Spectroscopic Characterization of Recombinant Cu,Zn Superoxide Dismutase from *Photobacterium leiognathi* Expressed in *Escherichia coli*: Evidence for a Novel Catalytic Copper Binding Site

Dolly Foti,[‡] Bruno Lo Curto,[‡] Giovanni Cuzzocrea,[‡] M. Elena Stroppolo,[§] Francesca Polizio,[§] Mariano Venanzi,[||] and Alessandro Desideri*[,§]

Department of Biological and Organic Chemistry, University of Messina, Italy, and Department of Biology and INFM, and Department of Chemical Science and Technology, University of Rome "Tor Vergata," Rome, Italy

Received December 9, 1996; Revised Manuscript Received March 31, 1997[⊗]

ABSTRACT: Cu,Zn superoxide dismutase from *Photobacterium leiognathi* has been cloned and expressed in *Escherichia coli*. The circular dichroism spectrum in the UV region of the recombinant protein indicates an higher content of random coil structure with respect to the eukaryotic enzymes. Investigation of the active site by optical, CD, and EPR spectroscopy indicates a different coordination geometry around the catalytic copper site with respect to the eukaryotic enzymes. In particular a different orientation of the metal bridging histidine is suggested. The pH dependence of the copper EPR spectrum shows the presence of a single equilibrium which is at least one unit lower than the p*K* value observed for the bovine enzyme. Despite such structural differences the catalytic rate of this enzyme is identical to that observed for the eukaryotic Cu,Zn superoxide dismutase, suggesting that the overall electric field distribution is similar to that observed in the eukaryotic enzymes.

Three major types of superoxide dismutases (SOD)[1] have been described. Bacteria have been generally found to contain a manganese type of SOD (Mn SOD), an iron type (Fe SOD), or both in the cytoplasm (Hassan, 1989), whereas copper−zinc has been considered to be characteristic of the cytosol of eukaryotic cells (Bannister et al., 1987). However, in recent years a few species of bacteria have been found to contain a Cu,Zn SOD which has been shown to be periplasmic (Puget & Michelson, 1974; Steinman, 1982, 1985; Benov & Fridovich, 1994; Battistoni & Rotilio, 1995). Analysis of the primary structure of Cu,Zn SOD from bacteria has been reported, and it has been suggested that this protein represents an evolutionary divergence from the eukaryotic Cu,Zn enzymes (Tainer et al., 1982; Bordo et al., 1994). Both the eukaryotic and the prokaryotic enzymes are dimers of about M_w 32 000, although a fully active monomeric Cu,Zn SOD has been recently purified from *Escherichia coli* (Battistoni & Rotilio, 1995; Battistoni et al., 1996a). Differences have been observed comparing the primary structure of the eukaryotic and the prokaryotic enzymes at the level of the N-terminal regions, at the residues involved in the subunit interactions, and at the residues that form the loop shaping the substrate electrostatic channel (Bordo et al., 1994).

The structure−function relationship of the eukaryotic cytoplasmic Cu,Zn SODs is now quite well understood from solution studies of native and site-directed mutated enzymes (Getzoff et al., 1992; Fisher et al., 1994; Polticelli et al., 1995a, 1996) and from the available three-dimensional structures (Tainer et al., 1982; Djinovic et al., 1992, 1994). The peculiarity of this enzyme is to catalyze a reaction limited by the diffusion of the substrate toward the active site (Bannister et al., 1987), which is controlled by the conserved distribution of the electric field generated by the enzyme (Desideri et al., 1992). The function of Cu,Zn SOD in bacteria is poorly understood. Since they are located in the periplasmic space it is reasonable to presume that Cu,Zn SOD may serve to protect against superoxide that is outside the cytoplasm or external to the bacterial cell. In fact it has been demonstrated that the absence of Cu,Zn SOD in *Caulobacter crescentus* affects neither cell viability nor the integrity of the cell envelope but protects a periplasmic or membrane-associated function requiring magnesium or calcium (Steinman, 1993). Recently the three-dimensional structure of Cu,Zn SOD from *Photobacterium leiognathi* has been reported (Bourne et al.,1996), and interesting differences with respect to the eukaryotic SODs, concerning the dimer interface region and the assembly of the electrostatic loop forming the active site, have been described. Crystals of the *E. coli* enzyme have also been obtained (Battistoni et al., 1996b), and determination of the three-dimensional structure is underway. Solution studies on functionally important residues of prokaryotic SODs have been few (Puget & Michelson, 1974; Martin & Fridovich, 1981; Borders & Fridovich, 1985; Benov & Fridovich, 1994). An NMR study of *Brucella abortus* Cu,Zn SOD suggested a different orientation of the conserved histidines coordinating

* Author to whom correspondence should be addressed at Department of Biology, University of Rome "Tor Vergata," Via della Ricerca Scientifica e Tecnologica, 00133 Rome, Italy. Tel: +39 6 7259 4376. FAX: +39 6 202 5450. E-mail: desideri@utovrm.it.

[‡] University of Messina.

[§] Department of Biology and INFM, University of Rome.

[||] Department of Chemical Science and Technology, University of Rome.

[⊗] Abstract published in *Advance ACS Abstracts*, May 15, 1997.

[1] Abbreviations: AMP, ampicillin; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); dNTPs, deoxynucleotide triphosphates; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; SOD, superoxide dismutase.

S0006-2960(96)03020-6 CCC: $14.00

the metals at the active site on the basis of the chemical shift observed in the bacterium with respect to the human enzyme (Chen et al., 1995).

In this work the Cu,Zn SOD from *P. leiognathi* has been cloned and expressed in *E. coli* by recombinant DNA techniques (Foti, 1995). The physicochemical properties of the enzyme have been investigated. In particular, the spectroscopic properties of the copper ion have been studied in the native enzyme, both in the presence of the competitive inhibitor azide and as a function of pH. The results provide evidence for a different coordination environment around the catalytic metal with respect to the eukaryotic enzyme, although identical activities are found for both *P. leiognathi* and eukaryotic SODs.

MATERIALS AND METHODS

Bacteria Strains. *P. leiognathi* ATCC strain 25521 was obtained from American Type Culture Collection. *E. coli* strain DH5α was a kind gift of C. Falcone (University of Rome, La Sapienza).

Clone Construction. The *P. leiognathi* was grown in a nutrient broth (Difco) in presence of 1.5% NaCl. The cells were collected by centrifugation and genomic DNA was isolated accordingly to Chen and Kuo (1993).

The following oligonucleotides

5′-GGGAATTCCATGAACAAGGCAAAAACGTTACT-3′,

5′-GGTCTAGATTATTGGATCACACCACACGCC-3′

complementary to the amino and the carboxy terminals, respectively, of the *P. leiognathi* Cu,Zn SOD sequence were synthetized. The 32-mer 5′ amplification primer contained an *Eco*RI restriction site upstream to the signal peptide sequence, while the 30-mer 3′ amplification primer contained an *Xba*I restriction site in order to clone the amplified fragment into the expression vector.

PCR was carried out in 100 μL of reaction buffer containing 0.1 μg of DNA, 100 pM concentration of each primer, 50 μM dNTPS, 1.5 mM $MgCl_2$, 2 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer). Prior to the addition of the enzyme and dNTPs, the reaction mixture was heated at 95 °C for 5 min and quickly cooled on ice. The sample was then subjected to 25 cycles of PCR consisting of denaturation at 90 °C for 3 min, annealing at 56 °C for 2 min, and extension at 72 °C for 3 min.

The amplification product was purified from agarose gel, digested with *Eco*RI and *Xba*I, and cloned into vector pUC18 previously digested with the same restriction enzymes. The obtained expression plasmids pUC (Ph.-Bc) was inserted into the *E. coli* strain DH5α. DNA sequence analysis, performed using Sequenase (Bio-Rad Laboratories), confirmed that the expressed gene corresponded to that of the Cu,Zn SOD of *P. leiognathi*.

Protein Expression and Purification. Recombinant clones were grown in standard LB medium containing AMP (100 μg/mL) for 5 h at 37 °C. The induction of Cu,Zn SOD expression was carried out overnight using 0.15 M IPTG (Sigma) and 0.25 mM $CuSO_4$.

Proteins contained in the periplasmic fraction were concentrated and extensively dialysed to remove sucrose against 50 mM Tris-HCl buffer, pH 7.4, and then injected onto an FPLC apparatus equilibrated with the same buffer and equipped with a HiLoad 16/60 Superdex 75 column (Pharmacia) and then onto Mono-Q 16/10 ion exchange column (Pharmacia) using a 20 mM Tris-HCl, 0−1 M NaCl, pH 7.4, gradient. The purity of the enzyme as judged by SDS−PAGE was >98%. The final yeld was 10 mg of protein per liter of culture.

Biochemical Characterization. Protein concentration was evaluated by the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard. Copper and zinc concentrations were determined with a Perkin-Elmer 3030 atomic absorption spectrometer equipped with a graphite furnace. Concentration of the cupric ion was evaluated by double integration of the EPR spectra using a 0.05 mM Cu-EDTA solution as standard. Molecular weight was determined by gel exclusion chromatography on a HiLoad 16/60 Superdex 75 column mounted on an FPLC system using myoglobin, bovine Cu,Zn SOD, and bovine serum albumin as standards.

Isoelectric points were determined by isoelectric focusing on 4% polyacrylamide gels containing 3% ampholines. Cu,Zn SOD bands were located by staining the gels with Coomassie Blue G250. Ampholines spanning the pH ranges 4−7, 6−8, and 7−10 were used.

The N-terminal sequencing was carried out by automated Edman degradation by using an Applied Biosystems model 473A pulsed liquid sequencer with on-line detection of phenylthiohydantoin (PTH) amino acids.

Activity Assay. The activity assay was carried out with the pyrogallol method (Marklund & Marklund, 1974), at 30 °C, in a 1 cm optical length cuvette with 1 mL of volume. The enzyme concentration in the cell was 0.5 μM. The activity was measured by the absorbance variation at 420 nm with a Perkin-Elmer λ9 spectrophotometer.

Spectroscopy. Spectrophotometric measurements were carried out with a λ9 Perkin-Elmer spectrophotometer. Circular dichroism spectra were carried out at 20 °C in a 0.1 cm cell, with a Jasco J500 spectropolarimeter equipped with a Jasco Dp-500 N processor. EPR spectra were recorded at 100 K on a Bruker ESP 300 spectrometer operating at 9 GHz with 100 kHz field modulation.

RESULTS

Expression. The cloned gene of *P. leiognathi* Cu,Zn SOD codes for a preprotein with a peptide leader sequence which directs the protein into the periplasma. In order to verify that the protein was correctly processed to give the mature protein, N-terminal sequencing of the Cu,Zn SOD of *P. leiognathi* expressed in *E. coli* has been carried out. Up to twenty residues of the recombinant protein have been sequenced, and they have been found to correspond to the sequence of the native *P. leiognathi* enzyme (Steinman, 1987).

Isoelectric focusing indicated the presence of three isoenzymes with the following p*I* values: 8.4 (relative population ∼95%), 7.9 (<4%), and 8.1 (<1%). These isomers were all of identical size, as determined by SDS electrophoresis and molecular exclusion chromatography, and displayed the same enzymatic activity, identical to that of the bovine enzyme, as assayed by the pyrogallol method. All the three isoenzymes contained two copper and two zinc atoms per dimer as determined by atomic absorption, allowing us to exclude that one of the three bands observed in the IEF gel

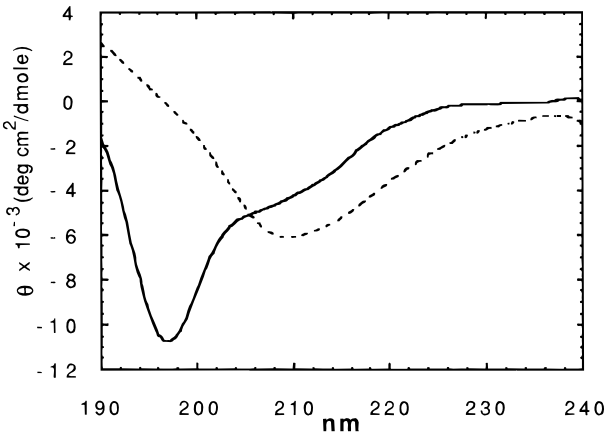

FIGURE 1: CD spectra in the 190−240 nm region of 1.65 μM *P. leiognathi* (—) and 3.10 μM bovine Cu,Zn SOD (---) in 10 mM Tris-HCl, pH 7.4 at 20 °C.

could be due to some demetalated form of the enzyme. Because of the identical activity, all of the reported characterizations have been carried out on the mixture of the three forms.

Circular Dichroism Spectroscopy in the UV Region. The CD spectrum of the *P. leiognathi* Cu,Zn SOD in the 190−250 nm region is reported in Figure 1. For comparison the CD spectrum of the bovine enzyme in the same region is also reported. The spectrum of the bovine enzyme, as well as that of the eukaryotic SOD investigated up to now (Mei et al., 1992), has a broad minimum at 210 nm, in line with the high content of β sheet present in the enzyme. The spectrum of the *P. leiognathi* enzyme still has a shoulder at around 210 nm but displays a deep minimum at 198 nm. This result indicates that, although a high content of β sheet is present in the *P. leiognathi* enzyme, the percentage of random coil structure is higher than that found in the bovine enzyme.

Optical and CD Spectroscopy. The UV absorption spectrum of *P. leiognathi* enzyme is characterized by a strong absorption centered at 280 nm (Figure 2a) because of the presence of one tryptophan residue per monomer. The calculated ϵ at 280 is 24 000 M^{-1} cm^{-1}. The electronic spectra of the bovine and *P. leiognathi* Cu,Zn SOD enzyme in the 350−750 nm region are reported in Figure 2b. The spectrum of the copper chromophore of the bacterial enzyme displays two bands with maxima at 680 and at 407 nm, respectively. The broad band centered at 680 nm has been demonstrated in the bovine enzyme to be actually composed by the partial overlap of two components which are due to the d−d transitions of the copper atom (Cupane et al., 1994). This band in the *P. leiognathi* enzyme has an extinction coefficient slightly higher (ϵ = 320 M^{-1} cm^{-1}) than that found in the bovine enzyme (ϵ = 300 M^{-1} cm^{-1}). Also the band at 407, which occurs at 421 nm in the bovine Cu,Zn SOD with an ϵ = 400 M^{-1} cm^{-1}, has a slightly higher extinction coefficient in the *P. leiognathi* enzyme (ϵ = 410 M^{-1} cm^{-1}). This band has been assigned to a ligand to metal charge transfer (LMCT) transition between the imidazole of His 61 and the copper (Pantoliano et al., 1982; Cupane et al., 1994). The blue shift and the slight variation in the extinction coefficient in the *P. leiognathi* enzyme could suggest a different orientation of the imidazole with respect to the metal which may cause a different overlap between the ligand and metal orbitals involved in the transition. This is supported by the CD spectra in the visible region of the bovine and *P. leiognathi* enzyme reported Figure 3. The d−d positive band at 610 nm in the bovine SOD (Pantoliano et al., 1982) is shifted to 605 nm in the *P. leiognathi* Cu,Zn SOD, with a molar ellipticity higher than that of the bovine enzyme. Some shifts are also observed in the 400−450 nm region (Figure 3), where the charge transfer band between the copper and the imidazolate bridge has been shown to occur. The greatest difference is observed at lower wavelength; in fact, the band at 340 nm of the bovine enzyme which has been assigned to a Cu−imidazole charge transfer band (Pantoliano et al., 1982) disappears in the *P. leiognathi* spectrum and a less pronounced shoulder appears at 320 nm (Figure 3).

EPR Spectroscopy. The copper EPR spectrum of bacterial Cu,Zn SOD is somewhat different from that of the bovine enzyme (Figure 4). The spectrum is rhombic and pH independent from pH 6.5 to 9, when a new species, which is completely formed at pH 11, starts to appear (Figure 4). Lowering the pH restores the original EPR spectrum, indicating that no denaturation is occurring since all of the modifications observed are fully reversible. On the other hand, increasing the pH to a value higher than 12 gives rise to a biuret type spectrum (Figure 4) which does not revert upon lowering the pH indicating the occurrence of protein

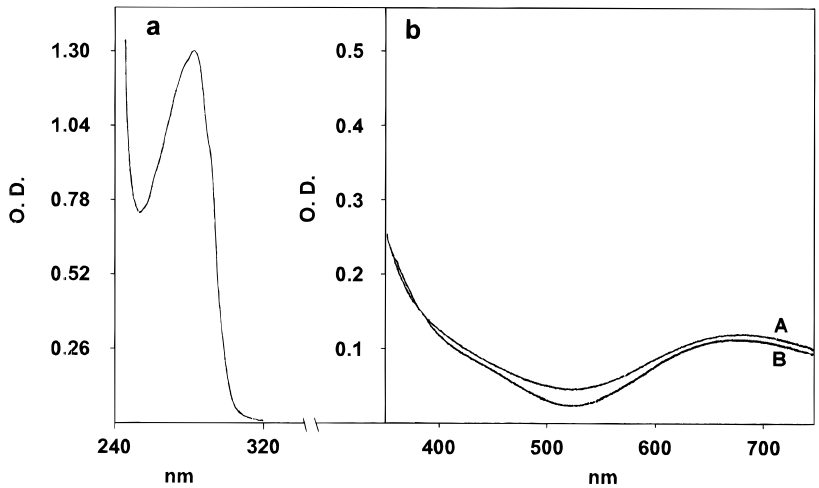

FIGURE 2: (a) UV spectrum, 240−320 nm, of 0.54 mM *P. leiognathi* Cu,Zn SOD in 20 mM Tris-HCl pH 7.4 at 20 °C. The cuvette optical pathlength was 0.1 cm. (b) Optical absorption spectra, in the 350−750 nm region, of 0.30 mM *P. leiognathi* (trace A) and 0.30 mM bovine Cu,Zn SOD (trace B) in 20 mM Tris-HCl, pH 7.4 at 20 °C. The cuvette optical pathlength was 1 cm.

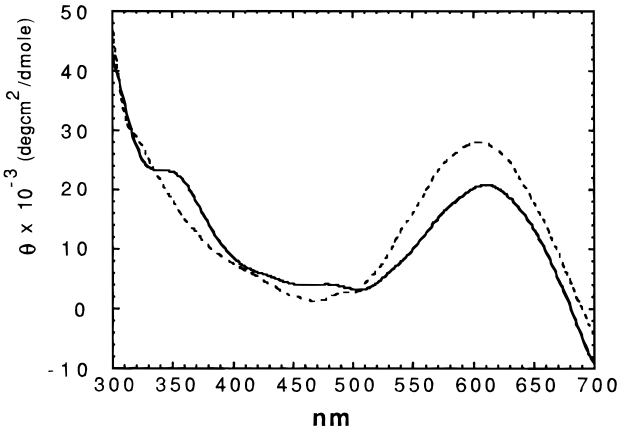

FIGURE 3: CD spectra at 20 °C in the 300−700 nm region of 0.27 mM *P. leiognathi* (---) and 0.21 mM bovine Cu,Zn SOD (—) in 10 mM Tris-HCl, pH 7.4.

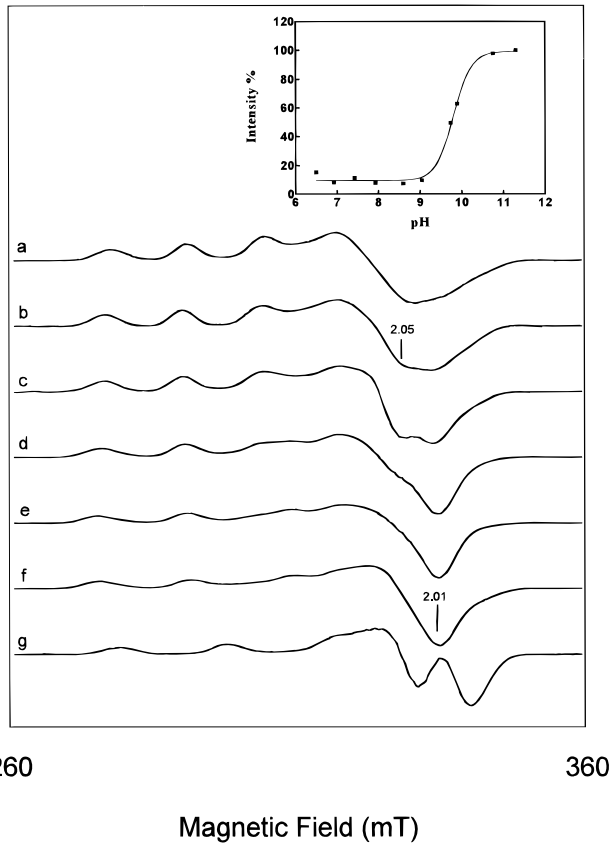

FIGURE 4: EPR spectra of (a) 0.1 mM bovine in 20 mM Tris-HCl, pH 7.4, and of 0.1 mM *P. leiognathi* Cu,Zn SOD as function of pH: pH (b) 7.40, (c) 9.05, (d) 9.90, (e) 10.70, (f) 11.30, and (g) 12.50. Spectral conditions: 20 mW, microwave power; 9.43 GHz, microwave frequency; 1.0 mT, modulation amplitude; $T = 100$ K. The inset shows the plot as function of pH of the difference in intensity between the $g = 2.01$ and the $g = 2.05$ EPR signal, normalized to the value obtained at pH 11.

denaturation. The reversible high-pH species has an axial shape similar but not identical to that displayed by the bovine enzyme (Rotilio et al., 1971). A barely defined superhyperfine structure with splitting of 14.5 G, as observed in the bovine Cu,Zn SOD (Rotilio et al., 1971), due to the interaction of the nitrogen nuclei with the unpaired electron of the copper atom, is observable in this species, in accordance with the finding that the metal coordinating ligands are identical to those of the eukaryotic enzyme (Bourne et al., 1996). For a quantitative analysis of the

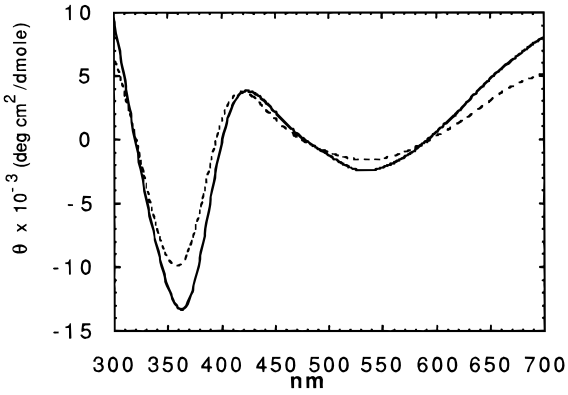

FIGURE 5: CD spectra in the 300−700 nm region of the azide-reacted *P. leiognathi*, 37.0 μM (—) and bovine, 81.0 μM (---) Cu,Zn SOD. Both spectra were recorded at 20 °C, in 10 mM Tris-HCl, pH 7.4.

transition monitored by the modifications of the spectroscopic properties of the copper at alkaline pH the difference in intensity at each pH between the $g = 2.01$ and $g = 2.05$ EPR signals (marked in Figure 4), normalized to the value observed at pH 11, is reported against pH. The curve fitting procedure (inset, Figure 4) indicates that the pH dependence of the spectra is governed by a single equilibrium with pK = 9.9. Such a pK value is more than one unit lower than the similar transition, from the neutral to the alkaline species, observed at 11.3 for the bovine enzyme (Calabrese et al., 1991; Rotilio et al., 1971). In the case of the bovine enzyme a pK at 11.3 has been also found in the pH dependence of the catalytic rate (O'Neill et al., 1988) and has been attributed on the basis of studies on chemically modified and site-directed mutated enzymes (Fisher et al., 1994; Polticelli et al., 1995b, 1996) to the cluster involving the invariant Arg 141 hydrogen bonded to a copper bound water molecule. A pK for the Arg 141 residue of approximately 0.5 unit lower than that for the corresponding arginine in the eukaryotic enzymes has been suggested for the *P. leiognathi* enzyme by chemical modification experiments with phenylglyoxal (Borders & Fridovich, 1985). The low pK value observed at the level of the copper atom (Figure 4) is in line with the low pK observed at the level of the Arg 141 (Borders & Fridovich, 1985) and suggests a different microenviroment, i.e., a different solvent exposure, of the active site in *P. leiognathi* with respect to the bovine enzyme.

Reaction with N_3^-. Addition of N_3^- to the *P. leiognathi* Cu,Zn SOD gives rise to a new absorption band at 375 nm (spectrum not shown), which has been assigned in the bovine enzyme to an azide-to-copper charge transfer band characteristic of an azide bound to an equatorial position of the copper coordination sphere (Morpurgo et al., 1973). This band in the *P. leiognathi* enzyme is characterized by a higher extinction coefficient ($\epsilon = 3100$ M^{-1} cm^{-1}) with respect to that of the bovine SOD ($\epsilon = 2550$ M^{-1} cm^{-1}), confirming a different orientation of the copper coordination sphere in the two enzymes which may produce a different overlap between the orbitals involved in the transition. The CD spectra (Figure 5) confirm the results observed in the optical absorption spectra: the band at 370 nm has a higher molar ellipticity in the *P. leiognathi* Cu,Zn SOD with respect to the bovine enzyme. Small differences are also observed at the level of the d−d bands, confirming a slightly distinct geometrical arrangement at the active site in the two enzymes. However, despite such differences the affinity constant calculated by

the increase of the absorbance at 375 nm, which represents the fraction of the azide-bound copper, as a function of anion concentration is similar, being 150 and 120 M^{-1} for the bovine and the *P. leiognathi* enzyme, respectively. The similar value could be taken as a good evidence for a conserved electric field distribution around the copper site of the two Cu,Zn SOD since the anion affinity constant value has been found to vary upon neutralization of the charged residue involved in the electrostatic guidance of the superoxide anion into the active site channel (Banci et al., 1988; Stroppolo et al., 1994). In line, the bovine and *P. leiognathi* SODs, as already said, display identical catalytic rates.

CONCLUSIONS

The results reported in this paper indicate that Cu,Zn SOD from *P. leiognathi* displays interesting structural differences when compared to the bovine enzyme. In particular,

(i) a higher content of random coil structure may be inferred from the comparison of the CD spectra; and

(ii) a different orientation of the metals bridging histidine and a different active site solvent accessibility may be inferred by the optical and CD properties of the copper in the native and azide-reacted enzyme and by the pH dependence of the copper EPR spectrum, respectively.

It is interesting to notice that, despite such differences, the catalytic rate of *P. leiognathi* Cu,Zn SOD is identical to that of the eukaryotic enzymes. The differences observed at the level of the metal site reflect a different geometrical arrangement around the copper but not a change of the number and kind of the metal ligands which are highly conserved in the evolution of this enzyme (Bordo et al., 1994). In eukaryotic SODs, site-directed mutagenesis experiments have shown that the catalytic rate may be modulated by changing the catalytic field distribution around the enzyme (Getzoff et al., 1992; Polticelli et al., 1994, 1995a) but not by perturbing the copper geometry (Bertini et al., 1989). The identical catalytic rate and the similar affinity constant for the competitive inhibitor azide observed in the bovine and *P. leiognathi* enzyme suggest that *P. leiognathi* maintains an electrostatic field distribution similar to that observed in the eukaryotic SODs (Desideri et al., 1992). The recently reported three-dimensional structure of the *P. leiognathi* Cu,Zn SOD (Bourne et al., 1996) indicates that, because of the peculiar assembly of the dimer, the loop involved in the electrostatic attraction of the substrate is different from that observed in the eukaryotic SODs, and interesting questions arise on how the strategy of the electrostatic attraction in prokaryotic Cu,Zn SOD has evolved in order to produce a catalytic rate identical to that of the eukaryotic enzymes.

REFERENCES

Banci, L., Bertini, I., Luchinat, C., & Halliwell, R. A. (1988) *J. Am. Chem. Soc. 110*, 3629−3633.

Bannister, J. V., Bannister, W. H., & Rotilio, G. (1987) *CRC Crit. Rev. Biochem. 22*, 111−180.

Battistoni, A., & Rotilio, G. (1995) *FEBS Lett. 374*, 199−202.

Battistoni, A., Folcarelli, S., Gabbianelli, R., Capo, C., & Rotilio, G. (1996a) *Biochem. J. 320*, 713−716.

Battistoni, A., Folcarelli, S., Rotilio, G., Capasso, C., Pesce, A., Bolognesi, M., & Desideri, A. (1996b) *Protein Sci. 5*, 2125−2127.

Benov, L. T., & Fridovich, I. (1994) *J. Biol. Chem. 269*, 25310−25314.

Bertini, I., Banci, L., Luchinat, C., Bielski, B. H. J., Cabelli, D. E., Mullenbach, G. T., & Hallewell, R. A. (1989) *J. Am. Chem. Soc. 111*, 714−719.

Borders, C. L., & Fridovich, I. (1985) *Arch. Biochem. Biophys. 241*, 472−476.

Bordo, D., Djinovic-Carugo, K., & Bolognesi, M. (1994) *J. Mol. Biol. 238*, 366−386.

Bourne, Y., Redford, S. M., Steinman, H. M., Lepock, J. R., Tainer, J. A., & Getzoff, E. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12774−12779.

Calabrese, L., Polticelli, F., Capo, C., & Musci, G. (1991) *Free Radical Res. Commun. 12−13*, 305−312.

Chen, W., & Kuo, T. (1993) *Nucleic Acids Res. 21*, 2260−2265.

Chen, Y. L., Park, S., Thornburg, W., Tabatabai, L., & Kintanar, A. (1995) *Biochemistry 34*, 12265−12275.

Cupane, A., Leone, M., Militello, V., Stroppolo, M. E., Polticelli, F., & Desideri, A. (1994) *Biochemistry 33*, 15103−15109.

Desideri, A., Falconi, M., Polticelli, F., Bolognesi, M., Djinovich, K., & Rotilio, G. (1992) *J. Mol. Biol. 223*, 337−342.

Djinovich, K., Gatti, G., Coda, A., Antolini, L., Pelosi, G., Desideri, A., Falconi, M., Marmocchi, F., Rotilio, G., & Bolognesi, M. (1992) *J. Mol. Biol. 225*, 791−809.

Djinovich, K., Battistoni, A., Carri', M. T., Polticelli, F., Desideri, A., Rotilio, G., Coda, A., & Bolognesi, M. (1994) *FEBS Lett. 349*, 93−98.

Fisher, C. L., Cabelli, D. E., Tainer, J. A., Hallewell, R. A., & Getzoff, E. D. (1994) *Proteins 19*, 24−34.

Foti, D. (1995) Ph.D. Thesis, University of Messina, Italy.

Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L., & Hallewell, R. A. (1992) *Nature 358*, 347−351.

Hassam, H. M. (1989) *Adv. Genet. 26*, 65−97.

Hataway, B. J., & Billing, D. E. (1970) *Coord. Chem. Rev. 5*, 143−207.

Lowry, O. H., Rosebrough, N. J., Farr, A. R., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.

Marklund, S., & Marklund, G. (1974) *Eur. J. Biochem. 47*, 469−474.

Martin, J. P., & Fridovich, I. (1981) *J. Biol. Chem. 256*, 6080−6089.

Mei, G., Rosato, N., Silva, N., Rush, R., Gratton, E., Savini, F., & Finazzi-Agro', A. (1992) *Biochemistry 31*, 7224−7230.

Morpugo, L., Giovagnoli, C., & Rotilio, G. (1973) *Biochem. Biophys. Acta 322*, 204−210.

O'Neill, P., Davies, S., Fielden, M., Calabrese, L., Capo, C., Marmocchi, F., Natoli, G., & Rotilio, G. (1988) *Biochem. J. 251*, 41−46.

Pantoliano, M. W., Valentine, J. S., & Nafie, L. A. (1982) *J. Am. Chem. Soc. 104*, 6310−6317.

Polticelli, F., Battistoni, A., Bottaro, G., Carri', M. T., O'Neill, P., Desideri, A., & Rotilio, G.(1994) *FEBS Lett. 352*, 76−78.

Polticelli, F., Bottaro, G., Battistoni, A., Carri', M. T., Djinovich-Carugo, K., Bolognesi, M., O'Neill, P., Rotilio, G., & Desideri, A. (1995a) *Biochemistry 34*, 6043−6049.

Polticelli, F., O'Neill, P., Costanzo, S., Lania, A., Rotilio, G., & Desideri, A. (1995b) *Arch. Biochem. Biophys. 321*, 123−126.

Polticelli, F., Battistoni, A., O'Neill, P., Rotilio, P., & Desideri, A. (1996) *Protein Sci. 5*, 248−253.

Puget, K., & Michelson, A. M. (1974) *Biochem. Biophys. Res. Commun. 58*, 830−838.

Redford, S. M., McRee, D. E., Getzoff, E. D., Steinmann, H. M., & Tainer, J. A. (1990) *J. Mol. Biol. 212*, 449−451.

Rotilio, G., Finazzi-Agro', A., Calabrese, L., Bossa, F., Guerrieri, P., & Mondovi', B. (1971) *Biochemistry 10*, 616−621.

Steinman, H. M. (1982) *J. Biol. Chem. 257*, 10283−10293.

Steinman, H. M. (1985) *J. Bacteriol. 162*, 1255−1260.

Steinman, H. M. (1987) *J. Biol. Chem. 262*, 1882−1887.

Steinman, H. M. (1993) *J. Bacteriol. 175*, 1198−1202.

Stroppolo, M. E., Polticelli, F., Costanzo, S., Lania, A., Galtieri, A., Polizio, F., Petruzzelli, R., & Desideri, A. (1994) *Comp. Biochem. Physiol. C 109*, 141−145.

Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., & Richardson, D. C. (1982) *J. Mol. Biol. 160*, 181−217.

BI963020F