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Structure and Reaction Mechanism of Nickel-containing Superoxide Dismutase

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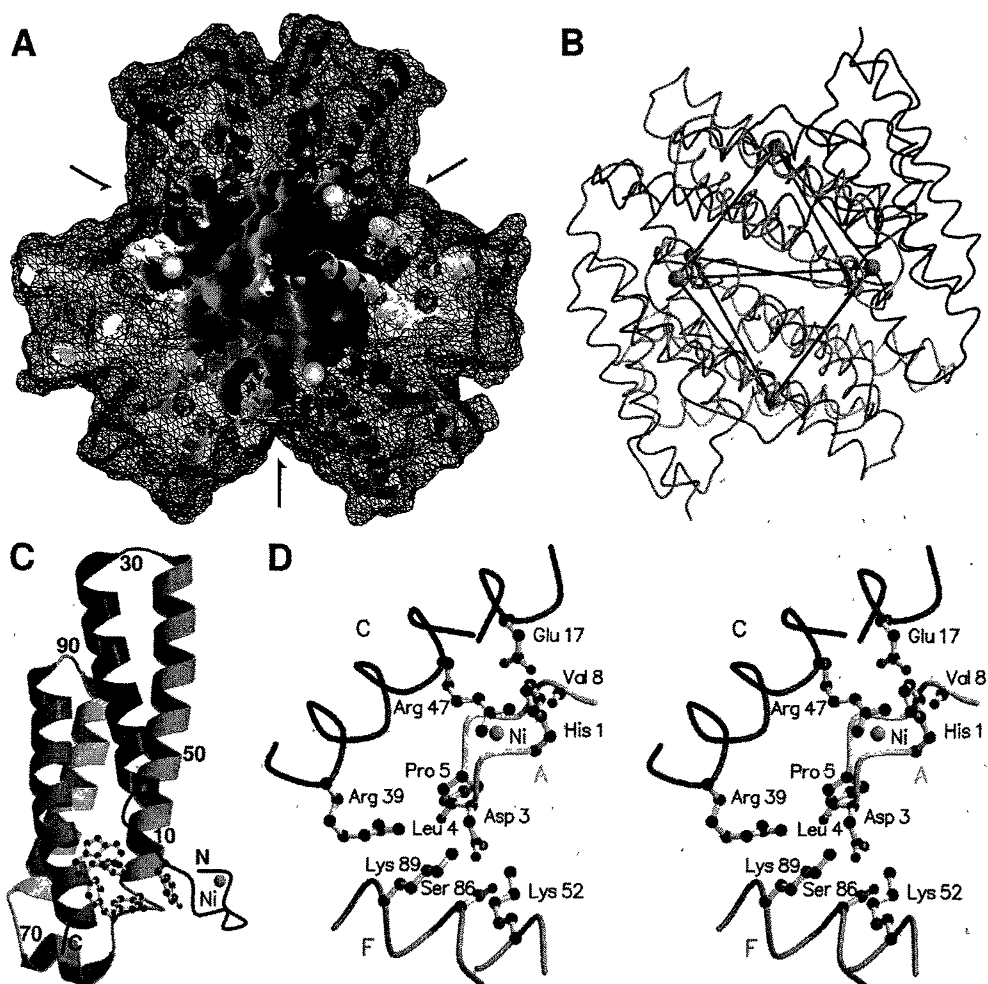
Nickel-containing Superoxide Dismutase (NiSOD) was prepared to homogeneity from *Streptomyces seoulensis* and was used for X-ray Absorption spectroscopy (XAS), Electron Paramagnetic Resonance (EPR) spectroscopy, Electron-Nuclear Double Resonance spectroscopy (ENDOR) and X-ray crystallography. The molecular mass of NiSOD subunit was determined to be 13.2 kDa by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) Mass Spectrometry. And the molecular mass of the native enzyme was determined to be 73 kDa by gel filtration chromatography and to be 84.9 kDa by sedimentation equilibrium using analytical ultracentrifuge. When the *sodN* gene of *Streptomyces seoulensis* was expressed in *Streptomyces lividans* TK24 or *Streptomyces coeolicolor* A3(2), a total of seven kind hybrid SOD bands appeared. This result, together with the gel filtration and sedimentation equilibrium data indicated that the quaternary structure of NiSOD is a homohexamer, not the previously reported homotetramer, which is novel among SODs.

The EPR spectrum of NiSOD as isolated exhibited resonances typical of a rhombically distorted $S = 1/2$ Ni(III) signal with unique g values (g_{xyz} = 2.306, 2.232 and 2.016) at 100 K. The large anisotropy and the average g value of 2.18 of NiSOD EPR spectrum indicate that the unpaired electron mainly resides in a metal-based orbital. The fact that g_x and g_y are larger than g_z and that g_z is nearly 2.00, together with the observation of a superhyperfine splitting of the g_z component, indicates a $(d_{z^2})^1$ electron configuration for Ni(III). The EPR spectrum could be simulated as an effective S = 1/2 system, using a Gaussian line-shape function and the following parameters; g_{xyz} = 2.306, 2.232, 2.016, A_{xyz} = 16.2, 17.7, 24.6 G and l_{xyz} = 28, 17, 7.8 G. At pH 4.0, the EPR spectrum showed g-values (g_{xyz} = 2.282, 2.24, 2.0155) which is more axial than that at pH 7.4, indicating that the active site structure has changed. In addition, the EPR spectrum taken at pH 5 and above pH 8 showed clear superhyperfine splitting in the g_y region.

The ^{61}Ni-substituted NiSOD was purified and searched for EPR signals at 100 K. When the resultant EPR spectrum was compared with that of the normal enzyme (natural abundance of ^{61}Ni is

1.2%), the introduction of an isotope with a nuclear spin (I = 3/2) induced a clearly resolved hyperfine structure at g = 2.016, unambiguously identifying that the EPR signal from NiSOD is due to Ni. When the EPR spectrum was taken from the ^{15}N (I = 1/2)-enriched NiSOD (natural abundance of ^{15}N is 0.366%), the three prominent lines in the g_z region of native NiSOD were changed to two prominent lines, indicating that the original triplet was originated from ^{14}N (I = 1) superhyperfine splitting. Also the introduction of ^{15}N induced a clear splitting in the g_y (g = 2.23) region because of $A(^{15}N)/A(^{14}N)$ = 1.4. The EPR spectrum of NiSOD purified from the cultures supplemented with 0.5% $(^{15}NH_4)_2SO_4$ and 0.01% natural histidine (mainly ^{14}N) as a nitrogen source, together with the ENDOR and Electron Spin



Echo Envelope Modulation (ESEEM) spectroscopy indicated that the N-donor ligand is the $N_{\varepsilon 2}$ atom of histidine imidazole group. The EPR spectrum of ^{33}S enriched NiSOD showed distinct line broadening in the g_z region resulting from a superhyperfine interaction with ^{33}S nucleus (I = 3/2), directly showing that more than one sulfur atom act as ligands for Ni.

The crystal structure of NiSOD demonstrated that NiSOD is a hexameric enzyme consisting of four-helix-bundle subunits. The hexamer exhibited a three-fold symmetry axis with three two-fold axes perpendicular to the three-fold axis. The subunit structure which comprises 117 residues in the mature enzyme revealed a four-helix bundle in the canonical all-antiparallel topology. The crystal structures of the resting NiSOD revealed that each Ni(III) ion is coordinated by the amino group of His1, the amide group of Cys2, and two thiolate groups, Cys2 and Cys6. Upon reduction, a systematic increase in bond lengths was observed. The water molecule closest to the Ni(III) in the resting form of the enzyme was absent in the reduced crystal form, the second water molecule still being present at a slightly shortened average distance of 4.0 Å.

The substitution of phenylalanine for tyrosine at position 9 was accompanied by little loss of activity, implying that other polar groups in the active site are sufficient to provide hydrogen bonds to the potential functionally important water molecule or that the water molecule is not essential for SOD activity. The maintenance of the SOD activity in Y9F and Y9W mutants, and the complete loss of activity in Y9A mutant could be explained by the hydrophobic nature of the side chain of amino acid residues which participate in an aromatic packing at the N-terminal side of the four-helix bundle. The activity of Y9W mutant enzyme was lower than that of Y9F mutant enzyme and this could be explained by the active site accessibility of the substrate superoxide anion, which might be more obstructed by the side chain of tryptophan than that of tyrosine upon approaching the active site. Glutamate 17, arginine 39, and lysine 52 appeared essential for the stability of active site loop. The non-denaturing PAGE of these mutant proteins expressed in *S. lividans* TK24 showed only four hybrid bands with SOD activity, indicating that at least three functional subunits are required for the hexameric NiSOD activity.

Some Actinomycetes other than *Streptomyces* species, were tested for the NiSOD activity. *Actinomadura citrea, Arthrobacter globiformis, Hongia koreensis,* and *Rothia dentocariosa* did not show the Ni-inducible SOD activity. *Microtetraspora glauca, Kitasatospora griseola, Kitasatospora setae, and Micromonospora rosaria* showed the Ni-inducible SOD activity. SODs were purified from *M. glauca, K. griseola,* and *M. rosaria,* and characterized by UV-visible absorption spectroscopy, EPR spectroscopy, and amino-terminal sequencing. The results indicate that the NiSOD exists not only in *Streptomyces* species but also exists in some other Actinomycetes.

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