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ORIGINAL ARTICLE

Exon 5 encoded domain is not required for the toxic function of mutant SOD1 but essential for the dismutase activity: identification and characterization of two new *SOD1* mutations associated with familial amyotrophic lateral sclerosis

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ABSTRACT

Two new mutations in the gene encoding cytoplasmic Cu,Zn superoxide dismutase (SOD1) have been discovered in patients with familial amyotrophic lateral sclerosis (FALS). These mutations result in the truncation of most of the polypeptide segment encoded by exon 5, one by the formation of a stop codon in codon 126 (L126Z) and the other by inducing alternative splicing in the mRNA (splicing junction mutation). These two mutants of SOD1 result in a FALS phenotype similar to that observed in patients with missense mutations in the *SOD1* gene, establishing that exon 5 is not required for the novel toxic functions of mutant SOD1 associated with ALS. These mutant enzymes are present at very low levels in FALS patients, suggesting elevated toxicity compared to mutant enzymes with single site substitutions. This increased toxicity likely arises from the extreme structural and functional changes in the active site channel, β-barrel fold, and dimer interface observed in the mutant enzymes, including the loss of native dismutase activity. In particular, the truncation of the polypeptide chain dramatically opens the active site channel, resulting in a marked increase in the accessibility and flexibility of the metal ions and side chain ligands of the enzyme active site. These structural changes are proposed to cause a decrease in substrate specificity and an increase in the catalysis of harmful chemical reactions such as peroxidation.

Keywords: Cu, Zn superoxide dismutase (SOD1), amyotrophic lateral sclerosis (ALS)

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder characterized by degeneration of large motor neurons in the motor cortex, brain stem and spinal cord (1). About 5–10% of ALS cases are familial, and the rest are sporadic (2). Familial ALS (FALS) is inherited in most cases as an autosomal dominant trait, and ~25% of FALS is caused by mutations in the gene for cytoplasmic Cu, Zn superoxide dismutase (SOD1) (based on the calculation of 230 FALS families in our collection) (3–5). Over 50 mutations located in exons 1, 2, 4 and 5 of the *SOD1* gene have been found in FALS families. Almost all of the mutations are missense mutations and result in single amino acid substitutions in the polypeptide chain of SOD1 (6). Several lines of transgenic mice that overexpress different *SOD1* mutants have a phenotype similar to that of ALS patients, suggesting that the phenotype observed in the transgenic mice

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is due to a gain of toxic function by mutant SOD1. The *SOD1* knockout mice do not develop the ALS phenotype, but their motor neurons are more vulnerable to axonal injury (7,8). Therefore, it has been postulated that the motor neuron degeneration in FALS is primarily due to a gain of toxic function by SOD1, although loss of dismutase function of SOD1 as a contributing cause cannot be excluded.

The total SOD1 activity in red blood cells from FALS patients is ~40–90% when compared to the controls (6). Several SOD1 mutants expressed in eukaryotic cell lines have a shorter half life than that of the wild type (8). These and the observations related to the position of the mutations in the wild type SOD1 structure collectively suggest that SOD1 mutants may have an altered structural conformation compared to the wild type. The alteration in conformation is the only common element in all SOD1 mutations and is probably required for the novel toxic property of mutant SOD1. In this study, we characterised two recently identified FALS-associated SOD1 mutations. The first mutation changes codon 126 into a stop codon and the second is an intronic mutation which results in the formation of a new splicing site. In the second mutation, the abnormal splicing of mRNA causes a frame shift in the downstream sequence. Our study suggests that a conformational change which includes changes in the active site is induced by these two mutations and a minimal amount of these mutant SOD1 may be sufficient to cause motor neuron degeneration in FALS.

MATERIALS AND METHODS

Patient samples

Two patients from unrelated families were included in this study. Both patients have a family history of ALS. The first patient (patient 1), a 58 year old male, has a 4-year history of progressive muscle weakness and atrophy. The second patient (patient 2), a 72 year old male, has slowly progressive symptoms of muscle weakness and atrophy. Both patients were diagnosed with ALS based on their history, physical and electromyographic examinations. Blood samples were obtained after informed consent.

DNA sequencing and RT-PCR

Genomic DNA was isolated from white blood cells of the patients using a DNA extraction kit (Gentra, NC). The extracted DNA was specifically amplified by intronic primers of the *SOD1* gene (5), and the amplified DNA was sequenced using an ABI 373 autosequencer. Total mRNA of transformed lymphoblastoid cells from patient 2 was extracted according to the published protocol for single step RNA extraction (9). To amplify the SOD1 mRNA, a forward primer 5′-GAC AAA GAT GGT GTG GCC GA-3′ (started at codon 90 in exon 4), and a reverse primer 5′-CTA CAG CTG GCA GGA TAA CA-3′ (started at the 59 bp downstream from termination codon) were synthesized according to the published sequence of *SOD1*. RT-PCR was performed according to the protocol from RT-PCR kit (Perkin Elmer, NJ). The DNA fragments amplified by RT-PCR were directly sequenced. Meanwhile, the amplified DNA fragments were cloned into pUC18 plasmid vector. DNA extracted from the transformed *Escherichia coli* clones was also sequenced in order to confirm the mutations found in the mRNA.

Western blot analyses of SOD1

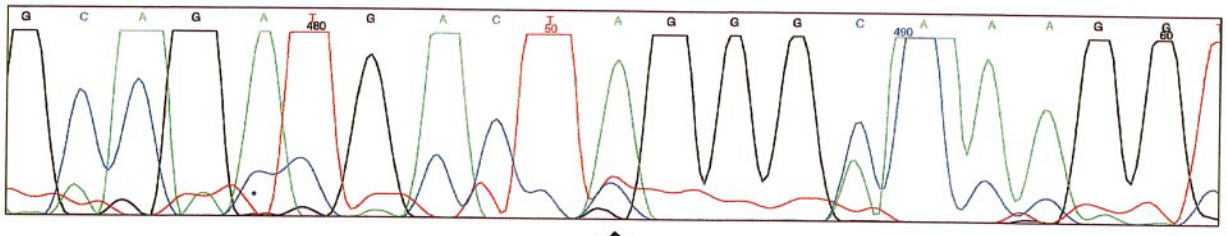
The wild type and mutant SOD1 polypeptides in red blood cells (RBC), transformed lymphocytes and the recombinant SOD1 expressed in *E.coli* were examined by Western blot. The RBCs were isolated from whole blood by centrifugation at room temperature. After removing the serum, the RBCs were washed three times with PBS (phosphate buffered saline, pH 7.2) and stored at –80°C. The cells were lysed by a freeze-thaw cycle and repeated at least two times. To remove hemoglobin, the samples were passed through a DEAE-cellulose column in 8 mM Tris-acetate buffer (pH 6.8). Proteins collected from this column were then analyzed by Western blot. The lymphocytes were collected from the whole blood using gradient centrifugation in a lymphocyte separation medium (Organon Teknika, the Netherlands). The collected lymphocytes were then transformed by Epstein-Barr virus according to published procedures (10). The transformed cells, maintained in cell culture medium (RPMI1640 plus 10% of bovine calf serum), were collected and washed three times with PBS to remove the residual medium. Cells were lysed by freeze-thaw cycles as described in the preparation of RBC lysate. After centrifugation at 4°C, the supernatant was collected for analyses in Western blot.

Samples for Western blot analyses were loaded onto a 15% SDS–polyacrylamide gel for electrophoresis under reducing conditions. The separated polypeptides in the gel were transferred onto a nitrocellulose membrane. The membrane was blocked by incubation with 3% bovine serum albumin (BSA) in PBS at 4°C overnight to reduce any nonspecific binding. The membrane was then incubated with a rabbit anti-human SOD1 antibody (1: 500 diluted in 1% BSA/PBS) at room temperature for 2 h. After washing the membrane in PBS three times, a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (1:1000 diluted in 1% BSA/PBS) was incubated with the membrane for another 1 h at room temperature. The membrane was then washed again three times in PBS. Color reaction was developed with the HRP substrate, 4-chloro-1-naphthol, for 5–15 min. When the control SOD1 band was clearly visible, the reaction was stopped by transferring the membrane into water.

Site-directed mutagenesis and recombinant SOD1 expression

The SOD1 cDNA was inserted into a prokaryotic expression vector pSE420 (Invitrogen Corporation, CA) at the *Nco*I restriction enzyme site. The orientation of the insertion and the sequence of SOD1 cDNA were confirmed by direct sequencing. A pair of oligonucleotide primers that contain the designed mutations according to the cDNA sequence of mutant *SOD1*, were synthesized for making each of the mutants. Mutation was made in the cDNA sequence of SOD1 by polymerase chain reaction (PCR) using a site-directed mutagenesis kit (Quick Change kit, Stratagene Corporation, CA). The PCR-amplified plasmid was digested by *Dpn*I which removes only methylated DNA. Subsequently, the amplified plasmid was transfected into competent *E.coli* (XL1-blue, Stratagene Corporation, CA) and mutation in the SOD1 coding sequence was confirmed by direct DNA sequencing.

A



120 123 126 127 **T to A**

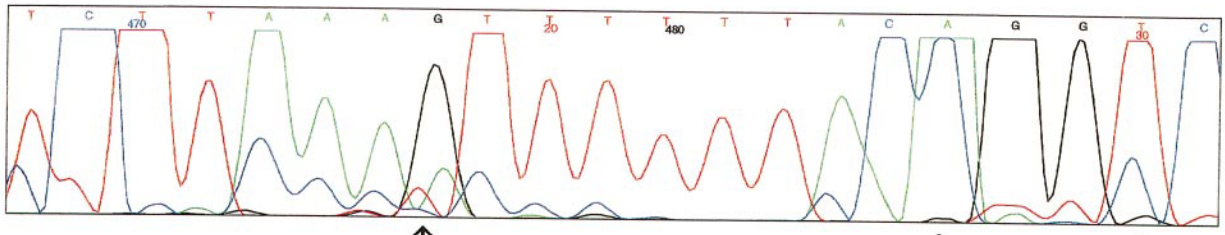
Val His Glu Lys Ala Asp Asp Leu Gly

GTC CAT GAA AAA GCA GAT GAC TTG GGC

↓

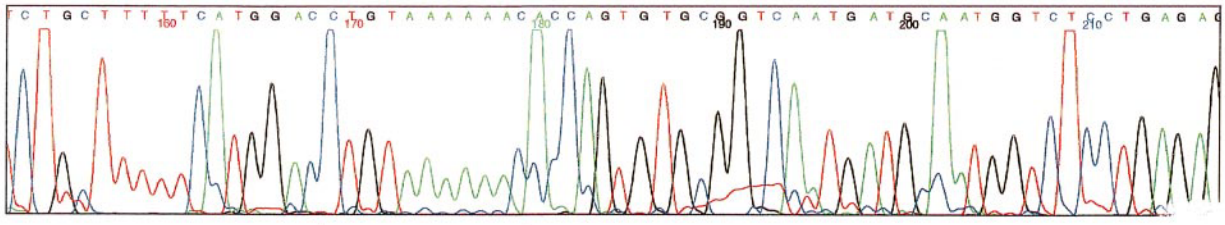
TAG

B



A to G **splicing site : AGGT**

C



10 bp insertion

115 118 121

Arg Thr Leu Val Val His Glu

CGA ACA CTG GTG GTC CAT GAA

118 123

CGA ACA CTG GTG **TTT TTT ACA G**GT CCA TGA

Arg Thr Leu Val **Phe Phe Thr Gly Pro** stop

Figure 1. DNA sequencing of patients' genomic DNA and mRNA. DNA was PCR-amplified from both patients' genomic DNA and the amplified fragments were directly sequenced. Patient 1 has a T→A transition in codon 126 of *SOD1* gene (**A**). Patient 2 has intronic mutation (A→G) at 11 bases upstream from the intron–exon junction of exon 5 (**B**). The DNA amplified from mRNA of the lymphoblastoid cells derived from patient 2 were cloned and amplified. Ten bases of DNA, TTT TTT ACA G, were found inserted between 118 (in exon 4) and codon 119 (in exon 5) of *SOD1* gene (**C**). This 10 base insertion changed the reading frame after codon 118 (exon 4). Five novel amino acid residues, Phe-Phe-Thr-Gly-Pro would be inserted in the polypeptide sequence of SOD1 before the formation of the stop codon at position 124 (C).

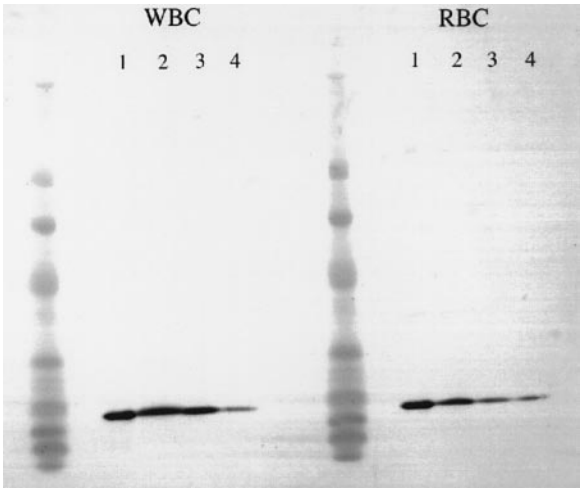


Figure 2. Western blot analyses of SOD1 expressed in the RBC and lymphoblastoid cells derived from patients. Proteins extracted from RBC and lymphoblastoid cells of patient 1 with L126Z (lane 3) or patient 2 with junction mutation (lane 4) of *SOD1* were separated in 15% SDS–PAGE and blotted with an anti-SOD1 antibody from rabbit serum (1:1000 diluted). The proteins from RBC were partially purified by using a DEAE-cellulose column before loading on the gel. A standard SOD1 sample (lane 1) and a SOD1 sample from a person with wild type *SOD1* gene (lane 2) were run in parallel as controls.

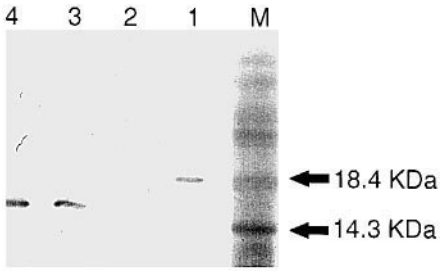


Figure 3. Western blot analyses of recombinant SOD1 expressed in *E.coli*. Equal amounts of total proteins were extracted from host cells that harbor the cDNA of wild type SOD1 (lane 1), L126Z (lane 3) and junction mutation (lane 4), and were loaded on a 15% SDS–PAGE. The proteins extracted from the host cells that do not contain the *SOD1* cDNA sequence were also loaded as a negative control (lane 2). The separated proteins were blotted with a rabbit anti-SOD1 antibody (1:1000 dilution) on a nitrocellulose membrane.

Expression of recombinant human SOD1 was induced by growing the host cells (*E.coli*, XL1-blue, strategene, CA) in Luria broth (LB) containing 2 mM isopropylthio-β-D-galactoside (IPTG) at 37°C. The bacterial pellet obtained from centrifugation, was washed with $T_{50}N_{30}$ (50 mM Tris-HCl, pH 8.0; 30 mM NaCl) to remove the residual LB. Cells were then resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, 100 mM NaCl, 1 mg/ml lysozyme and 5 mM phenylmethylsulfonylfluoride), and deoxycholic acid (4 mg for each g of *E.coli*) was then added to lyse the bacterial cells. Cell lysates were digested with DNase I (0.1 mg/ml) to remove the chromosomal DNA. After centrifugation at 4°C, the supernatant was collected for further analyses.

In vitro SOD1 activity assays

SOD1 dismutase activity was analyzed by zymogram gel analysis. The zymogram gel for SOD1 activity was prepared according to Beauchamp and Fridovich (11). The samples for analysis were prepared as described above. The gel was stained in 2.53 mM nitrobluetetrazolium (NBT) solution after electrophoresis and the colour reaction was developed in a solution containing 0.028 mM riboflavin and 280 mM TEMED (N,N,N′,N′-Tetramethylethylenediamine). The SOD1 activity was shown as a negatively stained band in contrast to the purple colour of the background staining.

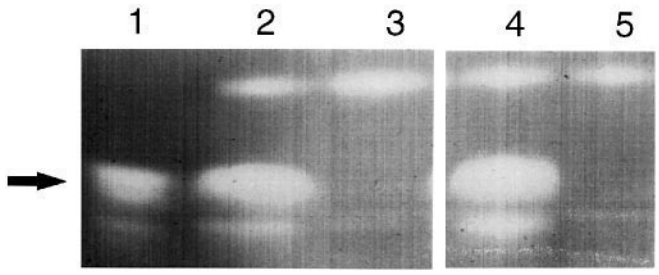


Figure 4. Analyses of the dismutase activity of L126Z and junction mutation on zymogram gel. A SOD1 zymogram gel of 12% acrylamide was prepared. The proteins extracted from *E.coli* that contains the cDNA of wild type SOD1 (lane 2 and 4), L126Z (lane 3) and junction mutation (lane 5) were run onto the SOD1 zymogram gel. A standard SOD1 (lane 1, Sigma) of 50 ng was included as a positive control.

Table 1. Solvent exposed surfaces and sphere accessibility of active site moieties

	Wild type SOD1	Junction mutant	L126Z mutant
Solvent exposure^{+} (Å^2)			
His46	5.3	28.5	14.9
His48	9.8	22.2	22.2
His63	18.5	27.9	27.9
His71	3.7	40.6	30.4
His80	23.1	23.6	23.6
Asp83	0.0	0.0	0.0
His120	15.1	34.5	34.5
Copper	3.4	3.4	3.4
Zinc	0.0	0.0	0.0
Sphere accessibility* (Å)			
Copper	1.7	5.7	5.7

$^{+}$Only the solvent exposed areas of the side chain atoms of the active site amino acids are tabulated here.
*The maximum radius of a sphere which can access this atom within the environment of the protein.

Analysis of SOD1 crystal structure

Crystallization, data collection, and preliminary refinement of wild type SOD1 have been described (12). For this work, the structural model was further refined against this data set using X-PLOR (13) and TNT (14), with manual fitting to σ_{A-} weighted (15) $2F_o$-F_c, F_o-F_c, and simulated annealed omit electron density maps using the program Xfit (16). The final model, containing five independent SOD1 dimers in the crystallographic asymmetric unit, consisted of 1530 amino acids, 10 Cu and 10 Zn atoms, two sulfate ions, and 383 water molecules. During the refinement, the bonds between the metal ions and their ligands were completely unrestrained. The final R value for this model was 17.3% (R_{free} =23.2%) for 70 263 reflections between 10.0–2.6 Å resolution (0_σ cutoff, 91.4%

complete). The geometry of the final model is good, with r.m.s. deviations from ideality (17) of 0.012 Å for bond lengths and 2.2° for bond angles. Solvent exposed surface areas were calculated using MS (18) with a probe radius of 1.4 Å. Maximal sphere accessibilities were calculated using MaxAccess (19).

RESULTS

DNA sequencing of patient 1 showed a mutation of T→A at SOD1 codon 126 (Fig. 1A). This mutation changes the codon TTG for Leu at residue 126 into a stop codon TAG (Fig. 1A). Therefore, this mutation (L126Z) terminates the SOD1 polypeptide at residue 125. DNA sequencing of the patient 2 revealed an intronic mutation from A→G in intron 4, 11 bp upstream from the junction of intron 4 and exon 5 of *SOD1* gene (Fig. 1B). This mutation changes the tetranucleotide AATT to AGTT, which is a splice junction sequence. In order to confirm the function of this new splicing sequence AGTT, the specific mRNA of SOD1 from the patient's lymphoblastoid cells was amplified by RT-PCR and analyzed by acrylamide gel electrophoresis. Two DNA fragments were amplified (data not shown). The short fragment had the sequence of wild type SOD1 (data not shown), while the longer DNA fragment had a 10 bp sequence inserted between the sequences of exon 4 and exon 5 (Fig. 1C). The inserted sequence is 3′-TTT TTT ACA G-5′. The insertion of this 10 bp would change the reading frame after codon 118 of *SOD1*. Five amino acid residues 'Phe-Phe-Thr-Gly-Pro' would be added into the mutant SOD1 sequence before the formation of the stop codon TGA (Fig. 1C). Thus, this intronic mutation causes a truncation of 35 amino acids from the C-terminus of SOD1 through alternative splicing of mRNA.

To examine the expression of L126Z and the splicing junction mutants, protein preparations from patients' RBC and lymphoblastoid cells were separated by SDS–PAGE, transferred to nitrocellulose membrane and blotted with an anti-SOD1 polyclonal antibody. A single band corresponding to the wild type SOD1 was detected in both samples (Fig. 2), while the polypeptide encoded by L126Z or splicing junction mutant was not detected in either the RBCs or lymphoblastoid cells by Western blot analyses. The L126Z and splicing junction mutants were also studied in the prokaryotic expression system. Expression of these recombinant SOD1 mutants as well as wild type SOD1 was examined by Western blot analyses using the anti-SOD1 polyclonal antibody. This antibody recognized a 17 kDa band expressed by the wild type *SOD1* (Fig. 3). A 15 kDa band expressed by L126Z or splicing junction mutants was also apparently recognized by the anti-SOD1 antibody (Fig. 3), suggesting a stable expression of these two mutants. However, the recombinant L126Z and splicing junction mutants did not show any dismutase activity on the SOD1 zymogram gel (Fig. 4).

To study the structure–function effect of L126Z and splicing junction mutants, we mapped the amino acid truncations caused by these two mutants on the X-ray crystal structure and estimated the structural changes resulting from these truncated mutant enzymes, especially in the active site of SOD1 (Fig. 5). The floor of the active site channel of SOD1 consists of the buried Cu and Zn atoms and their associated protein ligands (His46, His48, His63, His120 for Cu and His63, His71, His80, Asp83 for Zn). These active site moieties were examined in the context of the final refined crystal structure of wild-type SOD1 and these exon 5 mutations with the simplifying assumption of no conformational change accompanying the mutations. In particular, the solvent exposure and the accessibility of these moieties were calculated from the wild type structure and compared to the values calculated from model structures truncated after residue 123 (splicing junction mutant) and 125 (L126Z mutant). Although the two active site metal atoms do not undergo a major increase in solvent exposure upon truncation of the protein (Table 1), five of the seven side chains which form bonds to the metal ions experience large increases in solvent exposure, the exceptions being His80 and Asp83 which are situated toward the interior of the protein relative to the Zn atom. Moreover, the entire active site region has been opened by removing the loop forming one entire side of the channel that sequesters the Cu ion from accessibility to ligands larger than superoxide (O_2^{-}). As a result, the accessibility of the Cu ion is significantly increased (Table 1). The native site accommodates substrates of maximum radius 1.7 Å whereas the two ALS mutants can accommodate substrates of maximum radius 5.7 Å (Table 1).

DISCUSSION

The L126Z and splicing junction mutation are two unique *SOD1* mutations associated with FALS because they cause premature termination of SOD1 polypeptide at the C-terminus. The mRNA of mutant SOD1, at least for the splicing junction mutant, was detected by RT-PCR. However, the mutant proteins encoded by L126Z and the splicing junction mutation were not detected in either RBCs or lymphoblastoid cells by Western blot analyses. Since the anti-SOD1 polyclonal antibody used in the Western blot recognizes multiple epitopes in the sequences encoded by all five exons of *SOD1* (data not shown), the absence of the mutant SOD1 in the Western blot analyses can not be due to the loss of epitopes recognized by this antibody. Thus, the expression level of L126Z and splicing junction mutants in both RBCs and lymphoblastoid cells must be lower than the detectable range of our method (minimum 2 ng of SOD1). Based on the minimum amount detectable by our method and comparison of the density of standard SOD1 band to that of the control, the amount of L126Z and splicing junction SOD1 mutants if present must be at least 25–50 times lower than the wild type expressed in the same cells. A similar result was obtained by Watanabe *et al.* (20), when they studied the expression of the mutant SOD1 in the brain tissue of a FALS patient with a 2 bp deletion in codon 126. The decreased expression of these mutant proteins is most likely caused by the rapid degradation of mutant SOD1 after translation. It is expected that the mutant SOD1 have a rapid turnover rate and very low expression levels inside the cells. Most importantly, these observations suggest that extremely small amounts of SOD1 mutants, though undetectable by Western blot, can induce motor neuron degeneration in FALS patients. Thus, the L126Z and the splicing junction mutants must be highly toxic to motor neurons.

The mutant SOD1 polypeptides encoded by L126Z and splicing junction mutants are 28 and 30 amino acids shorter than the wild type, respectively. The truncated domain in both mutants includes almost the entire amino acid sequence encoded by exon 5. It is interesting that whereas at least 12

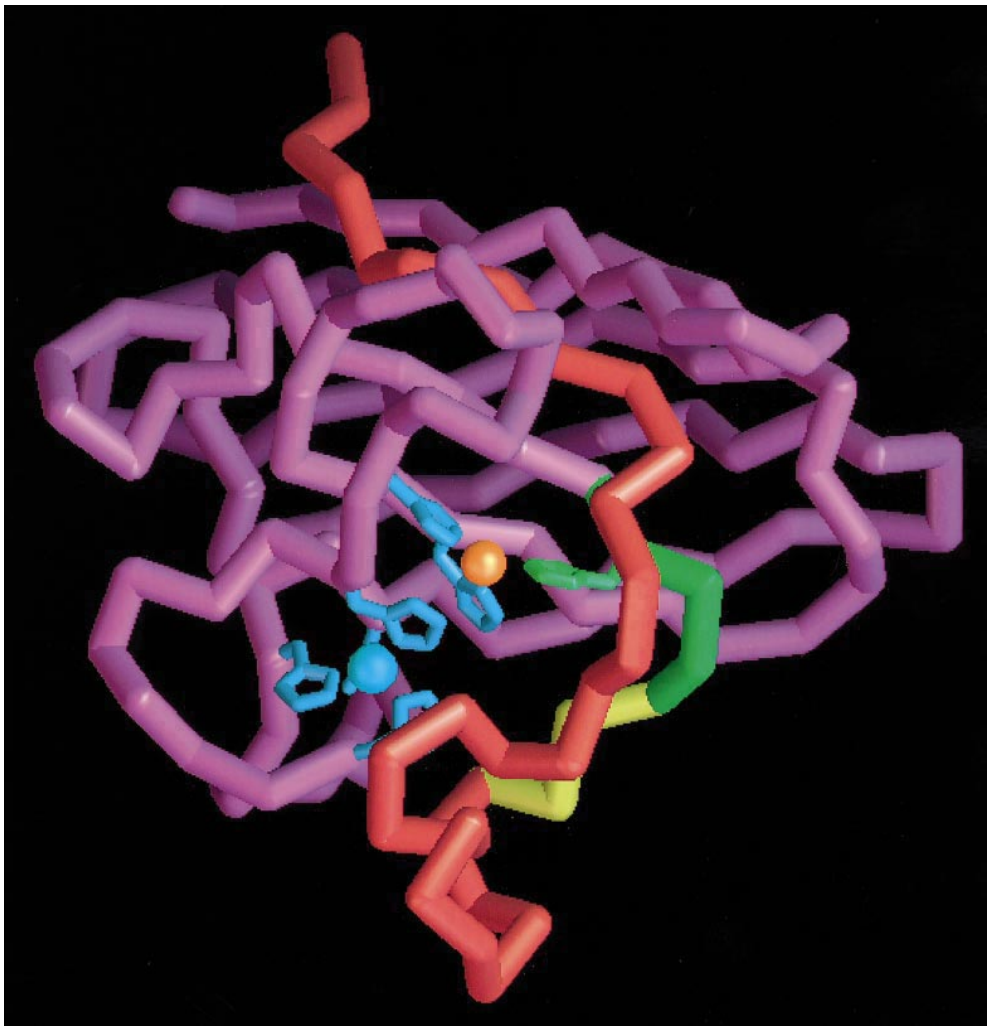


Figure 5. Two exon 5 deletion mutants associated with ALS dramatically open the entire active site channel as revealed by the human SOD1 alpha carbon trace (tubes). The active site copper ion (orange sphere) and zinc ion (blue sphere) become completely exposed by the removal of exon 5 (green, yellow, and red tubes), which forms the lid over the active site channel. The retained metal ion liganding residues (small cyan tubes) plus the His120 site (green small tubes), which is substituted by Phe in the splice junction mutant, will become completely exposed as both sides of the upper channel loop are removed in these remarkable new ALS mutations. The unchanged portion of the subunit fold (purple) retains seven of the eight β-strands plus six of the active site metal ion ligands. Amino acid residues 119–123 (green) have been altered in the splice junction mutant, residues 124 and 125 (yellow) are deleted in the splice junction mutant, and residues 126–153 (red) are deleted in both the junction mutant and the L126Z mutant.

different missense mutations in exon 5 of *SOD1* are known to be associated with FALS (6), the deletion of exon 5 encoded amino acids results in a similar phenotype. Within the context of the 'gain of toxic function' hypothesis, these new results establish that the exon 5 encoded domain is not an essential structure element for the toxic function of mutant SOD1, although this domain is essential for the dismutase function. Any structure–functional mechanism for the toxicity of mutant SOD1, therefore, must be due to conformational changes in other domains encoded by exons 1–4 of *SOD1*. Such structural changes may result in functional changes involved in substrate recognition, metal binding, dimer assembly or redox equilibrium. Similar changes in the structure and function of SOD1 may also occur in the mutant with missense mutations, assuming that they mimic to some degree, the structure fluctuations resulting from loss of exon 5. One type of conformational change likely to cause toxicity is the distortion of the active site of SOD1. Based on our analyses of the truncated domains on the X-ray crystal structure (21,22), the active sites of L126Z and splicing junction mutants are wide open and are able to accommodate ligands much larger than its natural substrate ($O_2^{\cdot -}$). The opened active channel likely provides increased accessibility to allow those molecules that normally are, at least partially excluded from the active site of SOD1, such as H_2O_2, $ONOO^-$ or ascorbic acid, to chemically interact with the metal ions. A second likely effect of this opening up of the SOD1 active site is that the metal atoms are held more loosely within the mutant proteins as the result of increased mobility of the surrounding amino acids. Both increased active site flexibility and accessibility should synergistically compromise SOD1 substrate specificity, with non-native substrates being readily able to access the active site. Thus, such increased Cu ion accessibility would allow the formation of molecules highly toxic to motor neurons. Increased peroxidase

activity of some mutant SOD1 has been reported, which is thought to be catalyzed through the Cu ion in the active site of SOD1, to form $OH^{\cdot -}$ radicals (23,24). The increased peroxidase activity of the mutants seems to be responsible for the induction of apoptosis in neuronal cell lines that harbor mutant SOD1 (25). These functional studies seem consistent with our structure–functional analyses of mutant SOD1 including these new exon 5 mutations. Thus, L126Z and splicing junction mutations likely generate SOD1 mutants that are highly toxic to motor neurons. Both mutants are expected to increase active site accessibility and compromise substrate specificity, which therefore provides a reasonable hypothesis for the structural basis underlying the toxic function of mutant SOD1.

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