pH-Dependent Properties of SOD Studied through Mutants on Lys-136

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The pH dependence of (i) the water ^1H NMR longitudinal relaxation rates, T_1^{-1}, at 0.03, 0.04, and 0.06 MHz for oxidized copper–zinc superoxide dismutase (Cu_2Zn_2SOD), (ii) the ^1H NMR shifts of oxidized Cu_2Co_2SOD, and (iii) the ^1H NMR shifts of reduced Cu_2Zn_2SOD have been measured for three mutants in which Lys-136 has been substituted with Ala, Gln, and Arg. Lys-136 is sitting in the protein active channel. The pK_a values of 11.5, on average, obtained for the oxidized form and 10.8, on average, for the reduced form are virtually the same for the mutants and the wild type protein. The latter pK_a is known to be due to deprotonation of His-63, which is not bridging the metal ions in the reduced species. It appears that the Lys residue cannot be responsible for the pK_a values of the oxidized form nor is capable of affecting the two pK_a's. After the role of Lys-136 was ruled out, the pK_a of the oxidized species was tentatively assigned to the semicoordinated water molecule, since all the copper histidine ligands remain coordinated. The affinity of the mutants for N_3^- is reduced in the case of Gln, essentially unchanged for Ala, and increased for Arg. SOD activities are similar to those of the wild type, except a slight increase for the Arg mutant. Both results are consistent with a positive charge relatively far from the active site.

Introduction

The enzyme superoxide dismutase (SOD, hereafter) acts as a scavenger of the O_2^- radicals, formed as byproduct of the O_2 metabolism in living organisms.[1,2] The activity of SOD is highly affected by the electrostatic interactions between the protein and the substrate O_2^-.[3-7] The active site of the oxidized enzyme is composed by a copper(II) ion which is coordinated by four histidine residues, one of which is bridging copper with zinc. The latter is coordinated by two other His residues and an Asp.[8,9] A water molecule occupies the fifth coordination position around copper with a copper–oxygen distance of about 2.8 Å.[9] From relaxation measurements of the water protons in solution a copper–oxygen distance of 2.5 Å is calculated[10] which is in accord with an EXAFS estimation.[11] The copper ion lies at the bottom of a shallow cavity.

The catalytic process may occur through a two-step mechanism[12]

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$

$$Cu^+ + O_2^- + 2H^+ \rightarrow Cu^{2+} + H_2O_2$$

although other mechanisms cannot be ruled out.[13,14] In non-saturating O_2^- conditions the rate-limiting step for the overall enzymatic reaction is the binding of substrate.[15] Its binding rate is controlled by diffusion of O_2^- in the active cavity. This cavity is characterized by the presence of several charged groups, such as Lys, Arg, Asp, and Glu. The positively charged groups are Arg-143, Lys-122, and Lys-136, and the negatively charged groups are Glu-133 and Glu-132 (as numbered in the human enzyme). These residues produce an overall electrostatic field which attracts the negatively charged superoxide ion and directs it toward the copper ion.[3-6] Electrostatic potential calculations have shown that the positive groups increase the rate of approach of superoxide above the diffusion limit.[6,7]

In the past some of these amino acid residues have been chemically modified in order to remove their charge or to decrease the effect of the charge. The positively charged Arg-143 was treated with phenylglyoxal,[16-18] and lysines were acylated.[19,20] Those experiments singled out the important role of Arg-143 in SOD activity. Recently, through site-directed mutagenesis, the positive group of Arg-143 has been substituted with neutral and negative groups.[21,22] In all cases the activity resulted largely reduced (to 10% or less). In the case of lysines, chemical modification is nonspecific. It has been found that elimination of the Lys positive charges reduces the overall activity.[19,20] The role previously attributed to Lys-136 on this ground[19,20] will be discussed in the present work through the investigation of mutants obtained through site-directed mutagenesis. The possibility to substitute a single group through site directed mutagenesis make

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(1) Fridovich, I. *Adv. Enzymol.* **1974**, *41*, 35.
(2) Fridovich, I. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1986**, *58*, 61.
(3) Koppenol, W. H. In *Oxygen and Oxy-radicals in Chemistry and Biology*; Rodgers, M. A. J., Powers, E. L., Eds.; Academic Press: New York, 1981; p 671.
(4) Getzoff, E. D.; Tainer, J. A.; Weiner, P. K.; Kollman, P. A.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 287.
(5) Klapper, I.; Hagstrom, R.; Fine, R.; Sharp, K.; Honig, B. *Proteins: Struct., Funct., Genet.* **1986**, *1*, 47.
(6) Sharp, K.; Fine, R.; Honig, B. *Science* **1987**, *236*, 1460.
(7) Sines, J. J.; Allison, S. A.; McCammon, J. A. *Biochemistry* **1990**, *29*, 9403.
(8) Tainer, J. A.; Getzoff, E. D.; Beem, J. S.; Richardson, J. S.; Richardson, D. C. *J. Mol. Biol.* **1982**, *160*, 181.
(9) Tainer, J. A.; Getzoff, E. D.; Richardson, J. S.; Richardson, D. C. *Nature* **1983**, *306*, 284.
(10) Bertini, I.; Briganti, F.; Luchinat, C.; Mancini, M.; Spina, G. *J. Magn. Reson.* **1985**, *63*, 41.
(11) Blackburn, N.J.; Strange, R. W.; McFadden, L. M.; Hasnain, S. S. *J. Am. Chem. Soc.* **1987**, *109*, 7162.
(12) Fee, J. A.; Gaber, B. P. *J. Biol. Chem.* **1972**, *247*, 60.
(13) Osman, R.; Basch, H. *J. Am. Chem. Soc.* **1984**, *106*, 5710.
(14) Rosi, M.; Sgamellotti, A.; Tarantelli, F.; Bertini, I.; Luchinat, C. *Inorg. Chem.* **1986**, *25*, 1005.
(15) Fee, J. A.; Bull, C. *J. Biol. Chem.* **1986**, *261*, 13000.
(16) Malinowski, D. P.; Fridovich, I. *J. Biol. Chem.* **1982**, *257*, 1349.
(17) Cocco, D.; Rossi, L.; Barra, D.; Bossa, F.; Rotilio, G. *FEBS Lett.* **1982**, *150*, 303.
(18) Bermingham-McDonough, O.; Mota de Freitas, D.; Kumamoto, A.; Saunders, J. E.; Blech, D. M.; Borders, C. L., Jr.; Valentine, J. S. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 1376.
(19) Cudd, A.; Fridovich, I. *J. Biol. Chem.* **1982**, *257*, 11443.
(20) Marmocchi, F.; Mavelli, I.; Rigo, A.; Stevenato, R.; Bossa, F.; Rotilio, G. *Biochemistry* **1982**, *21*, 2853.
(21) Beyer, W. F.; Fridovich, I.; Mullenbach, G. T.; Hallewell, R. A. *J. Biol. Chem.* **1987**, *23*, 11182.
(22) Banci, L.; Bertini, I.; Luchinat, C.; Hallewell, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 3629.

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this technique much more suitable than chemical modifications in order to investigate the role of specific residues such as lysines in the catalytic mechanism.

The catalytic activity of SOD is pH independent in the pH range 5–9. At higher pH values the catalytic rates decrease with a pK_a of 10.7 ± 0.1 (3σ).[23] The reasons for this loss of activity are still matter of debate. The deprotonation of Lys-136 has been proposed to be a cause of reduction of activity.[24] This residue, highly conserved among the enzymes from various species,[19,25] lies at about 13 Å from the copper ion and close to Glu-133.[4] Both residues point toward the entrance of the catalytic cavity. In those few cases where the positive charge at the 136 position is absent in naturally occurring systems, the negative charge at the 133 position is also absent.[25]

In order to clarify the role of Lys-136 in the enzymatic behavior of SOD and to understand the origin of the pH-dependent properties of SOD, we have undertaken a spectroscopic study on mutants at the Lys-136 position. Three different mutants have been obtained: Arg-136, a residue with the same charge of Lys, and Ala-136 and Gln-136, where neutral groups substitute the positively charged lysine. These studies parallel extensive activity measurements and structural studies as a function of pH performed by others.[26]

The characterization as a function of pH is reported for all the oxidized and some of the reduced forms of the enzyme. The results on the mutants are compared with those on the wild type.

Material and Methods

Samples Preparation. Human wild type SOD and its Lys-136 mutants were expressed in *E. coli*, under the guidance of R. A. Hallewell at Chiron Corp., and isolated as previously described.[27] Protein concentrations were determined by the Coomassie method[28] and by dry weight determination, the lower of the two values being accepted. Demetalation of the protein was achieved through dialysis against 10 mM EDTA at pH 3.8 in 50 mM acetate buffer[29] followed by extensive dialysis against 0.1 M NaCl in acetate buffer and then against acetate buffer alone.[30] The Cu_2Co_2 derivatives were obtained through stoichiometric addition of Co^{2+}, and then Cu^{2+}, to the apoprotein at pH 5.5. Metal uptake was followed spectrophotometrically.[31] The reduced form of the enzyme was obtained upon addition, to degassed samples, immediately before the experiment, of solid sodium dithionite to a final concentration of 10^{-2} M.[32] Activity measurements have been performed following the method of Paoletti et al. on—typically—10^{-7}–10^{-8} M SOD solutions.[33,34] Superoxide is generated by an EDTA–$MnCl_2$–O_2–mercaptoethanol mixture. Then the superoxide anion is reduced by NAD(P)H, acting as a scavenger. This reaction is monitored following the disappearance of the absorption at 340 nm, typical of NAD(P)H. Different amounts of SOD are added to inhibit the rate of NAD(P)H oxidation. One enzymatic unit is defined as the amount of enzyme that causes 50% inhibition (error is within 20%).

Spectroscopic Measurements. SOD samples for all spectroscopic measurements were 0.5–1 mM. Electronic spectra were recorded on a Cary 17D spectrophotometer. Circular dichroism spectra on the same solutions were recorded on a Jasco J500C spectropolarimeter. ^{1}H NMR spectra on Cu_2Zn_2SOD samples were recorded on a Bruker AMX 600 spectrometer. ^{1}H NMR spectra on Cu_2Co_2SOD were recorded on a Bruker MSL 200 as previously described.[35,36] The water proton relaxivity measurements were obtained on a field-cycling relaxometer, built by Dr. R. D. Brown and Dr. S. H. Koenig (IBM, Yorktown, NY) and installed at the University of Florence thanks to an agreement between the latter and the IBM T. J. Watson Research Center of Yorktown Heights, NY. The affinity for N_3^- has been measured on the Cu_2Co_2 derivative, following the ^{1}H NMR chemical shifts of the copper ligand proton signals upon addition of increasing amount of concentrated solutions (0.04–2 M) of sodium azide. As previously observed[37] azide binds to copper and is in fast exchange on the NMR time scale. From a simultaneous best fitting of the data (chemical shift values versus azide concentration) to a single equilibrium the affinity constant of N_3^- is obtained.

Results

Oxidized Form (Cu(II)). The electronic, CD, and EPR spectra of all the three mutants, at neutral pH, are essentially equal to those of the wild type, thus indicating that mutation on this position does not induce any sizeable change in the structure of the active site.

The enhancement of the water ^{1}H T_1^{-1} in the oxidized form with respect to the reduced form is a measure of the interaction of the water molecule(s) with the paramagnetic copper(II) ion.[10,35,38] As copper(II) is paramagnetic, the dipolar coupling of the unpaired electron with a nearby water proton yields a T_1^{-1} enhancement proportional to the inverse sixth power of the copper(II)–water protons distance. This value, for all three mutants, is the same of the wild type, within the experimental error (data not shown). The enhancement has been measured for the Gln-136 SOD, as a function of pH, at three magnetic fields (0.03, 0.04, 0.06 MHz), in a region of magnetic fields where the relaxation rates are almost field independent. For each pH the average value has been estimated. The water ^{1}H T_1^{-1} enhancement shows a sharp increase at high pH. A pK_a of 11.3 ± 0.4 (3σ) can be estimated that compares quite well with the value of 11.5 [39] measured for the wild type enzyme from the same type of measurements.

The derivatives with the zinc ion substituted by cobalt(II) are well suitable for ^{1}H NMR studies of the active site.[35] The substitution does not perturb the structure of the active site nor the enzymatic activity but allows the detection of sharp, well-resolved ^{1}H NMR signals for all the protons of the residues bound to both metal ions, because of the magnetic coupling between the two metal ions.[37,40,41] The ^{1}H NMR spectra of the mutants at position 136 are very similar to those of the wild type (Figure 1), thus confirming that no perturbations are induced at the active site.

The pH dependence of the chemical shifts of the ^{1}H NMR signals is shown in Figure 1. Unfortunately neither the wild type nor the mutant proteins are stable beyond pH 12 and the full sigmoid could not be obtained. From a nonlinear best fitting procedure,[42] the pH dependence of the shifts provides estimates of the pK_a values of 11.4 ± 0.5 (3σ) for the wild type, of 11.6 ± 0.6 for Ala-136 SOD, and 11.5 ± 0.2 for Arg-136 SOD. For Gln-136 SOD a slight increase in pK_a does not allow a best fit analysis. However from the observed pH dependence an estimate

(23) Banci, L.; Bertini, I.; Cabelli, D.; Hallewell, R. A.; Luchinat, C.; Viezzoli, M. S. *Inorg. Chem.* **1990**, *29*, 2398.
(24) O'Neill, P.; Davies, S.; Fielden, E. M.; Calabrese, L.; Capo, C.; Marmocchi, F.; Natoli, G.; Rotilio, G. *Biochem. J.* **1988**, *251*, 41.
(25) Getzoff, E. D.; Tainer, J. A.; Stempien, M. M.; Bell, G. I.; Hallewell, R. A. *Proteins: Struct., Funct., Genet.* **1989**, *5*, 322.
(26) Cabelli, D. E.; Getzoff, E. D.; Hallewell, R. A.; Tainer, J. A. Manuscript in preparation.
(27) Getzoff, E. D.; Cabelli, D.; Fisher, C. L.; Parge, H. E.; Viezzoli, M. S.; Banci, L.; Hallewell, R. A. *Nature* **1992**, *358*, 347.
(28) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.
(29) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049.
(30) Forman, H. J.; Evans, H. J.; Hill, R. L.; Fridovich, I. *Biochemistry* **1973**, *12*, 823.
(31) Fee, J. A. *J. Biol. Chem.* **1973**, *248*, 4229.
(32) Bertini, I.; Luchinat, C.; Monnanni, R. *J. Am. Chem. Soc.* **1985**, *107*, 2178.
(33) Paoletti, F.; Mocali, A. *Methods Enzymol.* **1990**, *186*, 209.
(34) Paoletti, F.; Aldinucci, D.; Mocali, A.; Caparrini, A. *Anal. Biochem.* **1986**, *154*, 536.
(35) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Benjamin/Cummings Publishing Co., Inc.: Menlo Park, CA, 1986.
(36) Hochmann, J.; Kellerhalls, H. P. *J. Magn. Reson.* **1980**, *38*, 23.
(37) Bertini, I.; Lanini, G.; Luchinat, C.; Messori, L.; Monnanni, R.; Scozzafava, A. *J. Am. Chem. Soc.* **1985**, *107*, 4391.
(38) Kowaleski, J.; Nordenskiöld, L.; Benetis, N.; Westlund, P.-O. *Prog. Nucl. Magn. Reson. Spectrosc.* **1985**, *17*, 141.
(39) Terenzi, M.; Rigo, A.; Franconi, C.; Mondovì, B.; Calabrese, L.; Rotilio, G. *Biochim. Biophys. Acta* **1974**, *351*, 230.
(40) Banci, L.; Bertini, I.; Luchinat, C.; Scozzafava, A. *J. Am. Chem. Soc.* **1987**, *109*, 2328.
(41) Banci, L.; Bertini, I.; Luchinat, C.; Piccioli, M.; Scozzafava, A.; Turano, P. *Inorg. Chem.* **1989**, *28*, 4650.

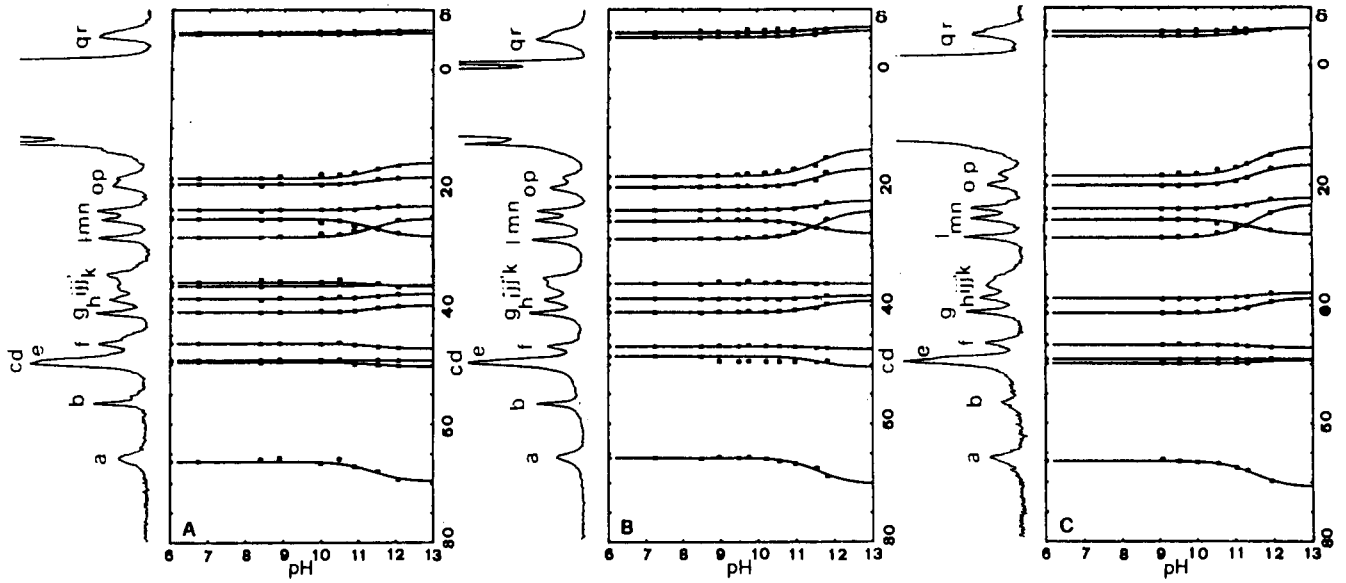

Figure 1. pH dependence of ^{1}H NMR signals of the Cu_2Co_2 derivative: (A) wild type SOD; (B) Ala-136; (C) Arg-136. The ^{1}H NMR spectra on the left of each panel are obtained at pH 6.0. The samples are in unbuffered water solution. The concentration is 1–2 mM. The proposed assignment of the signals is the following: (a) His-63 Hδ2; (b) His-120 Hδ1; (c) His-46 Hϵ1; (d) His-71 Hδ2; (e) His-80 Hδ2; (f) His-80 Hϵ2 (His-71 Hϵ2); (g) His-46 Hδ2; (h) His-120 Hϵ1; (i) Asp-83 Hβ1 (Asp-83 Hβ2); (j') Asp-83 Hβ2 (Asp-83 Hβ1); (j) His-71 Hϵ2 (His-80 Hϵ2); (k) His-48 Hδ1; (l) His-48 Hδ2; (m) His-46 Hϵ1; (n) His-120 Hδ2; (o) His-48 Hϵ1; (p) His-46 Hβ1; (q) His-46 Hβ2 (-); (r) -(His-46 Hβ2).

Table I. Affinity for N_3^- and Activity of Wild Type SOD and 136-Mutants

	N_3^- affinity (%)[a]	SOD activity (%)[b]		N_3^- affinity (%)[a]	SOD activity (%)[b]
WTSOD	100[c]	100[d]	Ala-136	126	80
Arg-136	350	160	Gln-136	54	120

[a] Estimated error is ±5%. [b] Estimated error is ±20%. [c] The N_3^- affinity constant for wild type is $K = 94 \pm 5$ M^{-1}. All the data are referred to pH 7.5. [d] The SOD activity is 19×10^3 U/mg as defined by Paoletti et al.[33,34]. All the data are referred to pH 7.4.

of the pK_a of about 12 is consistent with the data. Within the experimental error, the four pK_a's have the same value.

As it can be seen, most of the signals show only small, albeit measurable, changes in shift with pH. These results are consistent with the conclusion that the high-pH form has all the histidines bound to the metal ions, with the bridge between the two metal ions still intact.

The enzyme binds azide to the copper ion.[12,43] The affinity of azide for the Cu_2Co_2SOD derivative of the 136-mutants has been measured and compared with the same derivative of wild type SOD. The affinity data are reported in Table I together with the activity data, measured at pH 7.5 and 7.4, respectively. When the positive charge of Lys-136 is substituted by a neutral group of similar size like Gln, we observe a decrease in affinity for N_3^- by a factor of 2, while the activity is fully maintained. When Lys is substituted with the neutral but smaller Ala, the affinity for azide is slightly higher than the wild type and the activity is substantially maintained. Finally, when Lys is substituted by the positive and longer Arg, both azide affinity and SOD activity are increased, the former by as much as 3.5 times. The activity data are in good agreement with the pulse radiolysis data,[26] within the relatively large error of the present method.

Reduced Form (Cu(I)). Figure 2 shows the pH dependence of the C2 proton ^{1}H NMR signal of His-63, which in the reduced form is not bound to copper(I) ion. In the wild type enzyme this signal had been previously assigned through 2D NMR spectroscopy.[44] In contrast to the other C2 signals, the C2 signal of His-63 shows a marked pH dependence which provides a pK_a of 10.8 ± 0.1 (3σ).[45] The same measurement on the Gln-136 SOD indicates that the pK_a is not affected by the mutation (Figure 2). The pH dependence of this signal in the spectrum of $Cu^I_2Zn^{II}_2$-WTSOD has been interpreted as due to the deprotonation of His-63.[45] These data indicate that, also for the reduced form, the mutation has not affected the pH-dependent behavior of SOD. Apparently, the proton of NH of His-63 replaces one positive charge lost by copper upon reduction in such a way to provide an enzyme equally capable of attracting O_2^- inside the cavity.

Discussion

The activity profile of wild type SOD shows a pK_a of 10.7.[23] From spectroscopic measurements, two slightly different pK_a values have been obtained for the oxidized species and for the reduced species: 11.5 (present work) and 10.8,[45] respectively. In the latter case the observed pK_a could be ascribed to the deprotonation of the Zn-bound imidazole group of His-63. In the oxidized form the assignment of the group is uncertain. Indeed, His-63, in the oxidized form, has no ionizable NH since it bridges the two metal ions. The observed pK_a could be consistent with the deprotonation of the ϵ-amino group of Lys-136, as suggested previously.[20] However, the data on the Lys-136 mutants reported in this paper rule out this possibility. The deprotonation of Lys-136 does not affect the spectroscopic pK_a's, probably because it points toward the outside of the cavity. These results are strengthened by molecular dynamics calculations in solution:[46] the Lys-136 group appears to move out of the cavity, pointing toward Glu-132, i.e. far from the copper ion.

A reasonable candidate for the pH dependence of the oxidized form is the water molecule that is semicoordinated[47] to the copper

(42) Nonlinear best fitting was performed using a program written by one of us (C.L.) employing a standard analytical derivatives method (Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes. The Art of Scientific Computing*; Cambridge University Press: Cambridge, U.K., 1986) and implemented on a HP86B computer. The minimized parameters for N signals are one pK_a value and N limit shift values at alkaline pH.

(43) Rotilio, G.; Morpurgo, L.; Giovagnoli, C.; Calabrese, L.; Mondovì, B. *Biochemistry* **1972**, *11*, 2187.

(44) Bertini, I.; Capozzi, F.; Luchinat, C.; Piccioli, M.; Viezzoli, M. S. *Eur. J. Biochem.* **1991**, *197*, 691.

(45) Azab, H. A.; Banci, L.; Borsari, M.; Luchinat, C.; Sola, M.; Viezzoli, M. S. *Inorg. Chem.* **1992**, *31*, 4649.

(46) Banci, L.; Corloni, P.; Orioli, P. L. Submitted for publication.

(47) Gazo, J.; et al. *Coord. Chem. Rev.* **1976**, *21*, 93. Hathaway, B. J.; Billig, D. E. *Coord. Chem. Rev.* **1970**, *5*, 143.

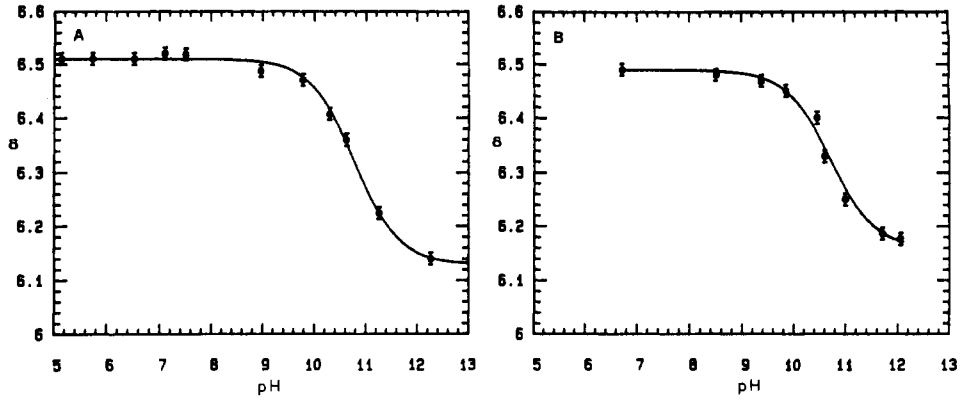

Figure 2. pH dependence of the chemical shift of the C2 proton of His-63: (A) $Cu^{I}Zn^{II}$ wild type SOD; (B) $Cu^{I}Zn^{II}$Gln-136 mutant. The samples are in unbuffered water solution.

ion.[38,48-50] At high pH the water ^{1}H T_1^{-1} [38,49,50] and ^{17}O T_2^{-1} [48] values increase with estimated pK_a's of 11.5 and 11.3, respectively. The water molecule may approach the copper ion and probably undergoes deprotonation. The decrease in metal–proton distance expected when a coordinated water becomes a coordinated hydroxide may easily outweight the loss of a proton and cause an increase in relaxation rate, due to the $1/r^6$ dependence of the relaxation enhancement.

In the pH range from 5 to 12, the dismutation rate of superoxide, as assayed by pulse radiolysis, is the same when catalyzed either by the Cu(II) or by the Cu(I) species.[51] Therefore both deprotonations, of His-63 and of the water molecule, could modulate the activity profile and be responsible of the pK_a observed at high pH. It may be difficult to detect the small differences in pK_a between the two species or it may be that the pK_a monitored through activity is somewhat different from that measured spectroscopically.

The present data lead us to rule out Lys-136 as responsible for the spectroscopically observed high pH pK_a's. In the light of this finding, we expect activity profiles with the same shape for the wild type and the 136 mutants. Recent pH-dependent activity profiles on the 136 mutants have been obtained through pulse radiolysis technique, which allows the collection of reliable activity data at pH values as high as 12.[26] It has been found that the pK_a of the activity profile is essentially maintained. Our prediction is thus confirmed that the activity-relevant pK_a is not affected at all by the nature of the residue present at position 136.

As a final comment it can be noted that azide affinity and activity are regulated in somewhat different ways by the electrostatic and steric properties of residue 136. In fact the activity does not change significantly by removal of the positive charge of lysine, thus suggesting its orientation outside the active cavity in solution, while it is increased by the Arg substitution, possibly because Arg may be oriented toward the active cavity. This picture is supported by the increased azide affinity for the Arg derivative, which suggests an electrostatic stabilization of the copper-bound anion and even the possibility of H-bonding to the anion as suggested to be the case for Arg-143.[52] The azide affinity for the neutral derivatives is decreased for the bulkier Gln and substantially similar to the wild type for the small Ala, suggesting some steric effect apparently more important for azide than for the smaller superoxide anion. It should be reminded, however, that both activity and affinity variations observed in this research are relatively small and, from a thermodinamic point of view, arise from very small free energy changes hardly accountable for on the basis of simple considerations.

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(48) Bertini, I.; Luchinat, C.; Messori, L. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 577.
(49) Banci, L.; Bertini, I.; Turano, P. *Eur. Biophys. J.* **1991**, *19*, 141.
(50) Boden, N.; Holmes, M. C.; Knowles, P. F. *Biochem. J.* **1979**, *177*, 303.
(51) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1983**, *87*, 1809. Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041.
(52) Bertini, I.; Lepori, A.; Luchinat, C.; Turano, P. *Inorg. Chem.* **1991**, *30*, 3363.