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PROBING THE STRUCTURAL BASIS FOR ENZYME-SUBSTRATE RECOGNITION IN Cu, Zn SUPEROXIDE DISMUTASE

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A full understanding of enzyme-substrate interactions requires a detailed knowledge of their structural basis at atomic resolution. Crystallographic and biochemical data have been analyzed with coupled computational and computer graphic approaches to characterize the molecular basis for recognition of the superoxide anion substrate by Cu, Zn superoxide dismutase (SOD). Detailed analysis of the bovine SOD structure aligned with SOD sequences from 15 species provides new results concerning the significance and molecular basis for sequence conservation. Specific roles have been assigned for all 23 invariant residues and additional residues exhibiting functional equivalence. Sequence invariance is dominated by 15 residues that form the active site stereochemistry, supporting a primary biological function of superoxide dismutation. Using data from crystallographic structures and site-directed mutants, we are testing the role of individual residues in the active site channel, including (in human SOD) Glu 132, Glu 133, Lys 136, Thr 137, and Arg 143. Electrostatic calculations incorporating molecular flexibility suggest that the region of positive electrostatic potential in and over the active site channel above the Cu ion sweeps through space during molecular motion to enhance the facilitated diffusion responsible for the enzyme's rapid catalytic rate.

KEY WORDS: Superoxide dismutase, structure-function, evolution, mutants, electrostatics.

INTRODUCTION

Cu, Zn superoxide dismutases (Cu, Zn SODs) are thermostable cytoplasmic enzymes, predominantly found in eucaryotes, that protect cells against the toxic effects of the superoxide radical, a by-product of aerobic metabolism. The enzyme acts in vivo as a dimer of identical subunits, each containing about 153 amino acids (depending on the species) with one Cu and one Zn ion. The Cu ion, an essential cofactor in catalysis, is cyclically oxidized and reduced during successive encounters with superoxide. Thus, Cu^{2+} accepts an electron from one superoxide radical to produce molecular oxygen and Cu^{+}, and donates an electron to a second superoxide radical to produce, together with two protons, hydrogen peroxide.[1] The rapid rate of both reactions, $2 \times 10^9 M^{-1} sec^{-1}$, is probably limited by the availability of substrate.[2] Precollision electrostatic guidance, indicated by experimental studies of the ionic strength dependence of the reaction rate[3] and by computational modeling of the molecular electrostatic field,[4] could increase the availability of substrate, making the reaction rate faster than by diffusion alone.

We report here a summary of our recent work geared toward understanding the structural basis for the mechanism of action of Cu, Zn SOD. First, we will discuss the

roles of sequence-conserved and functionally equivalent residues in the action and three-dimensional structure of Cu, Zn SOD, analyze the exon structure of the SOD gene in relation to the structural symmetry in the protein and discuss possible evolutionary pathways.[5] Next, we will look at several mutants that have been genetically engineered and their effects on the electrostatics of the enzyme.[6] Finally, we will show developments in designing computer graphic representations of the effects of motion on the electrostatic potential of Cu, Zn SOD.[7]

STRUCTURE OF CU, ZN SOD

The crystallographic structure of bovine Cu, Zn superoxide dismutase, refined to a resolution of 2 Å, shows that the structural core of the subunit is a flattened Greek key β-barrel motif consisting of eight antiparallel β-strands joined by seven turns or loops.[8] The catalytic copper ion is ligated to the surface of the β-barrel by four histidine residues and occurs at the base of a channel formed by two loops extending from the β-barrel. In addition to forming the superoxide channel, the two loops encode specific functional subdomains involved in precollision electrostatic guidance of the substrate (loop VII), zinc binding (loop IV), and dimer contact (loop IV).[4]

EVOLUTION OF CU, ZN SOD

Amino Acid Homology and Sequence Variation

The sequences of 15 Cu, Zn SODs were aligned and numbered against the human sequence.[9-27] A schematic of the β-strand and loop secondary structure numbered according to the human sequence (Figure 1) shows how the loops, symmetrical regions, and exons map onto the structrual elements. Although sequence changes between different Cu, Zn SODs cannot be used as a precise evolutionary clock,[10] species relatedness is reflected in the level of amino acid homology. Thus, among the mammals, rat and mouse show almost complete amino acid identity (97%). The human enzyme has an average of 82% amino acid identity with the other mammals. This falls to 67%, 61%, 56%, 54%, and 18% when human SOD is compared with swordfish, fruit fly, plants, fungi, and photobacterium, respectively (insertions and deletions are counted as mismatches).

The alignment of the 15 sequences shows that insertions and deletions occur in six of the seven loop and turn connections between the β- strands. Although some insertions and deletions may overlap the ends of β-strands, none is found within the body of these structural elements. These results from SOD indicate that β-strands are more permissive of sequence variation than of shifts in strand alignment. The absence of single residue insertions is somewhat surprising, since they could presumably be accommodated by β-bulge formation.[28] Larger insertions or deletions within β-strands may disrupt side chain packing interactions.

The two most sequence-variable regions in the Cu, Zn SOD molecule are between residues 19–36 and 88–105. Both of these segments consist predominantly of solvent-exposed antiparallel β-strand hairpins that are not buried underneath the active site channel or in the dimer interface. Alterations to these solvent-exposed residues may be less likely to reduce enzyme activity, whereas alterations to residues participating

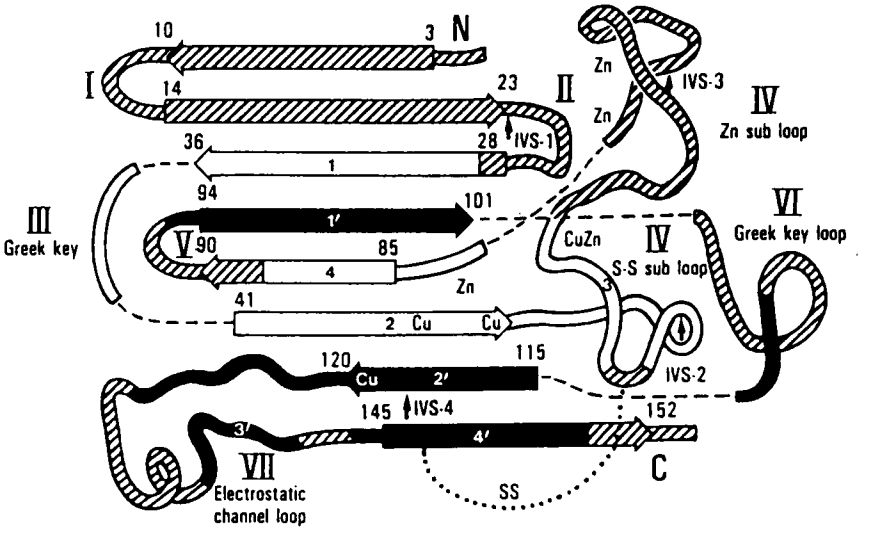

FIGURE 1 Schematic of the β-strand and loop secondary structure labeled to show how the loops, symmetrical regions, and exons from the sequence alignment map onto the structural elements. The positions of the N- and C-termini, Cu and Zn ligands, disulfide bond, and of the four intervening sequences are labeled. β-strands are shown as arrows and numbered at their N- and C-termini. Loops are labeled by Roman numerals. Greek key loops III and VI are so named because they represent the + 3 connections (named by how many strands they move over in the β-sheet) that form the classic Greek key design. Loop IV is subdivided into the S–S and Zn subloops at metal-bridging ligand His 63 (labeled CuZn). Loop VII includes 1.5 turns of α-helix (lower left) composed of residues 132-137. The twofold symmetry-related regions are coded here in white (with black labels 1, 2, 3, and 4) and black (with labels 1′, 2′, 3′, and 4′).

in the dimer contact or the active site are restricted because they are much more likely to inactivate the enzyme.

There are 23 invariant amino acids in the 15 species aligned. The invariant residues fall into these structure-function categories: active site (15 residues), dimer interface (4 residues), and β-barrel (4 residues). The metal-ligating residues account for seven of the invariant residues (His 46, 48, 63, 71, 80, 120, and Asp 83). Arg 143 forms part of the superoxide binding pocket above the catalytic Cu ion, where the positive charge of the Arg guanidinium group stabilizes the negatively charged superoxide substrate.[29] Arg 143 also appears to participate in short-range precollision electrostatic guidance of substrate.[4,30] Asp 124 is an invariant residue involved in correctly orienting the Cu- and Zn-ligating residues. By analogy with the bovine structure, Asp 124 forms strong, charged, hydrogen bonds both to Cu ligand His 46 and to Zn ligand His 71.[29] In addition, there are five invariant glycine residues (44, 61, 82, 138, and 141) that are involved in maintaining the structure of the active site, and Pro 66 that participates in the tight turn of the Zn-ligating region of loop IV, which directs the ligands His 63 and His 71 toward the Zn ion.

Two invariant residues and one variant residue of each subunit form all four of the hydrogen bonds across the dimer interface. Invariant Gly 51 and Gly 114 both form tight contacts and main chain hydrogen bonds across the dimer interface with the nonconserved Ile 151. The two invariant cysteines (Cys 57 and Cys 146) form a disulfide bridge stabilizing a region of loop IV (see Figure 1) involved in dimer contact by covalently joining it to the β-barrel.

Two glycines (Gly 16, Gly 147) and two bulky hydrophobic residues (Phe 45, Leu 106) are the invariant residues that appear to be involved in maintaining the stable Greek key β-barrel fold. Phe 45 forms one of only two cross-barrel connections, connecting β-strands directly across the β-barrel and tying the active site to the more regular side of the β-barrel. Phe 45 interacts with residue 18 across the barrel and anchors adjacent Cu-ligating residues His 46 and His 48. Leu 106 lies in Greek key loop VI, which crosses one end of the barrel. The side chain of Leu 106 points into the barrel creating a "cork" at the end that stabilizes the β-barrel by interacting with buried hydrophobic residues from the β-strands and with residues 112 and 113 (both usually Ile) from the other end of the Greek key loop.

Another level of amino acid conservation in proteins reflects the maintenance of an invariant function but not amino acid invariance. Such conserved residues or groups of residues show functional equivalence or stereochemical similarity. Perhaps the most common examples of functional equivalence in SODs are the selected replacements allowing large hydrophobic residues at the same position in the sequence alignment. The residues Ile, Leu, and Val are functionally equivalent at nine positions. Ile, Leu, or Val appear at positions 35, 47, 87, 104, 112, and Ile or Val appear at positions 18, 119, 148, 149. As might be expected, all of these side chains are buried from solvent either in the interior of the β-barrel, between Greek key loop VI and the β-barrel, or in the dimer interface. Although most cases of functional equivalence follow this simple pattern, occasionally more subtle examples can be detected. One example is the conserved (except in fungi) bulky hydrophobic residue Leu 38 that forms a "cork" at one end of the β-barrel, analogous to the invariant Leu 106 at the opposite end of the β-barrel. In *Neurospora* and yeast, Leu 38 is replaced with two amino acids. A particularly interesting example is Lys 136, which is important in electrostatic interactions with the substrate.[4] This residue can be replaced in its role by His 131, which can place a positive charge in a similar three-dimensional position by having its shorter side chain occur one turn earlier in the short α-helix of the electrostatic channel loop.

Structural Symmetry

Each Cu, Zn SOD subunit has an internal twofold axis of symmetry relating its α carbon positions. The structural symmetry in SOD led to the proposal that both halves of the primordial SOD were originally identical and encoded by one gene.[31] The primordial gene is believed to have duplicated and fused to form a single gene twice the size of the primordial gene that, after quite extensive further evolution, resulted in the present-day gene.

One of the earliest structures in Cu, Zn SOD evolution was probably a symmetrical six-stranded β-barrel, made from two identical polypeptide chains and containing the Cu ion at the axis of symmetry. One implication of this hypothesis is that the precursor molecule of the six-stranded barrel was a three β-strand monomer. At present, we are unable to trace the evolutionary pathway that resulted in the formation of this three β-strand structure. The fourth intervening sequence, IVS-4 at Val 118 (see Figure 1), is the only intron that maps to a buried position or interrupts a β-strand. IVS-4 may be a relic from the early evolution of the three β-strand structure. Thus, buried intron-exon junctions (which will tend to occur in core structures) may reflect relatively ancient events in protein evolution. If IVS-4 was present in the primordial gene it must have been lost from the 5′ (N-terminal) duplicated gene.

The lack of significant amino acid duplication in the present-day gene is perhaps evidence against the gene duplication hypothesis. Three-dimensional structure, however, is usually more conserved than the amino acid sequence during evolution, and the Cu, Zn SOD β-barrel may be more overdetermined than most structures. The Greek key β-barrel structures may be able to tolerate considerable sequence variation, provided local packing and the alteration of hydrophobic and hydrophilic side chains in the β-strands are retained. The structurally symmetrical amino acid duplications present in the eucaryotic SOD sequences occur primarily in a highly variable region of the sequence between residues 31 and 36, suggesting that they may not be very significant. Interestingly, the only sequence-invariant duplicated residues are His 48 and His 120, which ligate the activity-essential Cu ion. This led to the hypothesis that the other histidine ligands and the zinc domain were later evolutionary additions.[31]

Exons and Gene Duplication

Exons often correspond to structural or functional domains or subdomains of proteins[32] and introns probably direct the recombination of exons and their corresponding protein domains to facilitate protein evolution.[33] Intron-exon junctions frequently map to the protein surface, where insertions and deletions are least likely to disrupt the protein structure or function.[34] When the five exons of the human Cu, Zn SOD gene[35,36] are mapped to the protein structure, all but the last intron-exon junction (at Val 118) map to the protein surface.[35]

Exon I, corresponding to the first antiparallel β-hairpin, lies outside the structural symmetry, supporting the hypothesis that it was added after gene duplication. Before the addition of exon I, the first N-terminal β-strand of the six-stranded β-barrel was unable to pair with an adjacent β-strand until the fourth β-strand had been synthesized (see Figure 2, strands 1 and 1′ of the six-stranded monomer). In contrast, the first β-strands of both halves of the primordial dimer can together form a β-strand pair immediately and the N-terminal β-hairpin of the present-day eight-stranded β-barrel is able to form a β-strand pair immediately upon synthesis (see Figure 2). The ability to rapidly form a β-sheet nucleation site may reduce the likelihood of incorrect folding after sequence changes or the insertion of new subdomains.

Exon III corresponds to the zinc-binding subdomain and two of the ligating histidines. It also includes the structurally symmetrical region of residues 58–63, which appears to be inconsistent with addition of exon III after gene duplication. This 3, 3′ symmetry could result from the structural contraints for forming the superoxide channel, rather than from the gene duplication. Interestingly, exon III is the only exon that does not contribute any residues to the dimer interface, suggesting that addition of this Zn-binding loop may have occurred after the formation of the current dimer interface. The concept of exclusion of this structurally symmetrical region from the primordial gene is not entirely satisfactory, however, since 3 and 3′ connect parts of loops IV and VII (2 and 2′ symmetry) back to the β-barrel. Furthermore, it seems to make sense to enclose the primordial Cu ion in two such symmetrical loops to form a binding pocket for the superoxide. It is possible that symmetry 3 did not exist in the primordial SOD structure and may have evolved due to the structural constraints of forming the superoxide channel and binding pocket.

The present-day exon II was formed from part of one primordial duplicated gene by the introduction of exon III and its flanking intervening sequences (see Figure 2). Many genes with several introns will have exons similar to exon II: such exons are not

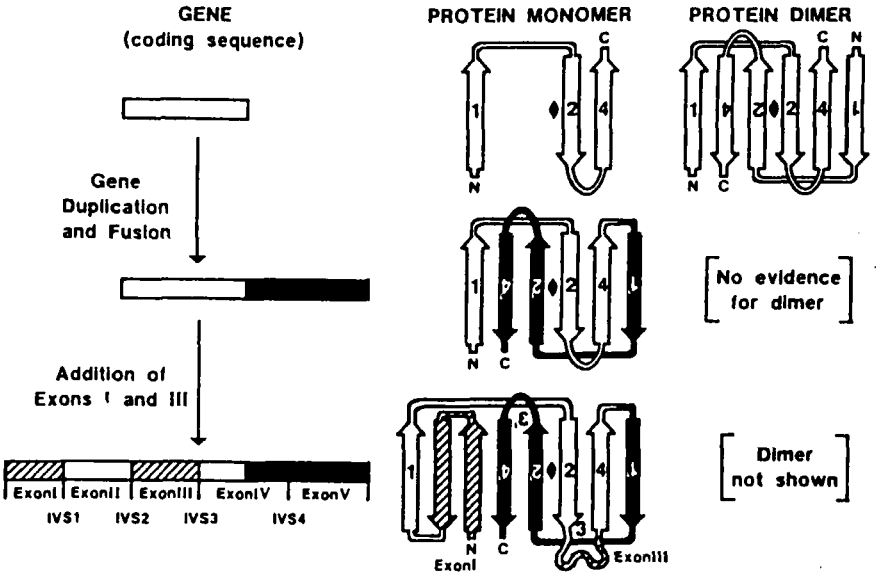

FIGURE 2 Proposed evolution of Cu, Zn SOD. The primordial SOD gene and protein structures are shown schematically on the top line and the present-day structures on the bottom line. The structurally symmetrical regions 1 and 1′, 2 and 2′, and 4 and 4′ are represented here entirely as β-strands (numbered arrows) connected by loop regions. The first loop in the primordial structure is the Greek key loop (connecting symmetry 1 and 2) that joins nonadjacent β-strands and forms a cross-barrel connection. The structurally symmetrical regions 3 and 3′, which are shown only on the present-day protein structure, contain no β-strands, and may have been modified from those in the primordial structure. The twofold axis of symmetry (containing the Cu ion) is indicated at the center (◆). Exons I and III are shown as more recent additions to the structure and both were added to the N-terminal symmetrical region. For simplicity, IVS-4 is shown only on the present-day gene structure, although it may date from the very early evolution of the gene.

necessarily structural or functional subdomains but are a reflection of useful sites in the protein structure able to accept new exons.

MUTATION STUDIES OF ELECTROSTATIC RESIDUES

Several mutations of Cu, Zn SOD have been made to study the contribution of single amino acids to the electrostatic behavior of the enzyme. An important sequence-invariant residue in all known Cu, Zn SOD sequences, Arg 143 (Arg 141 in bovine SOD) forms part of the superoxide binding site and has been implicated in local electrostatic stabilization of the substrate.[4,29] Chemical modification of bovine SOD Arg 141 with phenylglyoxal causes a 90% reduction in activity.[37] Mutations of the human SOD Arg 143 to Lys, Ile, Glu, Asp, or Ala all produce mutants with significantly reduced activity.[30] As expected for a residue important for electrostatic recognition, mutations to like charges (Lys^+) show lesser effects than mutations to uncharged (Ala, Ile) or oppositely charged residues (Glu^-, Asp^-). The Ile mutant of human SOD, although it has considerably less activity than the wild-type, does display increased stability. Examination of the bovine structure shows that Arg 141

overlies a hydrophobic cavity between a loop region and the β-barrel. The hydrophobic, branched isoleucine side chain may fill the cavity.

Other human SOD residues in the binding site that have been mutated to date include two threonines and an aspartate. Thr 137 (bovine 135), which forms the opposite side of the superoxide binding pocket from Arg 143 has been changed to Ile and Ala to investigate the importance of removing the polar group. It has also been mutated to Arg to investigate the effects of increasing the positive charge in the active site. All of these mutations have resulted in decreased activity. We are currently investigating a mutant with Thr 137 mutated to Arg and Arg 143 mutated to Ala to see if having an Arg on the other side of the pocket will result in an active enzyme. Thr 58 (bovine 56), which helps control access at the top of active site channel, has been mutated to Ile. Asp 124 (bovine 122), which forms two important charged hydrogen bonds stabilizing the Cu ligands, has been changed to Leu and Asn. Although much of the analysis remains to be done, this type of experiment promises to probe directly the role of specific residues in the mechanism of superoxide dismutation.

The long-range electrostatic forces involved in recognition and orientation of the negatively charged superoxide substrate were examined by mutations of Lys 136 (bovine 134) and Glu 133 (bovine 131), whose side chains form a salt-bridge. Calculations show that neutralization of these two residues individually or together resulted in the largest changes in the electrostatic field of any of the charged residues near the active site.[4] Brownian dynamics simulations on the bovine enzyme suggest that mutation of either Glu 133 (bovine 131) or Glu 121 (bovine 119), the negative residue that forms a salt-bridge with Lys 120, to a lysine would double the rate constant.[38] (Changes in conformation were not considered in this calculation.) Instead, mutation of Glu 133 in human SOD to either a Gln or Lys resulted in a significant decrease in activity (R. A. Hallewell, unpublished results). Therefore, Glu 133 may be important in preventing nonproductive binding of superoxide with Lys 136.

ELECTROSTATICS IN MOTION

Several researchers have developed static models of SOD using the published crystal structure. In our initial work on SOD, we calculated the electrostatic potential around the active site using a simple Coulombic relationship.[4] The direction of the electrostatic field was represented by arrows, color-coded according to the electrostatic potential. To look at the behavior of the field around the moving enzyme, we have conducted a normal mode analysis of SOD, where the time scales of movement are about 10^{-10} – 10^{-11} seconds, and devised computer graphical representations of the changes in the electrostatic field about a protein in motion.

From the two hundred normal modes (frequencies ranging from about $5\,cm^{-1}$ to $50\,cm^{-1}$) obtained from AMBER for the superoxide dismutase monomer, the twenty frequencies that had the largest movements in the residues Thr 56, Glu 130, Glu 131, Lys 134, Thr 135, and Arg 141, which lie around the rim of the active site, were chosen for a more focused analysis. Each mode was examined to characterize the type of motion around the active site channel to see whether a concerted rocking, twisting, or opening-and-closing was occurring. Electrostatic field vectors for several positions of a particular mode were calculated at points lying outside the protein surface on concentric spheres from 0 Å to 14 Å around the active site. Graphical analysis of one

vibrational mode, that at 9.8 cm^{-1}, showed two prominent features. First, there was a sweeping movement of the field across the volume of open space above the active site as the protein moved from one extreme of the normal mode to the other, arising from the concerted movement of the charged groups on the outer rim across and down toward the active site, then out and away, somewhat like jaws opening and closing. Second, as the side chains moved in toward the active site, the positive patch was focused just above the open copper site. This was caused by a deshielding of the copper ion as the protein structure opens up around it, increasing the positive charge over the active site.

The electrostatic potential at each point of a consensus spherical harmonic surface[39] was then calculated and the surface points color-coded according to the potential. The Cα chain was displayed simultaneously to facilitate the correlation of the motion of the molecule with changes in the surrounding electrostatic potential. The surface points remain fixed in position, but change color as the protein moves. Patches of charge in the dimer interface did not move much with this particular mode. The positive patch over the active site, however, moved back and forth.

These visualizations of molecular flexibility effects are not limited to the display of changes in electrostatic properties due to normal mode motions, but are equally applicable for displaying changes in any measurable chemical or molecular property due to any defined set of protein movements. The results from application of these methods to SOD suggest that modeling molecular motions may be important for studies of molecular recognition and interactions.

SUMMARY

Enzyme evolution is presumably driven to improve folding, stability to inactivation, and to optimize activity. The atomic sequences and structures contain an evolutionary record in terms of the observed three-dimensional location of conserved residues, internal symmetries, and intron-exon boundaries. This information has guided us in engineering and computational studies designed to test our analyses of the superoxide dismutase mechanism. It will also serve us as a guide to designing proteins with new attributes.

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