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Substrate-analog binding and electrostatic surfaces of human manganese superoxide dismutase

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ABSTRACT

Superoxide dismutases (SODs) are enzymes that play a key role in protecting cells from toxic oxygen metabolites by disproportionation of two molecules of superoxide into molecular oxygen and hydrogen peroxide via cyclic reduction and oxidation at the active site metal. The azide anion is a potent competitive inhibitor that binds directly to the metal and is used as a substrate analog to superoxide in studies of SOD. The crystal structure of human MnSOD-azide complex was solved and shows the putative binding position of superoxide, providing a model for binding to the active site. Azide is bound end-on at the sixth coordinate position of the manganese ion. Tetrameric electrostatic surfaces were calculated incorporating accurate partial charges for the active site in three states, including a state with superoxide coordinated to the metal using the position of azide as a model. These show facilitation of the anionic ligand to the active site pit via a 'valley' of positively-charged surface patches. Surrounding ridges of negative charge help guide the superoxide anion. Within the active site pit, Arg173 and Glu162 further guide and align superoxide for efficient catalysis. Superoxide coordination at the sixth position causes the electrostatic surface of the active site pit to become nearly neutral. A model for electrostatic-mediated diffusion, and efficient binding of superoxide for catalysis is presented.

1. Introduction

Superoxide dismutases (SODs) are essential antioxidant enzymes that protect cells from reactive oxygen species and promote health and longevity. In eukaryotes, MnSOD is found in the mitochondrial matrix and remediates superoxide generated by the electron transport chain (McCord, 2002). Mice with MnSOD knocked out die within the first 10 days of life and overexpression of MnSOD in fruit flies results in increased life span (Li et al., 1995; Sun et al., 2002). Mutations in human MnSOD are linked to neurological disorders, cancer, and late-onset diseases (Kim, 2010; Perry et al., 2007). In each enzymatic cycle, two superoxide ions are converted into molecular oxygen and hydrogen peroxide via cyclic reduction and oxidation at the active site metal (Holm et al., 1996). The following is an overview of the MnSOD enzymatic reaction.

$$Mn^{3+} + O_2^{\cdot-} \rightarrow Mn^{2+} + O_2$$
$$Mn^{2+} + O_2^{\cdot-} + 2H^+ \rightarrow Mn^{3+} + H_2O_2$$

Human MnSOD functions as a tetramer and is rate-limited only by diffusion of its substrates and products (Bannister and Bannister, 1987). The active site manganese ion is coordinated by His26, His74, Asp159, His163, and a single oxygen, typically thought to be a water or hydroxide ion (Fig. 1). These ligands are referred to as the "inner-sphere" residues. "Outer-sphere" residues surround the inner-sphere and include His30, Tyr34, Phe77, Trp78, Trp123, Gln143, Trp161 and, from across the dimer interface, Glu162. The substrate approaches the active site along a trajectory between His30 and Tyr34 and binds in the position opposite Asp159. Hydrophobic residues Phe77, Trp78, Trp123 and Trp161 form a hydrophobic cage around the base of the active site, trapping ligands and solvent to facilitate catalysis by the manganese ion (Hearn et al., 2001). His30, Tyr34 and Gln143 are thought to provide a hydrogen-bond network for proton donation to the active site in addition to orienting solvent for proton transfer (Edwards et al., 2001b; Leveque et al., 2000; Perry et al., 2009;

Abbreviation: SOD, superoxide dismutase.

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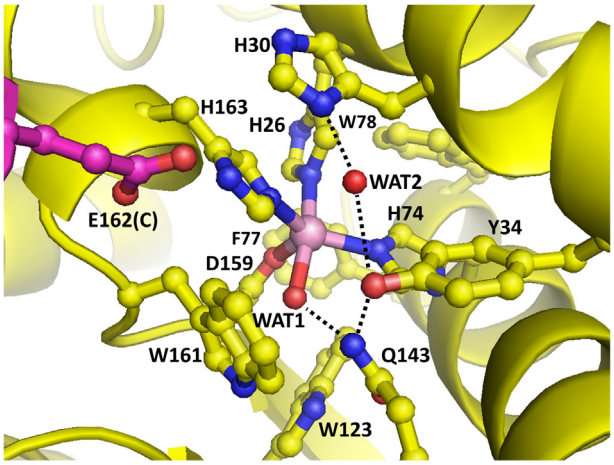

Fig. 1. Distorted five-coordinate trigonal bipyramidal active site geometry of native human MnSOD. A top view is shown as seen by approaching substrate. Sidechains of the manganese inner-sphere ligands and all residues within 7 Å of the metal are shown. The manganese is a pink sphere. Chain A is shown in yellow and chain C in magenta. Glu162 contributes to the active site from across the dimer interface. The hydrogen bond network to the manganese from bulk solvent (His30-WAT2-Tyr34-Gln143-WAT1) is indicated by dotted lines (PDB entry 5VF9). All figures were drawn using the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

Ramilo et al., 1999). Glu162 hydrogen bonds across the dimer interface to His163 from the adjacent subunit and contributes to stability and efficient catalysis (Quint et al., 2008). Both the inner and outer sphere arrangements are conserved among iron and manganese superoxide dismutases (Wintjens et al., 2004).

Two mechanisms for the activity of iron and manganese SODs have been suggested. The first is called the 5-6-5 mechanism and proposes superoxide coordinates to the active site metal and becomes molecular oxygen or hydrogen peroxide in a two-step fashion. Here the coordination state of the active site metal converts from five-coordinate trigonal bipyramidal to six-coordinate octahedral upon substrate binding and back to five-coordinate upon substrate release (Lah et al., 1995; Ludwig et al., 1991; Tierney et al., 1995). In this mechanism, anionic substrate-analogs are believed to bind in the same position as superoxide, opposite Asp159. The second mechanism observed with studies of thermochromism is called associative displacement. This mechanism proposes that six-coordinate anionic complexes represent an inactive form of the enzyme that is seen only at low temperatures. A five-coordinate complex represents the active form at physiological temperature, with anion binding displacing one of the manganese ligands, either bound water or Asp159 (Whittaker and Whittaker, 1996, 1997). The azide ion is a potent competitive inhibitor and is frequently assumed to act as a substrate analog to superoxide (Bull and Fee, 1985b; Misra and Fridovich, 1978). Published structures of azide in complex with MnSOD have been solved at room temperature for *Thermus thermophilus* (PDB entry 1MNG) and cryocooled for *Caenorhabditis elegans* (PDB entry 5AG2) (Hunter et al., 2015; Lah et al., 1995). Both show the azide binding end-on to the manganese ion at the sixth coordinate. An unpublished structure of the Y174F MnSOD-azide complex from *Escherichia coli* (PDB entry 1ZLZ) shows binding in the same manner as well, with the Y174F mutation breaking a hydrogen bond at the dimer interface. To date, the crystal structure of human MnSOD with bound azide has not been solved.

Here, the crystal structures of native human MnSOD and the human MnSOD-azide complex are reported and provide the binding position for azide in the active site. Electrostatic solvent accessible surface calculations were performed with these crystal structures to map the residues that are important for electrostatic guidance of the substrate to the active site. These surface calculations were conducted with three differing active site states, including one with superoxide coordinated using the azide binding site as a model.

2. Materials and methods

2.1. Protein purification and crystallization

Full length human MnSOD cDNA optimized for *E. coli* codons was cloned into the pACYCDuet-1 expression vector (Genscript) and transformed into the *sodA*$^{-}$*sodB*$^{-}$ strain of *E. coli*, which lacks endogenous Mn and FeSODs (Steinman, 1992). Cells were grown in Terrific Broth with 0.8% (v/v) glycerol and supplemented with 0.75 g L^{-1} $MnSO_4$ to provide the protein's active site manganese ion. Cell strain fidelity was maintained with 30 μg mL^{-1} kanamycin. Recombinant protein was expressed upon addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested by centrifugation and stored at -80 °C until purification. Cells were resuspended in 50 mM potassium phosphate (K_2HPO_4/KH_2PO_4), pH 7.8, prior to lysis using an Emulsiflex. Clarified lysate was incubated at 65 °C for one hour and precipitated proteins were removed by centrifugation. Soluble protein was dialyzed against 5 mM potassium phosphate, pH 7.8, and applied to pre-swollen diethylaminoethyl (DE52) cellulose resin (GE Healthcare). The protein-resin slurry was rocked for 1 h at 10 °C before vacuum filtration using Whatman #4 filter paper and a Büchner funnel. Resin was washed with an excess of 5 mM potassium phosphate, pH 7.8, then protein was eluted with 100 mM potassium phosphate, pH 7.8. Eluted protein was dialyzed against 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5, applied to a carboxymethyl (CM) Sepharose (GE Healthcare) column, and eluted with a NaCl gradient. Fractions were concentrated using 5 kDa molecular weight cut-off concentrators (Sartorius) to 21 mg mL^{-1}, as measured by NanoDrop ND-1000 spectrophotometer using an extinction coefficient of 43.43 L/mol^{-1} cm^{-1} at 280 nm. Human MnSOD crystals were grown from 1.8 M potassium phosphate, pH 7.8, by hanging-drop vapor diffusion at room temperature. Protein and reservoir solution were mixed at a 1:1 ratio to give a 2.0 μL drop and crystals appeared within 1 day. To obtain the azide complex, 2.0 μL of reservoir containing 200 mM sodium azide were added to drops of 6 day crystals. Data was collected 3 h after azide addition.

2.2. Data collection and structure determination

Crystals were briefly passed through a cryoprotectant solution consisting of 3.6 M potassium phosphate, pH 7.8, using a microloop (MiTeGen). The MnSOD-azide crystals also had 150 mM sodium azide in the cryoprotectant. Crystals were plunged into a 100 K stream of nitrogen gas provided by a Rigaku X-stream. X-ray diffraction data were collected using a Rigaku FR-E Cu Kα rotating-anode generator operating at 45 kV and 45 mA equipped with a R-AXIS IV^{++} detector. Data were processed using *HKL*-3000 for indexing, integration, and scaling (Minor et al., 2006). Native MnSOD and the MnSOD-azide complex were solved using Protein Data Bank coordinates **1JA8** that had the same unit cell dimensions and space group (Hearn et al., 2001). Following removal of solvent and active-site metals, simple molecular replacement was performed through rigid-body refinement and subsequent restrained-positional refinement using *REFMAC5* to 1.82 Å and 1.77 Å resolution for the native and azide complex structures, respectively (Murshudov et al., 2011). Using *Coot*, omit electron density maps were analyzed and the protein model was fit