J|A|C|S
COMMUNICATIONS

Published on Web 12/20/2003

# Expression, Reconstitution, and Mutation of Recombinant *Streptomyces coelicolor* NiSOD

Peter A. Bryngelson,[†] Sumonu E. Arobo,[†] Jennifer L. Pinkham,[‡] Diane E. Cabelli,[§] and Michael J. Maroney*,[†]

*Department of Chemistry and Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003, and Department of Chemistry, Brookhaven National Laboratory, Upton, New York 11973*

Received September 26, 2003; E-mail: mmaroney@chemistry.umass.edu

Superoxide dismutases (SODs) are enzymes that catalyze the conversion of superoxide to hydrogen peroxide and dioxygen (eq 1).[1,2]

$$2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Superoxide radical has been linked to various disorders ranging in severity from inflammatory to neurodegenerative diseases.[3−5] SODs protect organisms from the deleterious effects of superoxide and reactive oxygen species derived therefrom.[1,6] Various *Streptomyces* species are known to produce nickel-dependent SODs (NiSODs),[7,8] and more recently, genomic evidence for NiSODs has been found in cyanobacteria.[9] The NiSODs have several features in common with other SODs (e.g., MnSOD,[10] FeSOD,[11,12] and CuZnSOD[13]), including an active-site metal ion that undergoes one-electron redox chemistry and supports a catalytic rate near the diffusion limit. NiSOD also has a pH dependence identical to MnSOD despite the lack of amino acid sequence homology between NiSOD and any other SOD.[14] However, unlike Mn(II), Fe(II), or Cu(II), Ni(II) does not catalyze superoxide dismutation in aqueous solution,[1] presumably because of the lack of a redox couple at an appropriate potential. Thus, the protein appears to play a critical role in creating the redox-active nickel site. X-ray absorption spectroscopic (XAS) studies are consistent with an $S_3(O/N)_2$ ligand donor environment, with hyperfine coupling visible in the EPR spectrum of the resting oxidized protein establishing the presence of at least one N-donor ligand.[14] This ligand environment indicates that the appropriate redox potential for SOD activity is achieved in NiSOD by including S-donor ligands in the active site,[14] despite their well-known reactivity with $H_2O_2$ and $O_2$.[15,16] Here we report the expression in *Escherichia coli*, and in vitro processing and reconstitution of functional recombinant *S. coelicolor* NiSOD. Using this system, we have explored the functional roles of two potential nickel ligands (H1 and M28).

NiSOD is the product of the *sodN* gene, which encodes a protein with a 14 amino acid N-terminal extension that must be posttranslationally removed to obtain the mature protein with an N-terminal H residue. The sequence of the *sodN* gene indicates that only three S atoms exist in the mature polypeptide, C2, C6, and M28, thus identifying the N terminus as critical to nickel coordination and suggesting the possibility that H1 is a nickel ligand.[14] Preliminary studies (not shown) indicated that neither the *sodN* gene product expressed in *E. coli*, nor constructs with truncated N-terminal extensions would produce fully active NiSOD.[17] For this reason,

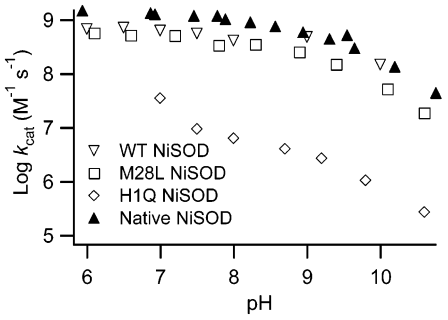


***Figure 1.*** Catalytic rate constants for NiSODs as a function of pH. (▽ = recombinant WT *S. coelicolor* NiSOD, □ = *S. c.* M28L-NiSOD, ◇ = *S. c.* H1Q-NiSOD, and ▲ = native *S. seoulensis* NiSOD.) Rates shown are based on nickel concentration of enzyme samples.

the *S. coelicolor sodN* gene[18] was subcloned as a C-terminal fusion to a 47-amino acid leader sequence containing a 6-His tag and a factor Xa cleavage site. The expressed protein was purified as described (see Supporting Information) and processed using factor Xa. The expression of the fusion protein and correct processing to yield apoNiSOD was confirmed by sequencing the DNA at the University of Massachusetts automated DNA sequencing facility and by the MWs of the products (ESI-MS − Calcd. for the fusion protein: 18 170 Da; Found 18 172 Da; Calcd. for apoNiSOD: 13 201 Da; Found: 13 202 Da). Edman N-terminal peptide sequencing of the processed peptide (Midwest Analytical, Inc., St. Louis MO) confirmed that the protein had the wild-type (WT) HCDLPCGV N terminus.

The apoNiSOD product is a monomeric protein that does not bind $Ni^{2+}$ ions, whereas the holoenzyme was previously reported to be tetrameric[8] (or hexameric, as found for native *S. seoulensis* NiSOD,[17] which has 91% amino acid sequence identity with *S. coelicolor* NiSOD). Reconstitution of apoNiSOD required treatment with DTT and dialysis with 1.0 mM $NiCl_2$. The DTT was necessary to reduce the cysteine residues, which are oxidized in apoNiSOD as determined by DTNB labeling, and establishes that the C2 and C6 are essential for specific binding of nickel. The resulting processed and reconstituted enzyme contained 0.88 equiv of Ni per subunit (graphite furnace AA, Bradford), was hexameric (ESI-MS), and spontaneously developed both the 378 nm UV−vis absorption and EPR spectrum that are characteristic of native, resting NiSOD.[14] The recombinant WT enzyme produced in this manner was fully active (using the xanthine oxidase assay[19] or direct measurement of the rate of $O_2^-$ disappearance using pulse-radiolytic generation of $O_2^-$[3,14,20] as shown in Figure 1).

Amino acid substitutions were made by site-directed mutagenesis using the appropriate mutagenic primers.[21] Mutants were expressed, isolated, and processed in vitro as described above for the WT

[†] Department of Chemistry, University of Massachusetts.
[‡] Department of Biochemistry and Molecular Biology, University of Massachusetts.
[§] Department of Chemistry, Brookhaven National Laboratory.


10.1021/ja0387507 CCC: $27.50 

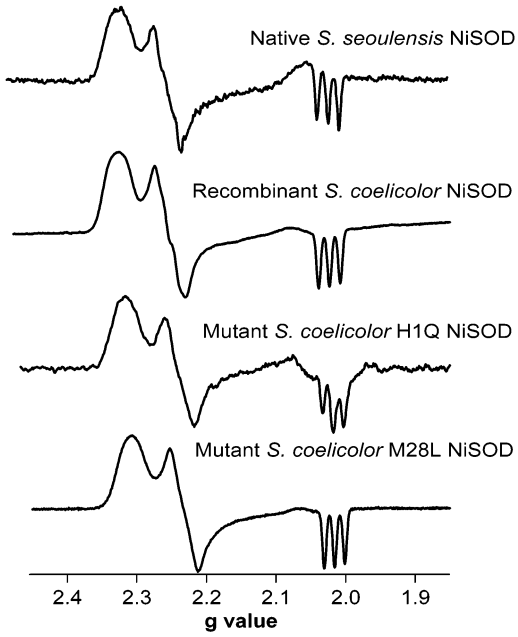


***Figure 2.*** X-band EPR spectra at 77 K of native oxidized *S. seoulensis* NiSOD ($g$ = 2.30, 2.24, and 2.01; $A_{zz}$ = 24.9 G assigned to one axial N-donor ligand);[14] and mutants.

recombinant protein. The expression and correct processing of the mutant fusion proteins to yield apo-M28L-NiSOD and apo-H1Q-NiSOD were confirmed by DNA sequencing and by the MWs of the products (Calcd. for the M28L-NiSODfusion protein: 18 152 Da; ESI-MS Found: 18 152; Calcd. for apoH1Q-NiSOD: 13 192 Da; ESI-MS Found: 13 190 Da). The reconstituted M28L-NiSOD contained 1.1 equiv of Ni per subunit. The reconstituted H1Q-NISOD contained 0.6 equiv of Ni per subunit.

The data in Figures 1 and 2 show that replacement of M28 by L does not affect the catalytic or EPR spectroscopic properties of the enzyme. The maximum value of $k_{cat}$ is unchanged, and the pH dependence of M28L NiSOD activity is identical to that of the WT enzyme. These results demonstrate that M28 is not important to the electronic structure or the catalytic properties of the active site.

In contrast, the H1Q mutation produces an enzyme that has activity that is 2 orders of magnitude lower than that of WT-NiSOD (Figure 1) and whose resting state contains mostly EPR-silent Ni(II) (spin integration = 0.06/Ni). Examination of a small population of enzymes in the Ni(III) state reveals that the signal is not altered and still exhibits the N-hyperfine coupling observed in WT enzyme. Thus, the H1 residue is not the N-donor ligand responsible for this hyperfine interaction. Nonetheless, the H1 residue is important to both the electronic structure and catalytic activity of the active site.

Preliminary attempts have been made to connect the functional consequences of the H1Q and M28L mutations to the structure of the Ni site using X-ray absorption spectroscopy (XAS) as a structural probe.[22] The M28L Ni K-edge X-ray absorption near edge structure (XANES) spectrum is not identical to WT, and the small perturbation observed relative to WT is consistent with a five-coordinate Ni site similar to that found in native *S. seoulensis* and WT *S. coelicolor* NiSODs (see Supporting Information). Since NiSOD contains only three S atoms, (C2, C6, and M28), the lack of a functional role for M28 indicates that the S-ligation previously observed by EXAFS analysis arises from only C2 and C6,[14] although a weak interaction with M28 like that found in blue copper proteins cannot be ruled out by the XANES data.[23] In the case of H1Q-NiSOD, the XANES analysis reveals a shift from five-coordinate geometry to four-coordinate planar geometry, although the reason for this change is obscured by the fact that *reduced* resting native *S. seoulensis* NiSOD has four-coordinate planar Ni(II) centers[14] and H1Q-NiSOD is mostly in the reduced state. While it is likely that the redox state change arises from the loss of H1 as a ligand, the loss of function for the H1Q mutation could also be attributed to a role in proton donation as found in CuZnSOD.[1]

**Acknowledgment.** We thank J-H Roe for the generous donation of purified *sodN* gene in the pET-3a expression vector. We acknowledge support from NSF MCB-021482 (M.J.M.) and from the donors of the Petroleum Research Fund, administered by the American Chemical Society. Pulse radiolysis studies were carried out at the BNL Center for Radiation Chemical Research which is funded under Contract DE-AC02-98CH10886 with the U.S. Department of Energy Division of Chemical Sciences, Office of Basic Energy Sciences. Trainee funding (P.A.B.) was provided by the NIH-Chemistry Biology Interface Program, T32 GM08515.

**Supporting Information Available:** Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Cabelli, D. E.; Riley, D.; Rodriguez, J. A.; Valentine, J. S.; Zhu, H. In *Biomimetic Oxidations Catalyzed by Transition Metal Complexes*; Meunier, B., Ed.; Imperial College Press: London, 1998; Chapter 10.
(2) Miller, A. F., Sorkin, D. *Comments Mol. Cell Biophys.* **1997**, *9*, 1−48.
(3) McCord, J. M. *Methods Enzymol.* **2002**, *349*, 331−341.
(4) Halliwell, B. In *Active Oxygen in Biochemistry*; Liebman, J. F., Ed.; Blackie Academic and Professional: New York, 1995; pp 313−335.
(5) Beyer, W.; Imlay, J.; Fridovich, I. *Prog. Nucleic Acid Res. Molec. Biol.* **1991**, *40*, 221−253.
(6) Miller, A. F.; Sorkin, D. *Comments Mol. Cell. Biophys.* **1997**, *9*, 1−48.
(7) Kim, E.-J.; Chung, H.-J.; Suh, B.; Hah, Y. C.; Roe, J.-H. *Mol. Microbiol.* **1998**, *27*, 187−195.
(8) Youn, H. D.; Kim, E. J.; Roe, J. H.; Hah, Y. C.; Kang, S. O. *Biochem. J.* **1996**, *318*, 889−896.
(9) Palenik, B.; Brahamsha, B.; Larimer, F. W.; Land, M.; Hauser, L.; Chain, P.; Lamerdin, J.; Regala, W.; Allen, E. E.; McCarren, J.; Paulsen, I.; Dufresne, A.; Partensky, F.; Webb, E. A.; Waterbury, J. *Nature* **2003**, *424*, 1037−1042.
(10) Borgstahl, G. E. O.; Parge, H. E.; Hickey, M. J.; Beyer, W. F.; Hallewell, R. A.; Tainer, J. A. *Cell* **1992**, 107−118.
(11) Tierney, D. L.; Fee, J. A.; Ludwig, M. L.; Penner-Hahn, J. E. *Biochemistry* **1995**, *34*, 1661−1668.
(12) Lah, M. S.; Dixon, M. M.; Pattridge, K. A.; Stallings, W. C.; Fee, J. A.; Ludwig, M. L. *Biochemistry* **1995**, *34*, 1646−1660.
(13) Tainer, J. A.; Getzoff, E. D.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 284−287.
(14) Choudhury, S. B.; Lee, J.-W.; Davidson, G.; Yim, Y.-I.; Bose, K.; Sharma, M. L.; Kang, S.-O.; Cabelli, D. E.; Maroney, M. J. *Biochemistry* **1999**, *38*, 3744−3752.
(15) Grapperhaus, C. A.; Darensbourg, M. Y. *Acc. Chem. Res.* **1998**, *31*, 451−459.
(16) Mirza, S. A.; Day, R. O.; Maroney, M. J. *Inorg. Chem.* **1996**, *35*, 1992−1995.
(17) Neimoth-Anderson, J.; Pinkham, J. L.; Maroney, M. J. Unpulished results.
(18) Roe, J. H. *sodN* gene of *S. coelicolor* made availible from Seoul National University.
(19) Crapo, J. D.; McCord, J. M.; Fridovich, I. Academic Press: San Diego, 1978; Vol. 53, pp 382−393.
(20) Searcy, D. G.; Whitehead, J. P.; Maroney, M. J. *Arch. Biochem. Biophys.* **1995**, *318*, 251−263.
(21) Mikaelian, I.; Sergeant, A. *Nucleic Acids Res.* **1992**, *20*, 376−376.
(22) Colpas, G. J.; Maroney, M. J.; Bagyinka, C.; Kumar, M.; Willis, W. S.; Suib, S. L.; Baidya, N.; Mascharak, P. K. *Inorg. Chem.* **1991**, *30*, 920−928.
(23) Scott, R. A.; Hahn, J. E.; Doniach, S.; Freeman, H. C.; Hodgson, K. O. *J. Am. Chem. Soc.* **1982**, *104*, 5364−5369.

JA0387507