Mutational analysis of lysine-coordinated superoxide ionophores on the surface of SOD1

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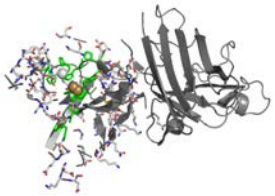

Amyotrophic Lateral Sclerosis (ALS) is a common neuromuscular disease without effective treatment characterized by the selective degeneration of motor neurons resulting in paralytic death. In a familial subset of ALS, mutant Cu/Zn superoxide dismutase (**SOD1**) enzymes initiate and sustain this disorder dose-dependently. SOD1 is a highly conserved 153-residue *β*-barrel homodimer that serves as the predominant intracellular scavenger of neurotoxic superoxide anion radical. Determining the gain-of-function mechanism of SOD1 toxicity for therapeutic intervention in ALS has been the subject of continuing research for over fifteen years.

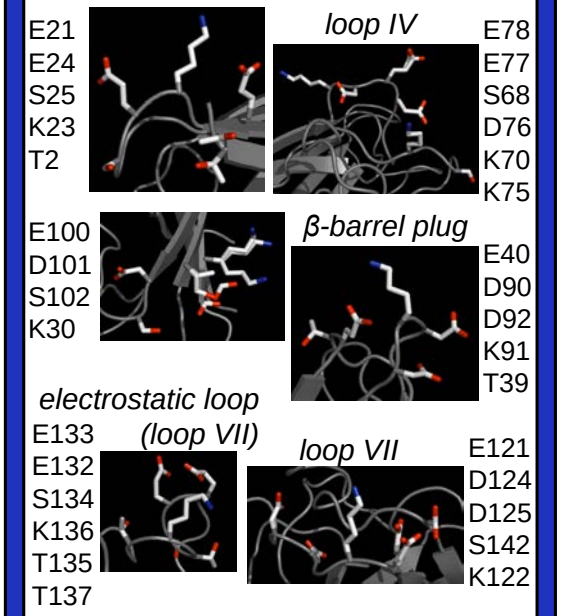

Fig 2. Cutaways showing ionophores (from referenced crystal structure PDB ID 2c9v).

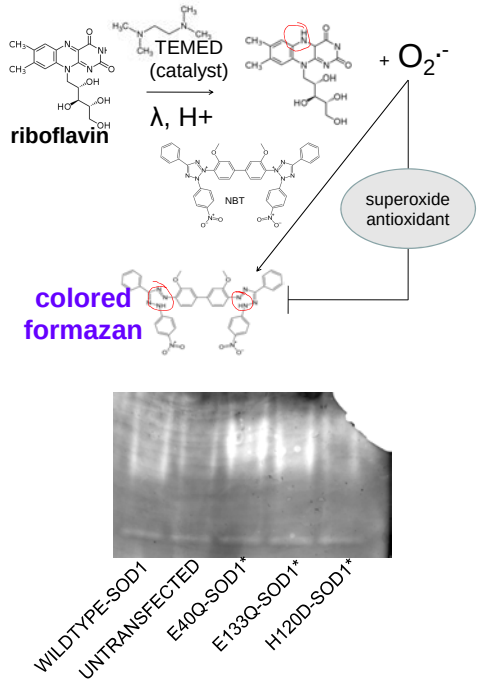

Fig 3. Mechanism of riboflavin - nitro blue tetrazolium superoxide radical antioxidant activity assay and representative native-PAGE demonstrating variable mutant SOD1 scavenging activities.

SUMMARY AND CONCLUSIONS

- We have discovered structural motifs on the surface of SOD1 that act as nodal ionophores for superoxide anion substrate.
- Using this paradigm we have cloned the enzymatically fastest species thus far assayed (E40Q), faster than the nucleophilic shift mutant E133Q at the electrostatic triad (Getzoff *et al.*)
- Unlike dozens of toxic SOD1 species thus far examined, E133V retains an oxidized intradisulfide and therefore does not aggregate at 48 h.

These early and exciting findings depict a superelectrophilic apo-SOD1 that acquires radical charge at focal lesions, nucleating polymerization and toxicity. This intermediate is cleared in the active intraoxidized/metallated state.

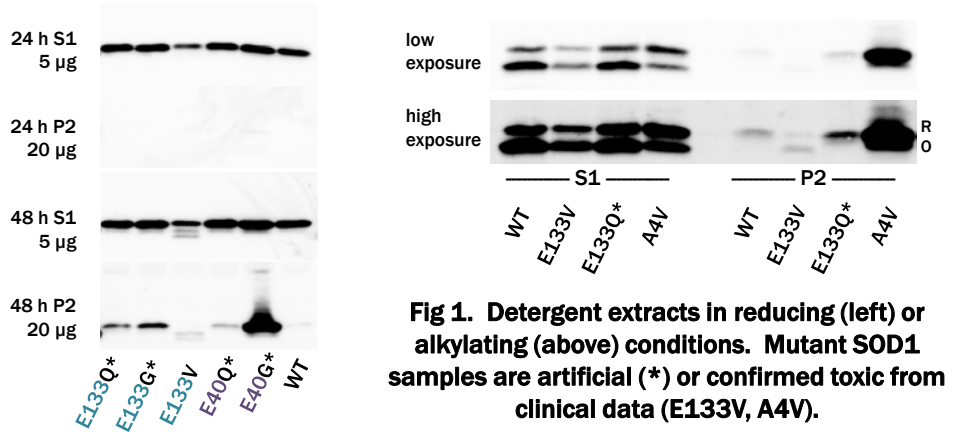

Fig 1. Detergent extracts in reducing (left) or alkylating (above) conditions. Mutant SOD1 samples are artificial (*) or confirmed toxic from clinical data (E133V, A4V).

REFERENCES

ED Getzoff et al. "Faster superoxide dismutase mutants designed by enhancing electrostatic guidance." 1992 *Nature* 358:347-351.

Beauchamp C and Fridovich I. "Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels." 1971 *Analytical Biochemistry* 244:276-287.

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MATERIALS AND METHODS

Cell culture and transfections. SOD1 cDNAs coded in the mammalian expression vector pEF-BOS were prepared by double CsCl/EtBr density gradient and confirmed by automated sequencing and agarose electrophoresis. 4 µg cDNA transfectant was prepared with Lipofectamine 2000 (Invitrogen) and added to confluent 60mm dishes of human embryonic kidney cells (HEK cells, line HEK293FT). Cells were passaged in high-glucose DMEM with 10% horse serum supplemented with L-glutamine. Fresh media was added 4h after transfection, and cell pellets were harvested to -80˚C for storage after rinsing three times in phosphate-buffered saline (PBS).

SOD1 aggregate extraction and immunoblotting. Cell pellets were thawed on ice and resuspended in 100 µL 1x TEN (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0, 100 mM NaCl). 100 µL 1x TEN with 1% Nonidet P40 (NP-40) and 1% protease inhibitor cocktail (PI, Roche) was then added for a final concentration of 0.5% NP-40. This mixture was then sonicated and centrifuged for 5 min at >100,000 x *g* in a Beckman Airfuge. The soluble portion was saved as *S1* and the pellet resuspended in 200 µL of rinse buffer: 1x TEN with 0.5% NP-40 and 1% PI. The pellet was sonicated and centrifuged again, this time discarding the soluble fraction. The pellet was finally resuspended and resonicated in 1x TEN with 0.5% NP-40, 0.25% SDS, 0.5% deoxycholate, and 1% PI and saved as *P2*. Protein concentrations were assayed by the BCA method (BSA standard). 5 µg S1 or 20 µg *P2* were brought up to 20 µL in 1x TEN with laemmli buffer (5% β-mercaptoethanol). Samples were boiled at 95 ˚C for 6 min and loaded on 18% tris-glycine acrylamide gels. Gels were transferred to nitrocellulose membrane for 2 h at 400 mA and then blocked for 15-40 mins in 5% lowfat dry milk in PBS-T (PBS with 0.1% Tween-20). Primary anti-m/hSOD1 antibody was added 1:5000 in milk from PBS-T for 1-16 h followed by 3x rinse in PBS-T alone and secondary goat anti-rabbit HRP at 1:2500 in milk from PBS-T for 1 h. Blots were rinsed again and visualized and pixel-quantified with ECL chemiluminescence on a Fujifilm LAS-3000. **Superoxide anion scavenging activity assay.** < 100 µg total cell lysate prepared from 10x pellet volume sonicated 0.1% NP-40 in 1x TN was run on 8% or 4-20% tri-glycine acrylamide gels in 1x TG with 20% methanol at 4˚C and 100 constant V for 4-6 h, without denaturant or reductant added. The gel was retrieved and soaked in 50 mM potassium bicarbonate buffer containing 65 µg/mL riboflavin and 280 µg/mL nitro blue tetrazolium, pH 7.6 (5). After incubating 2-40 m, the solution was aspirated and 0.1% TEMED in 50 mM potassium bicarbonate buffer was added to the gel. The gel was immediately exposed to white light from a bright box and imaged on a Agfa Duoscan, with contrast increased.