) or Lou Gehrig's disease, is characterized by progressive paralysis arising from degeneration of the upper and lower motor neurons.[1] It was once thought to be a rare disease but is now considered to be fairly common. The cumulative lifetime risk for MND is ∼1 in 1000, which is comparable with the occurrence rate of multiple sclerosis. The disease onset is variable, but usually patients succumb to the disease within 3−5 years after the diagnosis, making the number of living patients at any one time relatively small. The only currently approved agent for the treatment of MND is riluzole, but it is only weakly effective, providing a 9% gain in the chance of survival for 1 year after initiation of treatment.

Bob Brown, Teepu Siddique, and their teams provided a major breakthrough in 1993 when a link with the gene coding SOD1 (Cu−Zn superoxide dismutase) was discovered that accounts for approximately 2% of all cases and about 20% of all heredity cases.[2] There is strong evidence that the extent of metalation, both Cu and Zn, of mutant SOD1 is an important factor in the process of aggregation.[3−6] The Cu and Zn atoms have an essential role in enabling the formation of stable and well ordered "electrostatic" and "Zn binding" loop regions (Figure 1, Supporting Information). Structural data from X-ray crystallography have shown that in the metal deficient FALS SOD1 mutants apo-S134N, apo-H46R, and Zn-H46R[7,8] and G85R[9] these loops are disordered, leading the SOD1 functional dimers to combine, producing amyloid-like linear or helical filaments. Metal deficient wild-type SOD1 also showed evidence for the existence of amyloid-like structures, with near linear "zigzag" filaments being formed from alternating ordered−disordered dimers.[10] Molecular dynamics calculations of wild-type SOD1 have shown that large spatial and temporal fluctuations of the "electrostatic" and "Zn-binding" loops occur in the metal-deficient enzyme, exposing the β-barrels of the dimer to the external environment and allowing interactions with adjacent molecules as a first step toward aggregation.[11] The same loops have also been implicated by molecular dynamics calculations in the disruption of long-range communication in the dimer molecule of the metal-deficient H46R and G37R mutants.[12] It is possible that oxidative damage and aggregation work together to form the toxic properties of FALS SOD1.[13−15]

In the case of interface mutants, A4V and I113T, which are among the most severe mutants, structural data have shown the destabilization of the molecule,[16] leading to the suggestion that the dimers may readily dissociate into monomers[17] which can then oligemerize to form aggregates.[18] The introduction of an intersubunit disulfide bond, by mutating residue 148 in A4V, did completely abolish aggregation of the mutant.[19] Lansbury and co-workers have followed this by extensive in silico screening of some 1.5 million commercially available compounds to assess their binding at the dimer interface around residue 148 which was used for introduction of the disulfide bond.



1 2 3 4

Figure 1. Chemical sketch for 2-trifluoromethyl-4-aminoquinazoline (**1**), aniline (**2**), L-methionine (**3**), and uridine-5′-monophosphate (UMP) (**4**).

†The atomic coordinates have been deposited in the Brookhaven Protein Data Bank (accession numbers 2wyt, 2wyz, 2wz6, 2wz0, and 2wz5).

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[a] Abbreviations: SOD1, human Cu/Zn superoxide dismutase; FALS, familial amyotrophic lateral sclerosis; ALS, amyotrophic lateral sclerosis; MND, motor neuron disease; UMP, uridine-5′-monophosphate; ICOSA, International Consortium on SOD and ALS.

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The hundred top solutions were tried in aggregation assays, and their effects were compared with A4V and wild-type SOD1. Fifteen compounds were shown to significantly slow the aggregation of A4V in cell line studies,[20,21] with four of these being the most effective in reducing aggregate formation. Efforts to visualize these compounds in SOD1 or mutants have not been possible via cocrystallization, and as such, it remains unknown where these compounds bind and how they can be improved. Given that these compounds are uracil based, we attempted to soak uridine 5′-monophosphate (UMP) (Figure 1) in crystals of the L38V SOD1 that were readily available. Like A4V and I113T, L38V is also one of the most severe disease-causing mutations, where the residue forms a hydrophobic "plug" at one end of the β-barrel of each SOD1 monomer and is distant from the Cu or Zn atom or the dimer interface. The UMP molecule was clearly visible in the 1.7 Å resolution crystallographic structure, but surprisingly it was not at the interface but in a groove between the electrostatic and Zn-binding loops. A cocrystallization screening of a commercially available fragment library revealed another binding site near tryptophan 32 when 2-methoxy-5-methylaniline and L-methionine (Figure 1) were clearly visible in L38V soaked crystal structures. The same binding site was found to be occupied when G93A SOD1 crystals were soaked with a quinazoline based compound. Overlaying of these fragments/compounds found in this site, we call it the "druggable site",

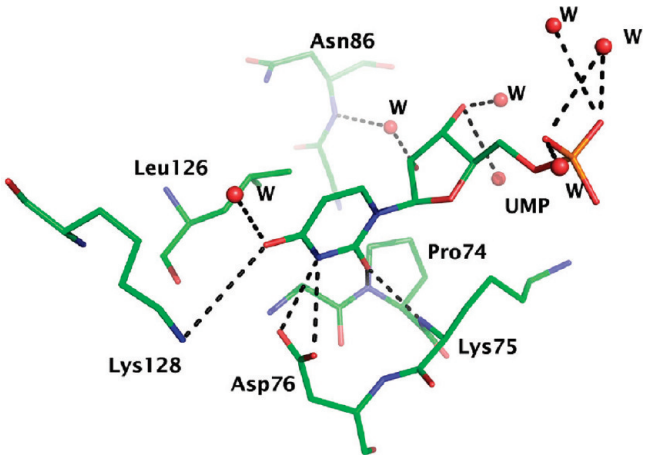

Figure 2. The UMP binding site is located in the groove between the electrostatic and Zn binding loops. H-Bonding network involving residues Lys128, Asn86, Asp76, Lys75, Pro74, and the water molecules are indicated by black dashes.

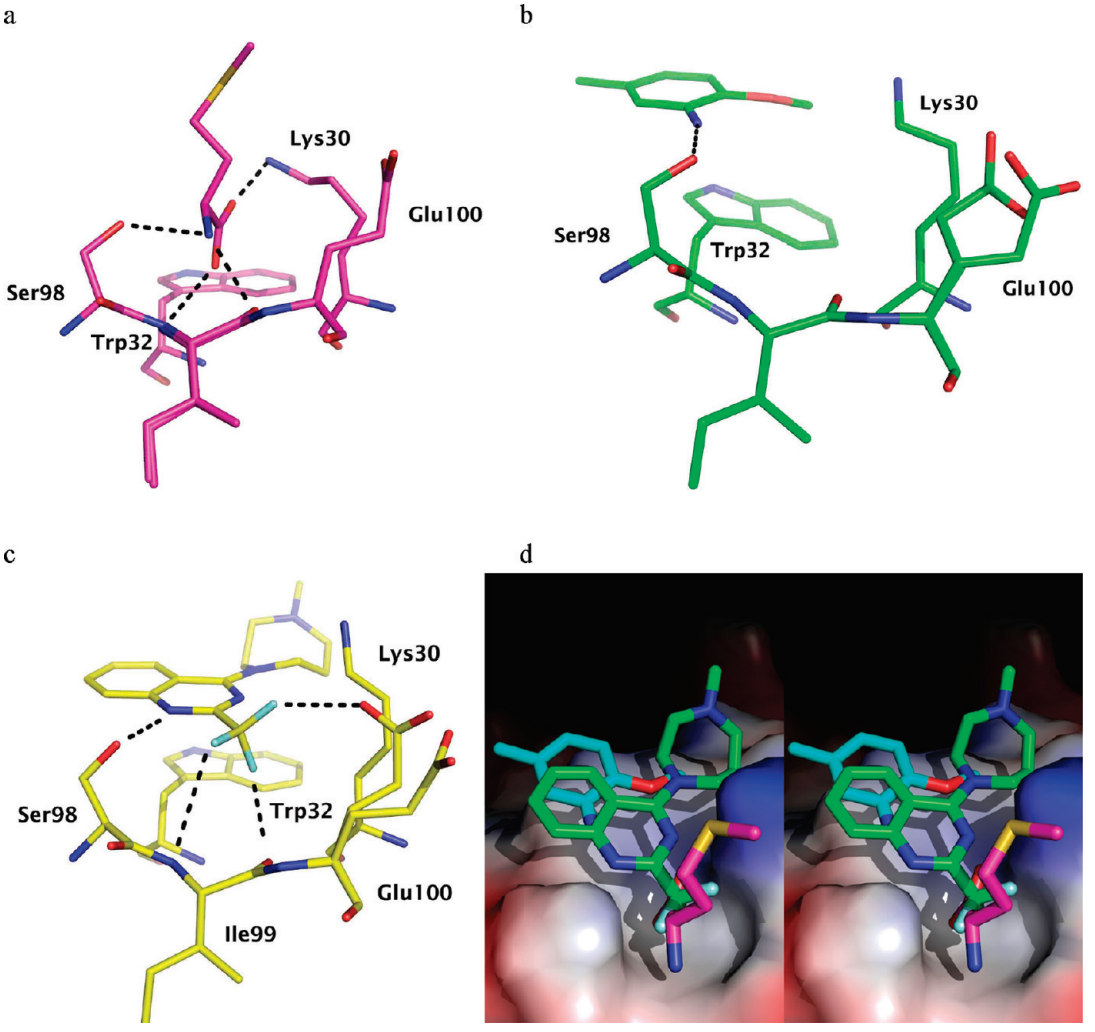

Figure 3. Crystallographic observation from cocrystallization of chemical complexes in what we have termed the "druggable" site adjacent to Trp32. Shown are structures revealing close interactions with compounds/fragments: (a) L-methionine; (b) aniline (2-methoxy-5-methylaninline); (c) 2-trifluoromethyl-4-aminoquinazoline; (d) stereo-overlay of the three molecules in this binding site. Hydrogen bonds are indicated by black dashes.

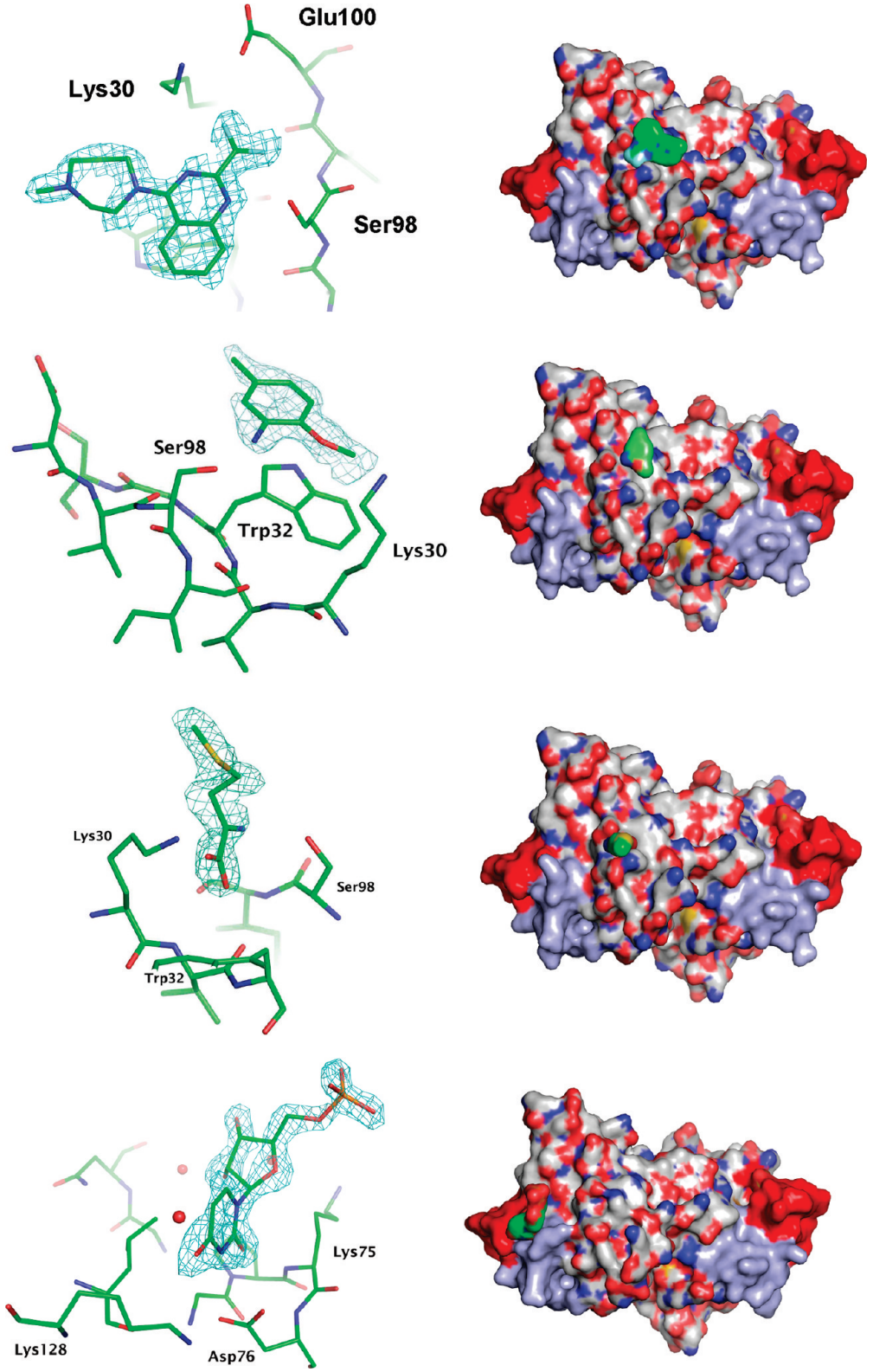

Figure 4. On the left: $2F_o - F_c$ electron density maps contoured at 1σ showing (a) quinazoline; (b) 2-methoxy-5-methylaniline (binding sites (a) and (b) are positioned above the Trp32 residue); (c) L-methionine, located close to Trp32 residue; (d) UMP with residues Lys128, Asp76, Lys75, Pro74, and water molecules. On the right is a model showing the ligand (green) locations on the SOD1 dimer surface. UMP binds in the groove between the electrostatic (pale-lilac) and Zn binding loops (red). A chloride ion is also found in this location in the native enzyme structure (see Supporting Information).

as a number of compounds reported here were found in this site where it is clear how the lead optimization can be undertaken. We thus present four ligand/fragment bound crystal structures of SOD1 FALS mutants and discuss the potential for two binding sites, which may be amenable to pharmacological agents.

UMP Binding Site

The UMP binding site in L38V is located in a groove between the electrostatic and Zn-binding loops on the surface of the protein (Figures 2 and 4). A chloride ion binding site has also been found close to this position (Supporting Information, Figure 3). Five hydrogen bonds to UMP are provided by Lys128, Lys75, Asp76, Pro74, and five additional hydrogen bonds are provided by water molecules. The UMP molecule is only bound directly to SOD1 by its pyrimidine base, and there is no direct contact between protein and the ribosyl group. The phosphate group of UMP has the weakest electron density and is hydrogen-bonded to three water molecules. The carboxylic group of Asp76 accepts two potential hydrogen bonds from the N3 pyrimidine atom, while one water molecule forms a hydrogen bond to the O4 atom that in its turn donates its hydrogen bond to another water molecule. The amide group of Lys75 forms a hydrogen bond to the pyrimidine O2 atom and water molecule. In the presence of UMP, the Cα atom of Lys75 moves toward the ligand by 0.6 Å and its side chain shifts away from the binding site to accommodate the binding.

"Druggable" Site

Crystallographic screening of RIGAKU's fragment library with readily available L38V crystals provided two hits and revealed clearly the binding site in proximity to Trp32, which we label "druggable" site[22] (Figures 3 and 4). Previously, oxidation of Trp32 has been shown to result in covalent aggregations of SOD1, and this "gain of function" mechanism may be linked to ALS.[23] The 1.65 Å resolution L38V/2-methoxy-5-methylaniline complex structure clearly showed alinine in electron density immediately over Trp32 and making contact with Ser98 (Figure 3b). The 1.5 Å resolution crystallographic structure of the L38V/L-methionine complex again shows clear density for L-methionine. The amino acid functionality of L-methionine in the structure protrudes deeply into the hydrophilic pocket adjacent to Trp32, with hydrogen bonds observed between the amino group and Ser98, and its carboxylate oxygens are hydrogen-bonded to O,N of Ile99 and NZ of Lys30 (Figure 4). The finding that an amino acid extends into this pocket may be of special significance because it suggests that this binding site is most probably biologically relevant. It is pretty likely that it gets occupied in vivo by one or more free amino acids or peptides (e.g., N-terminus of glutathione). A sample of 2-trifluoromethyl-4-aminoquinazoline (for which a patent application is pending[24]) was kindly provided by Drs. Dan Benjamin and Allen Reitz of Alsgen. We were successful in obtaining the structure of it bound in the G93A mutant. The resolution of this structure was again high (1.55 Å) allowing clear observation of the compound. The quinazoline ring of 2-trifluoromethyl-4-aminoquinazoline (Figure 3c) is located directly over the indole of Trp32; it has a single hydrogen bond with OG1 of Ser98, and its trifluoromethyl group extends down into a hydrophilic pocket where it makes two hydrogen bonds with OE1 and O of Glu100. Overlaying of 2-trifluoromethyl-4-aminoquinazoline, aniline, and L-methionine using the X-ray coordinates for all three structures (Figure 3d) provides helpful insight into how better compounds may be designed. When viewed from above the plane of Trp32, the phenyl ring of aniline is above the indole of Trp32 but shifted ~5 Å when compared to the quinazoline ring of 2-trifluoromethyl-4-aminoquinazoline. When viewed from the side, L-methionine occupies the hydrophilic cavity partially filed by the trifluoromethyl group of 2-trifluoromethyl-4-aminoquinazoline, and importantly, one of the fluorines overlays with the carbonyl of the carboxylate of L-methionine. By use of this direct information, compounds can be evolved optimizing various interactions while ensuring that solubility of lead compounds and their size are retained according to Lipinski's rule of five.

Direct observation of several compounds in FALS associated mutants of SOD1 in two binding sites is provided. This breakthrough should provide a good basis for knowledge-based lead optimization of compounds that are druggable for this debilitating disease.

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Supporting Information Available: FALS SOD1 mutation sites, chloride binding site, crystallization, data collection, refinement and model building, preparation of complexes, and quality of the crystallographic models. This material is available free of charge via the Internet at http://pubs.acs.org.

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