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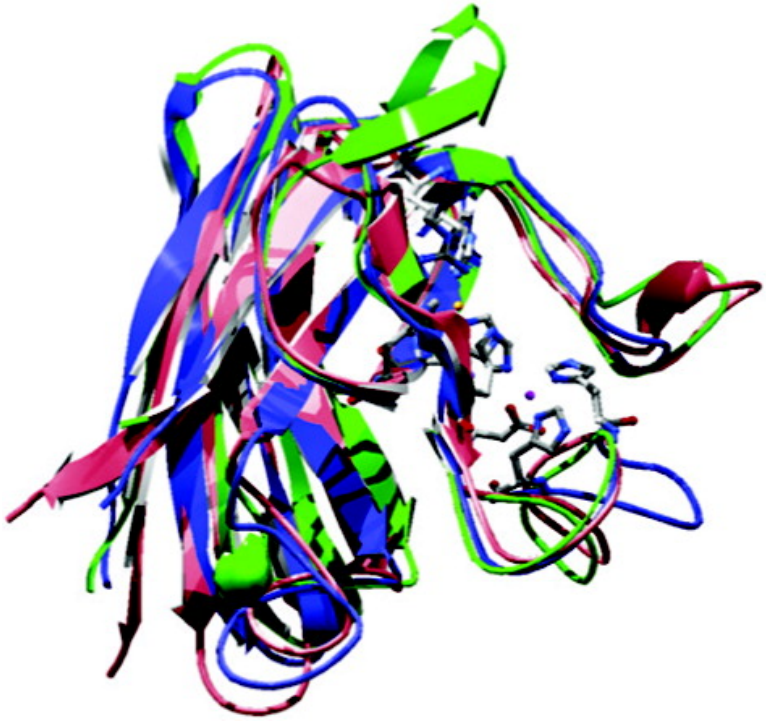

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From an Inactive Prokaryotic SOD Homologue to an Active Protein through Site-Directed Mutagenesis

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Abstract: It is known that several prokaryotic protein sequences, characterized by high homology with the eukaryotic Cu,ZnSODs, lack some of the metal ligands. In the present work, we have stepwise reintroduced the two missing copper ligands in the SOD-like protein of *Bacillus subtilis*, through site-directed mutagenesis. The mutant with three out of the four His that bind copper is not active, whereas the fully reconstituted mutant displays an activity of about 10% that of human Cu,ZnSOD. The mutated proteins have been characterized in solution and in the solid state. In solution, the proteins experience conformational disorder, which is believed to be partly responsible for the decreased enzymatic activity and sheds light on the tendency of several human SOD mutants to introduce mobility in the protein frame. In the crystal, on the contrary, the protein has a well-defined conformation, giving rise to dimers through the coordination of an exogenous zinc ion. The catalytic properties of the double mutant, which might be regarded as a step in an artificial evolution from a nonactive SOD to a fully functioning enzyme, are discussed on the basis of the structural and dynamical properties.

Introduction

Cu,Zn superoxide dismutase (SOD hereafter) belongs to a class of enzymes that play a major role in the physiological response to oxygen toxicity. It catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide[1,2] at a diffusion-limited rate enhanced by electrostatic guidance of the substrate to the active site.[3,4] The eukaryotic proteins, which are homodimeric enzymes containing one copper and one zinc ion per subunit,[5] are abundant in the cytoplasm, but they have also been found in the nucleus, in peroxisomes, lysosomes, and mitochondria.[6−8]

SOD are present also in several bacteria, where they have been mainly isolated from the periplasmic space.[9,10] A recent search of the complete genomes of 138 *Archea* and *Bacteria* located 57 putative homologues of human SOD in 48 organisms.[11] Most of these bacterial SOD homologues conserve the copper and zinc binding motifs, and the gene products are completely functional enzymes as found for the Cu,ZnSODs from *P. leiognathii*,[12] *E. coli*,[13] *S. thyphimurium*,[14] and *A. pleuropneumoniae*.[15] However, in 9 organisms, 11 sequences with identity of at least 30% with human SOD have been found, which however lack one or more copper ligands and therefore presumably do not have the capability to bind copper. One of these sequences is found in *Bacillus subtilis* (BsSOD) where two copper ligands are missing, one corresponding to His 48 in the human protein, and the other to the His residue that bridges the copper and the zinc ions in eukaryotic proteins (human His 63). These residues are replaced by a Tyr and a Pro, respectively. BsSOD has, however, the zinc site completely conserved and fully occupied by the zinc ion.[11]

The BsSOD protein has been extensively characterized in the zinc-containing form.[11] In solution, large regions, mostly

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(1) Mann, T.; Keilin, D. *Proc. R. Soc. London* **1938**, *126*, 303.
(2) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049−6055.
(3) Getzoff, E. D.; Tainer, J. A.; Weiner, P. K.; Kollman, P. A.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 287−290.
(4) Sines, J. J.; Allison, S. A.; McCammon, J. A. *Biochemistry* **1990**, *29*, 9403−9412.
(5) Getzoff, E. D.; Tainer, J. A.; Stempien, M. M.; Bell, G. I.; Hallewell, R. A. *Proteins: Struct., Funct., Genet.* **1989**, *5*, 322−336.
(6) Chang, L. Y.; Slot, J. W.; Geuza, H. J.; Crapo, J. D. *J. Cell Biol.* **1988**, *107*, 2169−2179.
(7) Keller, G.-A.; Warner, T. G.; Steimer, K. S.; Hallewell, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7381−7385.
(8) Sturtz, L. A.; Diekert, K.; Jensen, L. T.; Lill, R.; Culotta, V. C. *J. Biol. Chem.* **2001**, *276*, 38084−38089.
(9) Kroll, J. S.; Langford, P. R.; Loynds, B. M. *J. Bacteriol.* **1991**, *173*, 7449−7457.
(10) Steinman, D. H.; Ely, B. *J. Bacteriol*. **1990**, *172*, 2901−2910.
(11) Banci, L.; Bertini, I.; Calderone, V.; Cramaro, F.; Del Conte, R.; Fantoni, A.; Mangani, S.; Quattrone, A.; Viezzoli, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7541−7546.
(12) Redford, S. M.; McRee, D. E.; Getzoff, E. D.; Steinman, D. H.; Tainer, J. A. *J. Mol. Biol.* **1990**, *212*, 449−451.
(13) Pesce, A.; Capasso, C.; Battistoni, A.; Folcarelli, S.; Rotilio, G.; Desideri, A.; Bolognesi, M. *J. Mol. Biol.* **1997**, *274*, 408−420.
(14) Pesce, A.; Battistoni, A.; Stroppolo, M. E.; Polizio, F.; Nardini, M.; Kroll, J. S.; Langford, P. R.; O'Neil, J. D.; Sette, M.; Desideri, A.; Bolognesi, M. *J. Mol. Biol.* **2000**, *302*, 465−478.
(15) Forest, E.; Langford, P. R.; Kroll, J. S.; Getzoff, E. D. *J. Mol. Biol.* **2000**, *296*, 145−153.

involving loops, are in a molten globule state. This feature, for some aspects, resembles that of human apoSOD[16] rather than the copper-depleted protein.[17] Such partial conformational disorder and the associated mobility is intriguing also with respect to human FALS mutants, some of which have been found to display mobility around the mutation.[18] BsSOD protein crystallizes as a dimer, with an accessory zinc binding site on the surface of the protein, bridging two molecules. In this form, the protein is well folded and rigid.[11]

Bacillus subtilis mutants lacking the BsSOD coding gene (yojM) do not show growth alterations in normal conditions. Furthermore, under oxygen and copper stress conditions, the gene is not silent and the corresponding mRNA is normally detected in DNA arrays (Jennifer Cavet − University of Manchester, personal communication).

Based on the sequence analysis and on the phylogenetic tree,[11] BsSOD protein may be thought as a step of the evolution from a "no-copper superoxide dismutase" to fully active SODs. It could also be tempting to relate the mobility of FALS mutants with the mobility of these "aborted" SODs, as the "well-behaving" SODs are essentially rigid. With this in mind, we have tried to reconstitute SOD's activity through "artificial evolution" obtained by introducing the copper ligands with site-directed mutagenesis. We have cloned and expressed the single and the double mutants P104H and Y88H-P104H, which reintroduce one or both of the lacking copper binding histidines, reestablishing in the first case the ability to bind copper and in the second case the classical copper site of Cu,ZnSOD. We report here the structural and biochemical characterization of these two mutants showing that we have achieved our goal and recreated, with the double mutant, a partially active Cu,ZnSOD. The mechanistic and physiological implications of the results are discussed.

Experimental Section

Protein Expression and Characterization. The mutants P104H and Y88H-P104H of BsSOD were expressed in *E. coli* from the vector expressing the WT BsSOD protein.[11] The above mutations were obtained by using the Quick Change Site Mutagenesis kit (Stratagene). The presence of the mutations was confirmed via DNA sequencing analysis. Protein production and isolation was performed as reported for WT BsSOD.[11] The protein is obtained in good yield (~50 mg/L of culture). Samples were in unbuffered water solution with the exception of the samples for NMR spectra that were in 10 mM phosphate buffer, pH 5.0. The aggregation state of BsSOD was determined by gel filtration chromatography using proteins with known molecular weight as reference. The copper and zinc content was checked through atomic absorption analysis with a GBC 903 instrument. Activity assays were carried out by the pulse radiolysis method, which is based on the first-order rate of $O_2^{\bullet -}$ loss, monitored spectrophotometrically at 250 nm.[19] Enzymes are assayed in 10 mM formate, 10 mM phosphate, 10 μM EDTA, at pH 7.1 unless otherwise specified.

Electronic spectra were recorded on a Cary 50 spectrophotomer (Varian). EPR spectra were recorded at 180 K on a Elexsys E500 spectrometer (Bruker) equipped with X-band microwave bridge (microwave frequency 9.45 GHz) and an ER 4131 VT unit for temperature control. CD spectra were recorded on a Jasco J-810 spectrophotomer, at 298 K. Secondary structure elements were determined from the analysis of the spectra with the JASCO program (Spectra Manager Software from JASCO). NMR spectra were collected at 298 K on a 900 MHz Bruker Avance spectrometer.

Protein Crystallization and X-ray Structure Determination. Diffraction quality single crystals of BsSOD, of approximate size of $0.2 \times 0.2 \times 0.1$ mm, were obtained at 20 °C using the vapor diffusion technique. Only by adding extra zinc ions up to a 0.5:1 ratio does crystal growth occur and is reproducibly obtained.

The data were measured at DESY on the tunable beamline BW7A (Hamburg, Germany) at 100 K, and the crystals used for data collection were cryo-cooled using a solution containing 10% ethylene glycol in the mother liquor. The crystal diffracted to a maximum resolution of 1.9 Å (P104H BsSOD) and of 1.6 Å (P104H-Y88H BsSOD). The crystal spacegroup of P104H BsSOD was P1 with four molecules in the asymmetric unit, while the space group of P104H-Y88H BsSOD was $P2_12_12$ with two molecules in the asymmetric unit.

Both structures were solved by the molecular replacement technique using as a starting model one molecule of WT BsSOD (PDB ID 1S4I)[11] from where all of the water molecules and metal ions were omitted.

Data Deposition. The atomic coordinates of BsSOD mutants have been deposited in the Protein Data Bank (accession codes: 1XTL and 1XTM for P104H BsSOD and Y88H-P104H BsSOD, respectively).

Results and Discussion

Spectroscopic and Functional Characterization of the Mutants. To transform an inactive SOD lacking two copper ligands as it occurs in BsSOD-like protein, we have somehow mimicked evolution by introducing one and both copper ligands, thus creating the classical copper site of eukaryotic Cu,ZnSODs. For this purpose, we have produced two mutants of the SOD-like protein from *B. subtilis* (P104H BsSOD and P104H-Y88H BsSOD) and performed some spectroscopical and functional analysis. They have confirmed that both mutants retain the monomeric structure as in the case of WT BsSOD. Also, the secondary structure is not significantly affected by the mutations, as it can be derived from the CD spectra, which are very similar for the three systems.

Both mutants, P104H and Y88H-P104H BsSOD, are obtained from the culture medium with a regular zinc content (1 mol of Zn^{2+} per mol of protein). However, the two mutants behave differently with respect to copper content: the doubly mutated protein, after purification, presents an almost regular copper content (0.9 mol of Cu^{2+}/1.0 mol of protein), whereas the single mutant is deficient in copper (0.2 mol of Cu^{2+}/1.0 mol of protein). The copper site of the latter mutant can be, however, fully reconstituted in vitro, thus suggesting a lower affinity for copper, with respect to a regular copper binding site.

Interesting differences are observed in the visible region of the electronic spectra (Figure 1) where Y88H-P104H BsSOD shows an absorption at 680 nm, typical of the Cu,Zn SOD chromophore and due to d−d transitions of copper,[20] but with weaker absorbance ($\epsilon = 60$ M^{-1} cm^{-1}) than in human Cu,ZnSOD ($\epsilon = 150$ M^{-1} cm^{-1}). The single mutant P104H, on the contrary, has a bright red color and shows an absorption band at 461 nm ($\epsilon = 290$ M^{-1} cm^{-1}) with a shoulder at 545 nm and a broad band centered at 700 nm. The band at 461 nm, which accounts for the red color of the protein, may be ascribed to a phenolate-to-Cu^{2+} ligand-to-metal charge transfer, similarly

(16) Banci, L.; Bertini, I.; Cramaro, F.; Del Conte, R.; Viezzoli, M. S. *Biochemistry* **2003**, *42*, 9543−9553.
(17) Banci, L.; Bertini, I.; Cantini, F.; D'Onofrio, M.; Viezzoli, M. S. *Protein Sci.* **2002**, *11*, 2479−2492.
(18) Shipp, E.; Cantini, F.; Bertini, I.; Valentine, J. S.; Banci, L. *Biochemistry* **2003**, *42*, 1890−1899.
(19) Fielden, E. M.; Roberts, P. B.; Bray, R. C.; Lowe, D. J.; Mautner, G. N.; Rotilio, G.; Calabrese, L. *Biochem. J.* **1974**, *139*, 49−60.
(20) Pantoliano, M. W.; Valentine, J. S.; Nafie, L. A. *J. Am. Chem. Soc.* **1982**, *104*, 6310−6317.

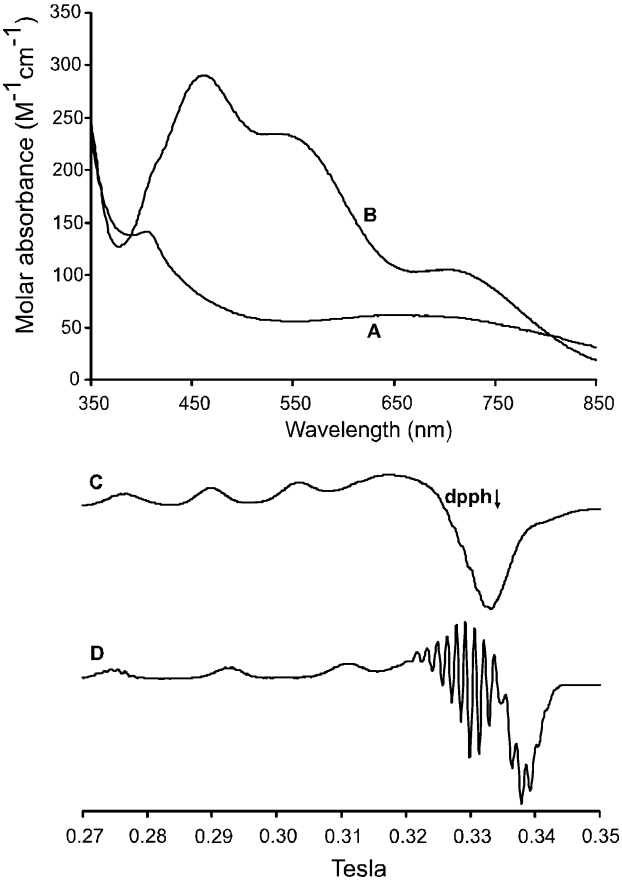

Figure 1. Electronic spectra of Y88H-P104H BsSOD (A) and P104H BsSOD (B). EPR spectra of Y88H-P104H BsSOD (C) and P104H BsSOD (D). The concentration of the samples is 2.7 mM.

to what occurs in galactose and glyoxal oxidases.[21,22] These features are consistent with Tyr 88 completing the copper coordination sphere (see later).

The double mutant shows an axial EPR spectrum (Figure 1C) with g values of 2.27 ($g_{||}$) and 2.07 ($g_{\perp}$) and $A_{||} = 136 \times 10^{-4}$ cm^{-1}, indicating a type II copper(II) in a puckered coordination.[23,24] The superhyperfine structure, which is resolved in the perpendicular feature of the spectrum, presents nine components, with a coupling constant of 12×10^{-4} cm^{-1}, consistent with the presence of 4 nitrogens coordinating the copper ion. The EPR spectrum of the single mutant P104H BsSOD (Figure 1D) is characterized by $g_{||} = 2.23$, $g_{\perp} = 2.02$, $A_{||} = 197 \times 10^{-4}$ cm^{-1}, indicative of a type II copper(II) in a tetragonal coordination sphere.[23,24] The perpendicular and the lowest field line of parallel signals are resolved into seven components with a splitting of 10×10^{-4} cm^{-1}, indicating a ligand hyperfine interaction arising from 3 equivalent nitrogen nuclei, belonging to His residues, coupled with the copper ion.

Both mutants have been tested for superoxide dismutase catalytic activity by pulse radiolysis. At low ionic strength (0.02 M), the observed rate constant (k_{obs}) of the single mutant P104H is 3.5×10^5 M^{-1} s^{-1} at pH 7.1, a value matching the rate

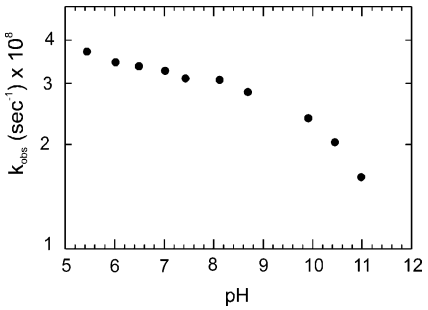

Figure 2. pH dependence of the catalytic rate constant of Y88H-P104H BsSOD. The enzyme was assayed in 10 mM formate, 10 mM phosphate, 10 μM EDTA.

constant for superoxide spontaneous dismutation[25] and which is about 4 orders of magnitude lower than those found for eukaryotic Cu,ZnSODs (between 2×10^9 and 4×10^9 M^{-1} s^{-1})[26] and prokaryotic Cu,ZnSODs (between 2×10^9 and 1×10^{10} M^{-1} s^{-1}).[14,15,27−29] The mutant is therefore inactive; this lack of activity can be reasonably ascribed to the dramatic change in copper coordination sphere; indeed a Tyr residue (Tyr 88) replaces one ligand to copper, stabilizing the 2+ oxidation state of copper, and possibly reducing the capability of copper to alternate between the 2+ and +1 oxidation state, which is required by the dismutation reaction. To catalyze the dismutation of superoxide, the Cu^+/Cu^{2+} pair in SOD should have a redox potential between that of the two pairs of the dismutation reaction ($E°$ $O_2^-/O_2 = -0.33$ V; $E°$ $O_2^-/H_2O_2 = +0.89$ V). In the P104H mutant, copper can be reduced only by strong reductants such as dithionite ($E° = -0.53$ V, pH 7), while weaker ones such as dithiothreitol ($E° = -0.33$ V, pH 7), active with regular SODs, are not able to reduce it. This is further evidence of the reduction of the copper redox potential.

The catalytic activity of the double mutant Y88H-P104H, in the same conditions (pH 7.1; $I = 0.02$ M), is 3.0×10^8 M^{-1} s^{-1}, about 10% of the dismutation rate of eukaryotic and other prokaryotic Cu,ZnSODs and significantly higher than the rates of some Cu(II) inorganic complexes.[30,31] The catalytic activity is similar to that of one of the monomerized eukaryotic SODs.[32] Also, the pH dependence of catalytic rates (Figure 2) resembles that of some eukaryotic Cu,ZnSODs.[33−35]

Structural Characterization of the P104H and Y88H-P104H BsSOD Mutants. WT BsSOD in solution is, for a large portion, in a molten globule state. This leads to a paucity of

(21) Whittaker, M. M.; Kersten, P. J.; Nakamura, N.; Sanders-Loehr, J.; Schweizer, E. S.; Whittaker, J. W. *J. Biol. Chem.* **1996**, *271*, 681−687.
(22) Whittaker, M. M.; Whittaker, J. W. *J. Biol. Chem*. **1988**, *263*, 6074−6080.
(23) Bertini, I.; Canti, G.; Grassi, R.; Scozzafava, A. *Inorg. Chem.* **1980**, *19*, 2198−2200.
(24) Bertini, I.; Scozzafava, A. Copper(II) as probe in substituted metalloproteins; In *Metal ions in biological systems*; Sigel, H., Ed.; Marcel Dekker, Inc.: New York and Basel, 1981; pp 31−74.
(25) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041−1100.
(26) Bertini, I.; Mangani, S.; Viezzoli, M. S. Structure and properties of copper/zinc superoxide dismutases; In *Advanced Inorganic Chemistry*; Sykes, A. G., Ed.; Academic Press: San Diego, CA, 1998; pp 127−250.
(27) Battistoni, A.; Rotilio, G. *FEBS Lett.* **1995**, *374*, 199−202.
(28) Spagnolo, L.; Toro, I.; D'Orazio, M.; O'Neill, P.; Pedersen, J. Z.; Carugo, O.; Rotilio, G.; Battistoni, A.; Djinovic-Carugo, K. *J. Biol. Chem.* **2004**, *279*, 33447−33455.
(29) Stroppolo, M. E.; Sette, M.; O'Neill, P.; Polizio, F.; Cambria, M. T.; Desideri, A. *Biochemistry* **1998**, *37*, 12287−12292.
(30) Latif Abuhijleh, A.; Woods, C. *Inorg. Chem. Commun.* **2002**, *5*, 269−273.
(31) Gonzalez-Alvarez, M.; Alzuet, G.; Borras, J.; del Castillo, A. L.; Garcia-Granda, S.; Montejo Bernardo, J. M. *J. Inorg. Biochem.* **2004**, *98*, 189−198.
(32) Bertini, I.; Piccioli, M.; Viezzoli, M. S.; Chiu, C. Y.; Mullenbach, G. T. *Eur. J. Biophys.* **1994**, *23*, 167−176.
(33) O'Neill, P.; Davies, S.; Fielden, E. M.; Calabrese, L.; Capo, C.; Marmocchi, F.; Natoli, G.; Rotilio, G. *Biochem. J.* **1988**, *251*, 41−46.
(34) Banci, L.; Bertini, I.; Cabelli, D. E.; Hallewell, R. A.; Luchinat, C.; Viezzoli, M. S. *Free Radical Res. Commun.* **1991**, *12−13*, 239−251.
(35) Fisher, C. L.; Cabelli, D. E.; Tainer, J. A.; Hallewell, R. A.; Getzoff, E. D. *Proteins: Struct., Funct., Genet.* **1994**, *19*, 24−34.

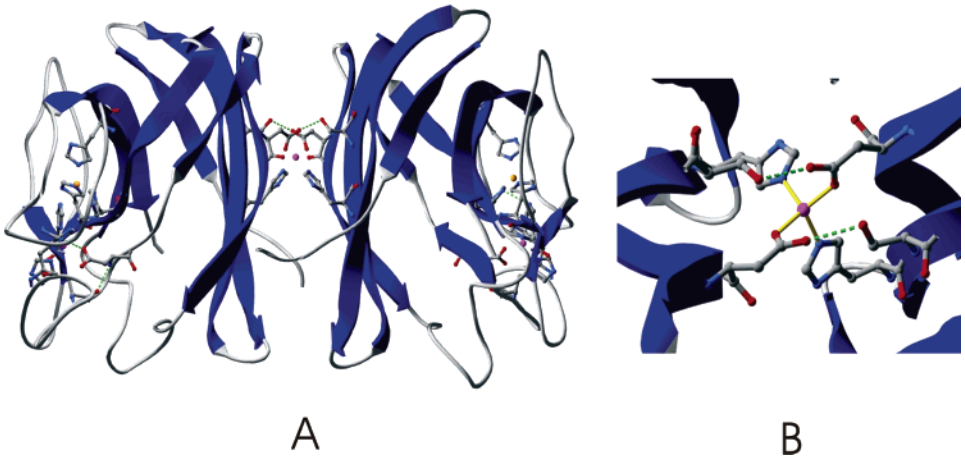

Figure 3. (A) The covalently linked BsSOD dimer. The protein is represented as a blue ribbon with the metal sites residues and metal ions represented as ball-and-sticks. The copper ions are copper colored, while the zinc ions are magenta. The exogenous zinc sits on a noncrystallographic two-fold axis passing between the two BsSOD subunits. Part (B) shows a close-up of the exogenous zinc coordination highlighting the two H-bonds, which occurs between the Asp137 and Ser52 side chains.

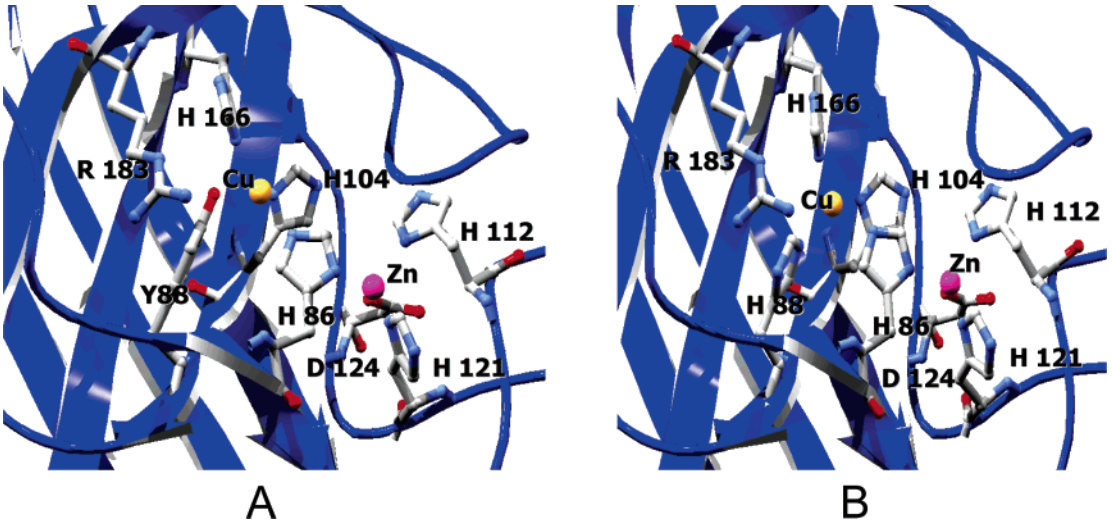

Figure 4. The Cu,Zn site of the P104H BsSOD mutant (A), and of the Y88H-P104H BsSOD mutant (B). The color code of the metal ions is as in Figure 3.

NOESY cross-peaks in the NMR spectra.[11] The mutants do not show in solution any further increase in conformational order as it can be concluded by similar, NOE-poor, NMR maps. Also, further addition of zinc ion, which favors crystal formation, does not contribute to define unique molecular conformation but, above 0.5 equiv of zinc per protein, induces protein precipitation. BsSOD has, however, the very peculiar behavior of easily crystallizing despite the disordered state in solution and to acquire a well-ordered conformation in the crystal. So, to further characterize the mutants, we have determined their crystal structures.

The two BsSOD mutants crystallize in different space groups, but in both crystals the BsSOD molecules are arranged in dimers held together by the coordination of an exogenous zinc ion, bound to His 71 and Asp 137 located at the monomer surface (Figure 3) in a tetrahedral arrangement.[11] The protein−protein interaction is further stabilized by the presence of intermolecular H-bonds.[11] The zinc-bridged dimers of both BsSOD mutants in the crystal are further assembled in long chains, where several direct and water-mediated H-bonds occur between the dimers along the chain. The dimers arrangement, as well as their organization in the crystal, are similar to those already observed in the crystals of WT BsSOD.[11] BsSOD is not unique in presenting an accessory metal binding site on its surface as analogous sites have been found in other proteins.[15,36,37]

The two BsSOD mutants have an identical backbone conformation that is also identical to that of WT BsSOD, as indicated by the RMSDs with the latter structure of 0.45 and 0.52 Å (backbone atoms) for the single mutant and for the double mutant, respectively.

The point mutation P104H, reintroducing the histidine bridging zinc and copper (His 104, corresponding to the HSOD His 63), allows the reconstitution of the Cu,Zn dinuclear center in BsSOD (Figure 4A). In this mutant, copper occupies a slightly distorted square planar site being bound to His 86, Tyr 88, His 104, and His 166 (Figure 4A). No electron density is observed for a ipotetical water molecule, which, on the contrary, is present close to the copper ion (∼2.5−3.2 Å Cu−OH_2 distance) in most prokaryotic and eukaryotic Cu,ZnSOD enzymes.[26] The presence of a coordination bond between Tyr 88 OH and copper produces the red color of the protein, due to the Cu-tyrosinate charge

(36) Tanaka, Y.; Tsumoto, K.; Nakanishi, T.; Yasutake, Y.; Sakai, N.; Yao, M.; Tanaka, I.; Kumagai, I. *FEBS Lett.* **2004**, *556*, 167−174.
(37) Solovyov, A.; Gilbert, H. F. *Protein Sci.* **2004**, *13*, 1902−1907.

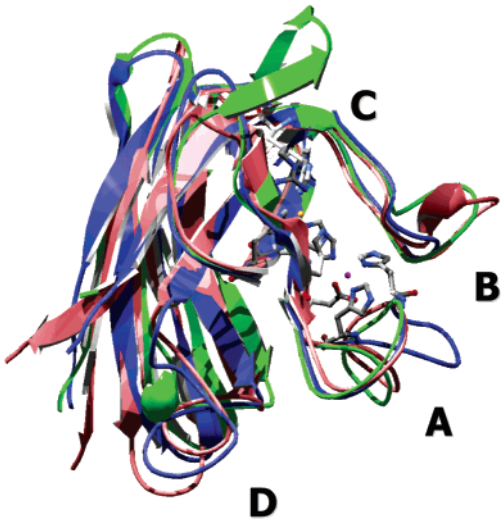

Figure 5. Least-squares superposition (on backbone atoms) of Y88H-P104H BsSOD (blue) with *P. leiognathi* (1YAI) SOD (green) taken as an example of all prokaryotes Cu,ZnSODs and human SOD (pink) taken as an example of eukaryotic Cu,ZnSOD. The differences in the Zn-subloop (A), in the electrostatic loop (B), and in the Greek-key loop (D) are evident as well as the β-hairpin insertion on top of loop IV (C).

transfer. The zinc site is identical to that present in all SODs with the zinc ion bound to His 112, His 121, Asp 124, and the bridging His 104.

The second mutation, Y88H, fully restores the metal site of prokaryotic and eukaryotic Cu,ZnSODs (Figure 4B) with copper coordinated by the nitrogens of four histidines. His 104, which bridges copper and zinc, is at 2.5 Å (Cu−Nϵ) from copper in subunit A and at 3.2 Å in subunit B. Similar long Cu−His distances have been observed in crystals of eukaryotic and prokaryotic SODs and have been interpreted as indicative of the copper ion being, partially or completely, in its Cu(I) state.[38−40] The other Cu−His distances in subunit A have some variability (1.9−2.4 Å), while they are homogeneous in subunit B (2.0−2.1 Å). A water molecule is present close to the copper ion in both subunits, at a Cu−O distance varying between 2.8 Å (subunit A) to 3.2 Å (subunit B). EPR and electronic spectra indicate that the copper site in this mutant has the same electronic and structural properties of a "classical" copper zinc SOD.

Comparison with the available structures of eukaryotic and prokaryotic Cu,ZnSODs shows that these mutants and WT BsSOD have some relevant differences highlighted in Figure 5; that is: (i) loop II (residues 64−66, BsSOD numbering) in BsSOD has a conformation and length similar to that of most prokaryotic, but different from eukaryotic SODs, (ii) as in the other prokaryotic SODs, in the disulfide subloop of loop IV (residues 80−102, BsSOD numbering), the turn does not have a α-helix structure at variance with eukaryotic SODs, (iii) the tip of the Zn subloop of loop IV (residues 113−119, BsSOD numbering) adopts a different conformation with respect to both the eukaryotic and the prokaryotic SODs due to a change of the backbone dihedral angles of Gly113 through Gly119 (Figure 5A), and (iv) loop VII (electrostatic loop, residues 168−181 BsSOD numbering, residues 120−144, HSOD numbering) is

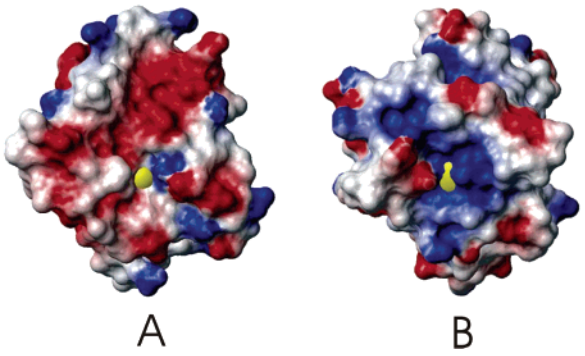

Figure 6. Electrostatic potential mapped onto the molecular surface of the (A) Y88H-P104H BsSOD and (B) HSOD subunits. Charged residues are colored as blue positive and red negative. The active site channel entrance is oriented perpendicular to the page. The yellow spheres indicate the copper ion.

shorter in BsSOD than in prokaryotic and eukaryotic SODs and lacks the second α-helix turn present in eukaryotic SODs (Figure 5B). Furthermore, the prokaryotic Cu,ZnSODs, which are fully active and for which the structure is available, contain, at variance with both BsSOD and eukaryotic SODs, a fully solvent exposed insertion at the turn of loop IV corresponding to BsSOD residues 94−98.[15] This insertion adopts a β-hairpin fold, which extends the turn by about 13 Å, and always contains the charged KDGK sequence (Figure 5C). These charged residues, which are absent in eukaryotic SODs, have been suggested to play a role in the electrostatic recognition and guidance of substrate in prokaryotic SODs and compensate in prokaryotic proteins, on an alternative location, the deletion of four residues in the electrostatic loop of eukaryotic SODs.[41] The absence of this KDGK sequence in BsSOD can contribute to lowering SOD activity of the Y88H-P104H BsSOD mutant. Other relevant differences between BsSOD and active prokaryotic SODs are the different extension and conformation of the Zn-subloop of loop IV (residues 115−120, BsSOD numbering) and of the Greek-key loop (residues 140−160, BsSOD numbering) where a positively charged insertion (KKGSK, residues 148−152, BsSOD numbering) is present in BsSOD (Figure 5D). However, this insertion is located in a different region with respect to the KDGK insertion observed in other prokaryotic SODs and far from the copper site.

All of these sequence variations around the active channel, which is formed by the electrostatic loop VII and by a portion of loop IV, produce in BsSOD an extensive negative electrostatic potential surface on the route of the negatively charged superoxide substrate to the reaction site (Figure 6). The entrance of the catalytic cavity in BsSOD presents the three negatively charged residues Asp 97, Glu 99, and Glu 111 (BsSOD numbering), which in the structure of the other SODs (with the exception of *E. coli* SOD) are replaced by positive or neutral residues, thus resulting in a dramatically different charge distribution on the protein surface between BsSOD and other fully active SODs (Figure 6).

Extensive characterization of the role of each amino acid of the active site channel in modulating the activity of eukaryotic SOD is available in the literature,[3,35,42−45] providing evidence

(38) Blackburn, N. J.; Hasnain, S. S.; Binsted, N.; Diakun, G. P.; Garner, C. D.; Knowles, P. F. *Biochem. J.* **1984**, *219*, 985−990.
(39) Hough, M. A.; Hasnain, S. S. *J. Mol. Biol.* **1999**, *287*, 579−592.
(40) Stroppolo, M. E.; Nuzzo, S.; Pesce, A.; Rosano, C.; Battistoni, A.; Bolognesi, M.; Mobilio, S.; Desideri, A. *Biochem. Biophys. Res. Commun.* **1998**, *249*, 579−582.
(41) Bourne, Y.; Redford, S. M.; Steinman, H. M.; Lepock, J. R.; Tainer, J. A.; Getzoff, E. D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12774−12779.
(42) Getzoff, E. D.; Cabelli, D. E.; Fisher, C. L.; Parge, H. E.; Viezzoli, M. S.; Banci, L.; Hallewell, R. A. *Nature* **1992**, *358*, 347−351.

that the conformational and electrostatic properties of the residues in the electrostatic loop and in the active site channel regulate the accessibility to the copper site and play a major role in determining the efficiency of the enzyme. It is worth noting that replacement of the positive Arg 143 (human SOD numbering) with neutral or negative groups causes a drop of activity to 10% and 4%, respectively, thus indicating a major role of this residue in attracting and docking the superoxide anion in the copper site.[46,47] It has been also shown that simple protein monomerization, without affecting the overall protein charge, produces a reduction of SOD activity to 35% of that of WT HSOD, mainly as a consequence of a different conformation of Arg 143 side chain, which moves further from the copper ion.[48] In BsSOD, which is monomeric in solution, the corresponding Arg (Arg 183) maintains the optimal orientation of the guanidinium group for attracting the superoxide anion toward the copper site. However, as noted above, the length and amino acid composition in the active site channel are different with respect to the active SODs, thus producing an extensive negative surface.

All of these differences in charged residues around the active site could be responsible, at least partially, for the decrease in the positive electrostatic potential that attracts and drives the superoxide anion toward the copper ion,[35,42,49] thus determining a reduction in the catalytic rates.

Finally, relevant for rationalizing the enzymatic efficiency of BsSOD mutants is the fact that this protein in solution, i.e., in the conditions where the catalytic rates have been measured, is extensively in a molten globule state. In particular, the loops defining the active channel experience extensive conformational disorder, which might have a dramatic effect on the catalytic rates. Taking into account its diffusion coefficient (100 $Å^2$ ns^{-1}),[50] superoxide can cover the depth of the SOD active channel (10 Å) in a time of the order of a few nanoseconds. The residues of the active channel display conformational variability with correlation times slower than 10 ns, that is, much slower than the time needed for O_2^- diffusion. This might significantly affect its diffusion, as the exchange process would produce different conformations in the channel itself. Some of these conformations would be not optimal for the enzymatic reaction, thus reducing the overall catalytic rate.

Concluding Remarks

Our experiments have shown that it is possible to restore a significant SOD activity in BsSOD just by reintroducing the two missing His residues, thus providing the protein with the ability to bind copper. The existing differences in catalytic activity can be attributed to the different charge distribution due to the different nature of the residues around the active site of BsSOD with respect to fully active prokaryotic and eukaryotic enzymes as well as to the extensive conformational disorder at the catalytic site.

Bacillus subtilis mutants lacking the BsSOD coding gene (yojM) do not show any difference in growth and survival time with respect to native organisms, even in oxidative stress conditions, at different concentrations of metals, from excess to absence of metal ions (Dr. Jennifer Cavet, School of Biological Sciences − University of Manchester, personal communication). This experimental evidence rules out the possibility that the protein can act as a partner of some other chaperones or cofactors because this would imply that the upstream proteins involved should be nonvital and redundant as well.

The large conformational variability observed for loops in solution and the reduced extent and the loose packing of the β strands are indicative of the propensity of the BsSOD polypeptide chain to be unstructured. The introduction of the copper ligands does not decrease this tendency. It is intriguing to speculate that such polypeptide chain and this SOD-like fold has an intrinsic tendency to conformational disorder, with evolution possibly producing stable SODs. Indeed, the fact that about 60% of bacteria lack a Cu,Zn SOD, whereas 100% eukaryotes possess Cu,Zn SOD, indicates that the direction of evolution goes from lacking or having an incomplete Cu,Zn SOD to possessing a fully functional enzyme, going from bacteria to eukaryotes.

Finally, it should be kept in mind that mutations in the human protein have been shown to reintroduce some mobility and conformational disorder.[18]

Independently of whether the prokaryotic SOD-like proteins missing the metal ligands are evolutionary steps toward stable SODs, this research shows the tendency to conformational disorder of the polypeptide, which is highly homologous to stable SOD. Metal ions do not change this tendency, although they restore part of the activity. In this frame, it is instructive to keep in mind that FALS-related SOD mutants experience increased mobility in some regions of the protein.[18]

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Supporting Information Available: Information on X-ray structure determination. Table 1: Data collection statistics for the P104H and P104H-Y88H mutants of BsSOD. Table 2: Structure refinement statistics for the P104H and P104H-Y88H mutants of BsSOD. Crystallographic (CIF) data are also available. This material is available free of charge via the Internet at http://pubs.acs.org.

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(43) Banci, L.; Bertini, I.; Hallewell, R. A.; Luchinat, C.; Viezzoli, M. S. *Eur. J. Biochem.* **1989**, *184*, 125−129.
(44) Bertini, I.; Banci, L.; Luchinat, C.; Bielski, B. H. J.; Cabelli, D.; Mullenbach, G. T.; Hallewell, R. A. *J. Am. Chem. Soc.* **1989**, *111*, 714−719.
(45) Banci, L.; Bertini, I.; Cabelli, D.; Hallewell, R. A.; Luchinat, C.; Viezzoli, M. S. *Inorg. Chem.* **1990**, *29*, 2398−2403.
(46) Banci, L.; Bertini, I.; Luchinat, C.; Hallewell, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 3629−3633.
(47) Bertini, I.; Banci, L.; Luchinat, C.; Hallewell, R. A. The exploration of the active-site cavity of copper−zinc superoxide dismutase. In *Annals of the New York academy of sciences*; Blanch, H. W., Klibanov, A. M., Eds.; New York Academy of Science Book: New York, 1988; pp 37−52.
(48) Banci, L.; Bertini, I.; Viezzoli, M. S.; Argese, E.; Orsega, E.; Choi, Y. C.; Mullenbach, G. T. *J. Biol. Inorg. Chem.* **1997**, *2*, 295−301.
(49) Klapper, I.; Hagstrom, R.; Fine, R.; Sharp, K.; Honig, B. *Proteins: Struct., Funct., Genet.* **1986**, *1*, 47−59.
(50) Sharp, K.; Fine, R.; Honig, B. *Science* **1987**, *236*, 1460−1463.