Investigation of a New Cu,Zn Superoxide Dismutase Mutant: The Thr → Arg 137 Derivative†

Lucia Banci,‡ Ivano Bertini,*,‡ Diane Bauer,§ Robert A. Hallewell,∥ and Maria Silvia Viezzoli‡

Department of Chemistry, University of Florence, 50121 Florence, Italy, Chiron Corporation, Emeryville, California 94608, and Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, U.K.

Received September 16, 1992; Revised Manuscript Received January 11, 1993

ABSTRACT: The preparation and biophysical characterization of a mutant of superoxide dismutase in which the native Thr 137 has been substituted with a positive Arg residue are reported. Thr 137 forms, together with Arg 143, a bottleneck at the entrance to the active-site Cu ion. The geometry of the Cu ligands shows only minor changes after the above substitution. However, the enzymatic activity of the Arg 137 mutant is smaller than that of the wild type at physiological ionic strength and approaches that of wild type in the limit of zero ionic strength. The binding constant of the anion N_3^-, which had previously been shown to be a good probe of the O_2^- substrate, is increased about 20-fold in the mutant with respect to the value found in the wild type. These results are discussed on the bases of the whole charge of the cavity and the possible change in the conformation of the active-site channel.

Cu,Zn superoxide dismutases (SOD),[1] are dimeric enzymes of about 153 amino acids per subunit, mainly found in the peroxisomes of eukaryotes (Fridovich, 1974, 1986; Keller et al., 1991), that protect against superoxide toxicity (Fee, 1977) by dismuting the free radical to O_2 and H_2O_2. Each subunit contains one Cu and one Zn ion. The Cu ion dismutes O_2^- by a cyclical mechanism, first accepting an electron from one O_2^- to produce O_2 and then donating an electron to a second O_2^- to produce, together with two protons, H_2O_2 (Fridovich, 1974, 1986; McCord & Fridovich, 1969; Fee, 1981; Valentine & Pantoliano, 1981).

The active-site Cu ion is coordinated by four histidines (His 48, His 46, His 120, and His 63); one of these, His 63, bridges the copper and zinc sites. The four imidazolic nitrogens have a distorted square planar geometry, with the nitrogen of His 48 slightly below the plane formed by Cu^{2+} and the other three nitrogens (Tainer et al., 1982, 1983; Parge et al., 1992). Furthermore, a water molecule is close to the copper ion at a Cu–O distandce of 0.27 nm (Tainer et al., 1982); slightly different distances have been obtained from EXAFS (Blackburn et al., 1984) and water proton NMRD measurements (Bertini et al., 1985; Gaber et al., 1972). Zinc is coordinated in a distorted tetrahedral arrangement by Asp 83, His 80, His 71, and His 63.

The active-site Cu ion is partially buried at the base of a channel which contains several positively charged residues (Figure 1) thought to be involved in the electrostatic guidance of the superoxide to the active site (Getzoff et al., 1983). Two residues form a bottleneck at the entrance of the cavity, just above the copper ion. They are the positively charged Arg 143 and the neutral hydrophilic Thr 137 (Tainer et al., 1983). The observed catalytic rate for the dismutation process is 2–3 × 10^9 s^{-1} M^{-1} at physiological pH (Fee & Bull, 1986). The rapid reaction rate, one of the fastest reported for an enzyme,

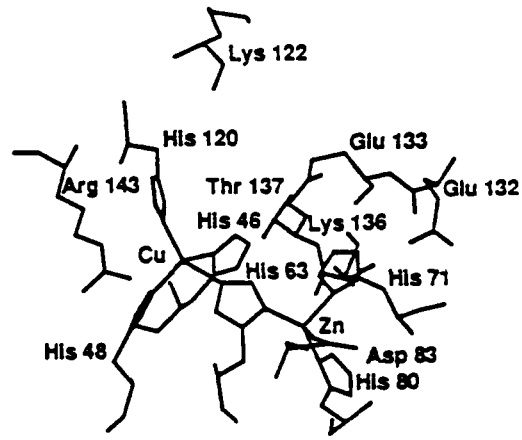

FIGURE 1: Schematic view of the active site and of relevant residues in the channel of human superoxide dismutase.

is the result of the charged groups present in the region of the active-site channel, which increase the affinity for the substrate (Fridovich, 1974, 1986; Getzoff et al., 1983), and of structural and dynamic properties of the active channel (Banci et al., 1992b). The rate-limiting step for the enzymatic reaction is the binding of substrate, which is limited by the diffusion of superoxide through the channel toward the copper ion (Fee & Bull, 1986). The charged groups in the active channel create the correct electrostatic field for guiding the superoxide to the copper site (Getzoff et al., 1992; Banci et al., 1992a). Indeed, mutation of charged residues in the active cavity dramatically changes the activity. Substitution of Arg 143 with groups of different charge, like Ile or Glu, causes a large decrease in activity (Beyer et al., 1987; Banci et al., 1988). Therefore, Arg 143 is reasonably supposed to create a local positive electrostatic field which attracts the superoxide anion toward copper and, by hydrogen bonding with it, promotes interaction with the Cu ion.

Another charged residue involved in electrostatic guidance of the substrate, but more distant from the active site, is Glu 133. Its negatively charged side chain is involved, together with Glu 132, Thr 137, and Lys 136, in a hydrogen-bonding network (Getzoff et al., 1992). The neutralization of the negative charge with the substitution of Glu with Gln, without

† This work was supported in part by NIH Grant GM39345 (R.A.H.).
‡ University of Florence.
§ Chiron Corporation.
∥ Imperial College of Science, Technology and Medicine.
[1] Abbreviations: SOD, Cu,Zn superoxide dismutase (EC 1.15.1.1); EXAFS, extended X-ray absorption fine structure; NMRD, nuclear magnetic relaxation dispersion; MD, molecular dynamics; DEFT, driven equilibrium Fourier transform; WT, wild type.

0006-2960/93/0432-4384$04.00/0

altering the structural properties of the active channel, results in a 3-fold increase in enzymatic activity (Getzoff et al., 1992; Banci et al., 1992a).

The neutral hydrophilic Thr 137 is involved in an extensive H-bond network in the active channel. The investigation of some mutants at this position, where the neutral Thr 137 has been changed to other neutral residues like Ser 137, Ala 137, and Ile 137, has revealed that the nature of the residue in 137 position determines the presence of water in the cavity (Bertini et al., 1989; Banci et al., 1990c). However, this property is not related to the enzymatic activity and may affect only the geometry of the chromophore. Thus, when the water molecule close to the copper ion is removed, as in the Ile 137 mutant, the spectroscopic data are interpreted as being due to an almost planar CuN_4 chromophore, with all of the histidines at about the same distance from copper (Banci et al., 1990a,b).

We have now substituted the neutral Thr 137 with a positively charged group, Arg. If the activity of SOD were essentially determined by electrostatic interactions, we might expect, in the case of this mutant, an increase in the enzymatic rates. Otherwise, if a different behavior is detected, we could obtain further information on the complex role of the various residues in the catalytic activity and on the factors determining the enzymatic behavior.

EXPERIMENTAL SECTION

Isolation and Purification of the Enzyme. The Arg 137 mutant of human SOD was contructed, expressed, and purified from yeast cells, as previously described (Hallewell et al., 1987, 1989). The mutant contains, in addition, a double modification at residues 6 and 111 (cysteines in the wild-type enzyme). These two Cys residues were substituted with Ala and Ser, respectively, and result in a protein with higher thermostability (Lepock et al., 1990; Hallewell et al., 1991) and active-site and catalytic activities identical to those of WT SOD (Parge et al., 1992). The mutant gene was sequenced in its entirety to confirm the correct sequence.

Protein concentrations were determined by the absorption at 265 nm, calculated with $\epsilon = 15\,900\ M^{-1}\ cm^{-1}$ (Fee & Gaber, 1972). Copper and zinc contents were determined by atomic absorption analysis using a GBC 903 instrument.

Metal Substitution. The apoproteins were obtained by extensive dialysis against 10 mM EDTA, in 50 mM acetate buffer, at pH 3.8 (McCord & Fridovich, 1969). The chelating agent was removed by dialysis against 100 mM NaCl in the same buffer and then against acetate buffer alone. The Cu_2-Co_2 derivatives were obtained by adding first Co^{2+} and then Cu^{2+}; the metal uptake was followed spectrophotometrically (Fee, 1973; Banci et al., 1987a).

Spectroscopic Measurements. All spectroscopic measurements, with the exception of ^1H NMR spectra, were carried out in pure water at pH 5.5. Electronic spectra were performed on a Cary 17D spectrophotometer; molar absorption was calculated on the basis of copper content. EPR spectra at room temperature were recorded on a Bruker ER200 at 9.8 GHz. ^1H NMR spectra were recorded at 200 MHz on a Bruker MSL200 instrument with the modified DEFT pulse sequence in order to suppress H_2O and bulk protein signals (Becker et al., 1969; Hochmann & Kellerhalls, 1980). Water proton NMRD measurements were performed on a Koenig-Brown relaxometer.

Activity Measurements. To ensure the accuracy of the results, the enzymatic activity of wild-type SOD and of the Thr 137 → Arg mutant was measured by two indirect assays set up, respectively, by Fridovich (McCord & Fridovich, 1969) and by Paoletti et al. (Paoletti et al., 1986, 1990; Paoletti & Mocali, 1990). Both assays utilize a system generating superoxide anions and a superoxide-dependent indicator reaction. The essence of the assays is the competition between SOD and the indicator reaction for O_2^-. SOD inhibits the rate of the indicated reaction, and 1 enzymatic unit is defined as the amount of enzyme that causes 50% inhibition. In the Fridovich assay, superoxide is produced by a xanthine oxidase/xanthine system. Ferricytochrome *c* is added and the superoxide anions reduces it. This reaction is monitored by the increasing absorption at 550 nm, typical of the reduced form of cytochrome *c*. Superoxide dismutase competes with ferricytochrome *c* for the substrate. The amount of SOD is varied until 50% inhibition is reached. In the method of Paoletti et al., superoxide is generated by the mixture EDTA–$MnCl_2$–O_2–mercaptoethanol as reported (Paoletti et al., 1990). The superoxide anion then reduces the species NAD(P)H, a reaction that is monitored following the disappearance of the absorption at 340 nm which is typical of NAD(P)H. The amount of SOD is varied until 50% inhibition is reached. Active protein concentrations for the assays were determined using Cu contents from atomic absorption measurements, and not from protein concentration, because Cu is the essential cofactor in the reaction. The standard assay is performed in 60 mM triethanolamine–diethanolamine buffer, pH 7.4, corresponding to physiological ionic strength (about 0.15 M). Ionic strength (I) was calculated by the equation, $I = {}^1/_2\sum_i m_i z_i^2$, where m_i is the molar concentration of each ionic species in solution and z_i is the charge of each ion.

RESULTS

Biophysical Measurements on Cu,Zn Enzymes. The electronic and CD spectra of the Cu,Zn Arg 137 mutant show minor variations with respect to wild-type SOD. In the visible region of the CD spectrum (data not shown), we observe two bands (13.4×10^3 and $16.7 \times 10^3\ cm^{-1}$) that fall in the same range of the corresponding d–d transitions assigned to the wild-type protein (Banci et al., 1990b). The electronic spectrum of the mutant shows a broad band characteristic of the electronic copper transitions in SOD with the maximum centered around $15.6 \times 10^3\ cm^{-1}$, slightly blue-shifted with respect to the wild-type SOD ($14.7 \times 10^3\ cm^{-1}$) (Figure 2).

The EPR spectrum (X-band, 9.8 GHz), recorded at room temperature, is reported in Figure 3 and is characterized by $A_\parallel = 141 \times 10^{-4}\ cm^{-1}$, $g_1 = 2.26$, $g_2 = 2.06$, and $g_3 = 2.02$. These values compare with $A_\parallel = 142 \times 10^{-4}\ cm^{-1}$, $g_1 = 2.26$, $g_2 = 2.07$, and $g_3 = 2.00$ (Brigg & Fee, 1978). In the Arg 137 mutant, g_2 and g_3 are slightly closer than in the wild type.

The water ^1H NMRD profiles, recorded between 0.01 and 50 MHz (data not shown), of the Arg 137 mutant and of the wild type are quite similar and indicate that the correlation time, τ_c, which is dominated by the electron correlation time of the copper ion, does not vary much in the mutant. The height of the plateau between 4 and 10 MHz, proportional to the amount of water interacting with the paramagnetic center (Bertini & Luchinat, 1986), shows that no changes occur in the degree of hydration and in the hydrophilicity of the cavity.

Biophysical Measurements on Cu,Co Enzymes. The ^1H NMR spectrum of copper–cobalt-substituted Arg 137 has been recorded at 200 MHz and is reported in Figure 4. The spectrum, which shows signals from the protons of the metal ion ligands, has been assigned through comparison with the spectrum of the same derivative of the wild-type enzyme and by the analysis of the dependence of the chemical shifts of the signals upon addition of increasing amounts of N_3^-. The

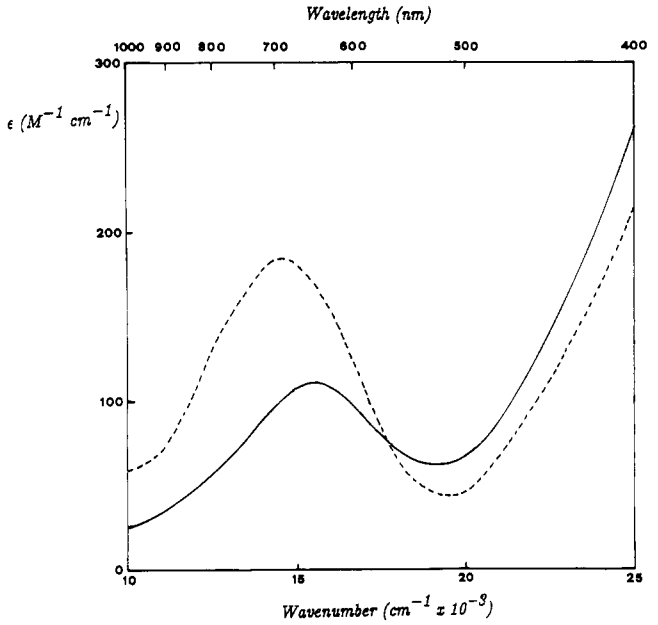

FIGURE 2: Electronic spectra of wild-type SOD (– –) and Arg 137 mutant (—). The samples are in water solution, pH 5.5.

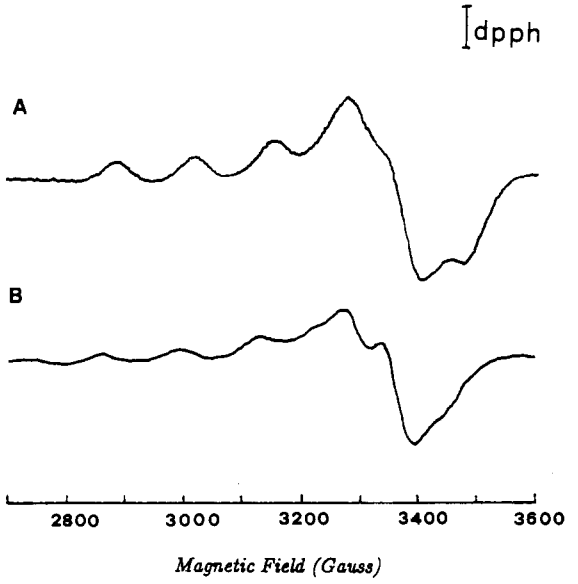

FIGURE 3: Room-temperature 9.8-GHz EPR spectra of (A) wild-type SOD and (B) Arg 137 mutant.

chemical shifts of the ^{1}H NMR signals are reported in Table I and compared with the shifts of the wild type and other mutants on position 137. In the case of Arg 137, a larger downfield shift with respect to the wild type is experienced by the signals belonging to His 48 (human labeling), and other smaller variations are observed for the signals belonging to the other residues. The spectrum, quite similar to that of the Ala 137 mutant (Banci et al., 1990a), indicates slight movements of the residues coordinated to the metal ions, especially Cu^{2+}.

Affinity toward N_3^-. The anion binding on the Cu,Zn enzyme of the WT form has been studied extensively through electronic spectroscopy (Pantoliano et al., 1982), EPR (Rotilio et al., 1971), and ENDOR (Huttermann et al., 1988), and the cobalt-substituted derivative has been studied through NMR (Banci et al., 1987b, 1990a). In the present study, the interaction of Arg 137 SOD with the anion N_3^- has been investigated by following the titration of the Cu_2Co_2 derivative through ^{1}H NMR spectroscopy. Azide binds copper in SOD

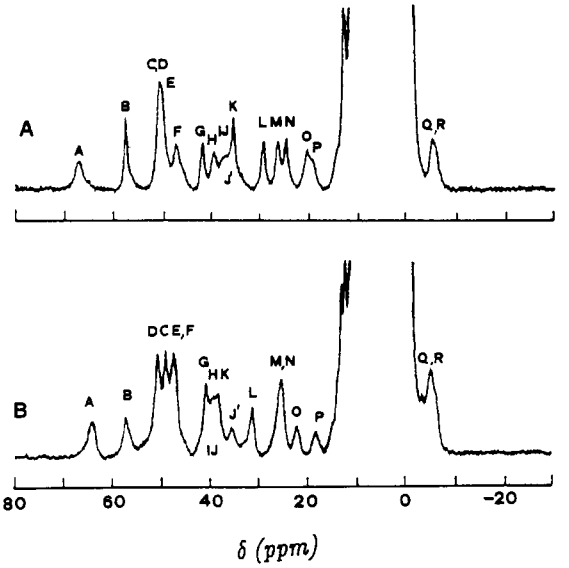

FIGURE 4: ^{1}H NMR spectra (200 MHz) of (A) Cu_2Co_2WT SOD and (B) Cu_2Co_2 Arg 137 SOD. The spectra are recorded at 300 K. The samples are in 20 mM Hepes, pH 7.5.

Table I: Chemical Shifts (ppm) of the ^{1}H NMR Signals of the Wild Type of the 137 Mutant Cu_2Co_2 SOD Derivatives at 200 MHz and 300 K.

signal	WT	Ser 137	Ala 137	Arg 137	Ile 137	assignment
A	66.3	67.8	65.9	64.1	64.7	His 63 Hδ2
B	56.9	56.8	56.9	57.2	57.6	His 120 Hδ1
C	50.0	50.9	48.6	48.6	48.8	His 46 Hε2
D	49.6	49.8	50.2	50.6	50.2	His 71 Hδ2
E	49.2	49.8	48.6	47.3	47.7	His 80 (71) Hε2
G	41.4	40.8	40.5	40.6	47.7	His 46 Hδ2
H	39.1	37.9	38.7	38.3	43.8	His 120 Hε1
I	36.9	36.0	36.3	39.1	36.5	Asp 83 Hβ1 (β2)
J′	36.0	36.0	36.3	39.1	36.5	Asp 83 Hβ2 (β1)
J	35.6	34.5	35.5	35.2	36.5	His 71 (80) Hε2
K	35.0	34.5	36.3	38.3	38.5	His 48 Hδ1
L	28.8	28.3	29.9	31.2	37.9	His 48 Hδ2
M	25.8	26.7	25.5	25.1	20.2	His 46 Hε1
N	24.1	23.5	24.5	25.1	23.8	His 120 Hδ2
O	19.8	20.3	21.4	22.1	22.4	His 48 Hε1
P	18.9	19.4	18.5	18.2	16.7	His 46 Hβ1
Q	–5.7	–4.8	–5.3	–5.3	–5.3	His 46 Hβ2 (–)
R	–5.7	–4.8	–5.3	–6.1	–7.3	(–) His 46 Hβ2

and is in fast exchange on the NMR time scale (Fee & Gaber, 1972; Bertini et al., 1990). Through the addition of increasing amounts of N_3^- the shifts of the signals can be followed, and a simultaneous fitting of their pattern allows us to estimate the affinity constant. We calculate a constant of $(1.9 \pm 0.1) \times 10^3\ M^{-1}$, which is about 20 times higher than that measured for wild-type SOD ($94 \pm 5\ M^{-1}$) at the same pH (7.5) (Bertini et al., 1989).

Activity Measurements. The activities of the wild-type and mutant enzymes have been compared by the two assays described in the Experimental Section. Using both methods the specific activity of the mutant, at physiological ionic strength, is approximately 30% that of the wild-type protein (27% ± 3% and 31% ± 3% by the methods of Fridovich and Paoletti, respectively). The finding that the mutant has reduced activity compared to the wild type is important because the mutant has increased positive charge close to the copper ion.

The activity of Thr 137 → Arg is affected by ionic strength. Measurements have been performed at values of I ranging from 0.1 to 0.3 M by adding NaCl. The ionic strength effects are larger than in the case of WT SOD on account of a more

positively charged active cavity. At the limit of zero ionic strength, the activity rates for the two proteins extrapolate to similar values, with the activity of the Thr 137 → Arg mutant never being larger than that of WT. This finding is again in contrast with a simple electrostatic model, which would have predicted a larger activity for the mutant in the limit of low ionic strength.

DISCUSSION

The differences observed in the electronic and EPR spectra of Cu,Zn Arg 137 SOD indicate minor geometrical changes toward a less distorted chromophore with respect to wild-type SOD. The effect is more evident in the ^{1}H NMR spectrum of the Cu_2Co_2 derivative: the signals assigned to His 48 (Banci et al., 1989a) in the Arg 137 mutant experience larger downfield shifts than those in wild-type SOD. This suggests a possible straightening of the Cu–N(His 48) bond.

In the other mutants at the 137 position, such as Ala 137 and Ile 137, the more regular arrangement of the histidines in relation to copper is accompanied by a weaker interaction of copper(II) with water (Banci et al., 1989b); this effect is most pronounced for Ile 137. However, in the case of Arg 137, water ^{1}H NMRD measurements show that the copper(II)–water distance is not affected by the mutation.

An approximately 20-fold increase in the affinity for N_3^- is observed for the Thr 137 → Arg mutant, whereas the dismutation rate is the same as for the wild type or decreases. Previous studies with other mutants showed the opposite effect: increases or decreases in azide affinity parallelled changes in enzyme activity. These previous studies with N_3^-, which does not react catalytically but which mimics the substrate, were used as evidence that the effects of a particular mutant on activity were primarily electrostatic (Banci et al., 1988, 1990c, in press; Bertini et al., 1989). The substantial increase in affinity of N_3^- is consistent with the increase in the positive charges within the active cavity.

The activity of the protein is reasonably dependent on the ionic strength, as expected for a charged cavity. Therefore, it is difficult to quantify the decrease in activity upon substitution at position 137. However, the binding affinity of N_3^- and the activity diverge in the present mutant. The catalytic behavior compared with the N_3^- affinity for the Thr 137 → Arg mutant therefore suggests that the mutation may change the structure of the active-site channel or affect aspects of the catalytic mechanism.

One possibility is that stabilization and precise positioning of the superoxide next to the copper ion are important for efficient catalysis. In support of this, it was found that substitution of Arg 143 with the longer and more flexible side chain of Lys reduces activity 2-fold (Beyer et al., 1987). Thus Arg in position 137 may reduce activity by altering the position of Arg 143 next to the copper ion by mutual repulsion of their positive charges. It is also possible that Arg 137 hydrogen bonds to some incoming superoxide radicals and reduces the probability of productive interactions of superoxide with Arg 143 or the copper ion.

It has recently been shown that Thr 137 is involved in a hydrogen-bonding network with Glu 132, Glu 133, and Lys 136 (Figure 1), and it has been suggested that this network may be involved in aspects of the catalytic mechanism (Getzoff et al., 1992). The substitution of Glu 132 and/or Glu 133 with neutral Gln residues increased the positive charge of the channel and dismutation activity, while maintaining the hydrogen-bonding network. Substitution of Glu 133 with Lys further increased the positive charge of the channel, but probably disrupted the residue interaction network: the dismutation activity increased to a smaller extent than with the neutralizing Gln substitutions. In the case of the Thr 137 → Arg mutation drastic changes may occur in the structure of the channel, producing the disruption of the hydrogen-bonding network present in the channel.

MD calculations on the wild-type protein (Shen & McCammon, 1991; Banci et al., 1992) have shown that the active-site channel has high mobility. Preliminary MD calculations on the present system indicate that the electrostatic loop is dramatically affected by this mutation (L. Banci et al., submitted). Indeed, according to the calculations, while the rest of the protein stays very close to the structure of wild type, Lys136, Glu132, and Glu133 move so that they are much further from the copper ion and are pointing toward the surface of the protein, breaking all of the hydrogen-bonding network. Therefore, this entire side of the channel seems to be significantly affected by the substitution of Thr 137 with Arg.

The present data, together with data on other mutants previously reported, suggest that precise positioning of positive charge next to the copper(II) ion is important for activity. Indeed, the Arg 143 to Lys substitution reduces activity by 55% (Beyer et al., 1987). The results on the present mutant may also indicate that, in addition to electrostatic effects, conformational properties of the channel are important in determining the catalytic properties of SOD. In contrast, the structure of the copper site, where the reaction occurs, seems to be less important for the functional properties of the protein (Banci et al., 1990c).

ACKNOWLEDGMENT

We thank Mr. Zhicheng Xia for technical assistance during NMRD measurements and Mr. Stefano Rocchi for technical assistance during ^{1}H NMR experiments. The Koenig-Brown relaxometer was installed at the University of Florence thanks to an agreement between the latter and the IBM Watson Research Center, Yorktown Heights, NY.

REFERENCES

Banci, L., Bertini, I., Luchinat, C., Monnanni, R., & Scozzafava, A. (1987a) *Inorg. Chem. 26*, 153.

Banci, L., Bertini, I., Luchinat, C., & Scozzafava, A. (1987b) *J. Am. Chem. Soc. 109*, 2328.

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

Banci, L., Bertini, I., Luchinat, C., Piccioli, M., Scozzafava, A., & Turano, P. (1989a) *Inorg. Chem. 28*, 4650.

Banci, L., Bertini, I., Hallewell, R. A., Luchinat, C., & Viezzoli, M. S. (1989b) *Eur. J. Biochem. 184*, 125.

Banci, L., Bencini, A., Bertini, I., Luchinat, C., & Piccioli, M. (1990a) *Inorg. Chem. 29*, 4867.

Banci, L., Bencini, A., Bertini, I., Luchinat, C., & Viezzoli, M. S. (1990b) *Gazz. Chim. Ital. 120*, 179.

Banci, L., Bertini, I., Cabelli, D., Hallewell, R. A., Luchinat, C., & Viezzoli, M. S. (1990c) *Inorg. Chem. 29*, 2398.

Banci, L., Carloni, P., La Penna, G., & Orioli, P. L. (1992) *J. Am. Chem. Soc. 114*, 6994.

Banci, L., Cabelli, D. E., Getzoff, E. D., Lee, P., Hallewell, R. A., & Viezzoli, M. S. *J. Inorg. Biochem.* (in press).

Banci, L., Carloni, P., Orioli, P. L., submitted.

Becker, E. D., Ferretti, J. A., & Farrar, T. C. (1969) *J. Am. Chem. Soc. 91*, 7784.

Bertini, I., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA.

Bertini, I., Briganti, F., Luchinat, C., Mancini, M., & Spina, G. (1985) *J. Magn. Reson. 63*, 41.

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

Bertini, I., Banci, L., Luchinat, C., Piccioli, M. (1990) *Coord. Chem. Rev. 100*, 67.

Beyer, W. F., Jr., Fridovich, I., Mullenbach, G. T., & Hallewell, R. (1987) *J. Biol. Chem. 262*, 11182.

Blackburn, N. J., Hasnain, S. S., Binsted, N., Diakun, G. P., Garner, C. D., & Knowles, P. F. (1984) *Biochem. J. 219*, 985.

Brigg, R. G.; Fee, J. A. (1978) *Biochim. Biophys. Acta 537*, 86.

Fee, J. A. (1973) *J. Biol. Chem. 248*, 4229.

Fee, J. A. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M., & Fridovich, I., Eds.) pp 173–192, Academic, London.

Fee, J. A. (1981) in *Metals ions in biological systems* (Sigel, H., Ed.) pp 259–298, Marcel Dekker, Inc., New York.

Fee, J. A., & Gaber, B. P. (1972) *J. Biol. Chem. 247*, 60.

Fee, J. A., & Bull, C. (1986) *J. Biol. Chem. 261*, 13000.

Fridovich, I. (1974) *Adv. Enzymol. 41*, 35.

Fridovich, I. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 61–97.

Gaber, P., Brown, R., III, Koenig, S. H., & Fee, J. A. (1972) *Biochim. Biophys. Acta 271*, 1.

Getzoff, E. D., Tainer, J. A., Weiner, P. K., Kollman, P. A., Richardson, J. S., & Richardson, D. C. (1983) *Nature 306*, 287.

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

Hallewell, R. A., Mills, R., Tekamp-Olson, P., Blacher, R., Rosenberg, S., Otting, F., Masiarz, F. R., & Scandella, C. (1987) *Biotechnology 5*, 363.

Hallewell, R. A., Laria, I., Tabrizi, A., Carlin, G., Getzoff, E. D., Tainer, J. A., Cousens, L. S., & Mullenbach, G. T. (1989) *J. Biol. Chem. 264*, 5260.

Hallewell, R. A., Imlay, K. C., Lee, P., Fong, N. M., Gallegos, C., Tainer, J. A., Getzoff, E. D., Cabelli, D. E., Olson, P., Mullenbach, G. T., & Cousens, L. S. (1991) *Biochem. Biophys. Res. Commun. 181*, 474.

Hochmann, J., & Kellerhalls, H. (1980) *J. Magn. Reson. 38*, 23.

Huttermann, J., Kappl., R., Banci, L., & Bertini, I. (1988) *Biochim. Biophys. Acta 956*, 173.

Keller, G.-A., Warner, T. G., Steimer, K. S., & Hallewell, R. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7381.

Lepock, J. R., Frey, H. E., & Hallewell, R. A. (1990) *J. Biol. Chem. 265*, 21612.

McCord, J. M., & Fridovich, I. (1969) *J. Biol. Chem. 244*, 6049.

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

Parge, H. E., Hallewell, R. A., & Tainer, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6109.

Paoletti, F., & Mocali, A. (1990) *Methods Enzymol. 186*, 209.

Paoletti, F., Aldinucci, D., Mocali, A., & Caparrini, A. (1986) *Anal. Biochem. 154*, 536.

Paoletti, F., Mocali, A., & Aldinucci, D. (1990) *Chem.-Biol. Interact. 76*, 3.

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

Shen, J., & McCammon, J. P. (1991) *Chem. Phys. 158*, 191.

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

Tainer, J. A., Getzoff, E. D., Richardson, J. S., & Richardson, D. C. (1983) *Nature 306*, 284.

Valentine, J. S., & Pantoliano, M. W. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) p 291, Wiley, New York.