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An investigation of Cu_2Zn_2 superoxide dismutase and its Ile-137 mutant at high pH

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Abstract. The activity profile of the Cu_2Zn_2HSOD *Ile*-137 mutant has a pK_a of 9.6, i. e. one unit lower than the wild type (WT). This property has allowed us to investigate the inactive high pH form of the enzyme before denaturation occurs. The electronic and EPR spectra do not change with the above pK_a. The ^1H NMR spectrum of the Cu_2Co_2-analog reveals slight decreases in the hyperfine shifts of the protons of *His*-48 at high pH, which are consistent with a water molecule becoming closer to the copper ion, as detected through water ^1H T_1^{-1} NMR measurements. The affinity of azide at high pH is lower than at low pH, though still sizeable. The WT follows the same pattern up to pH $\simeq$ pK_a. It appears that the drop in activity is not related to any major change involving the metal coordination sphere, but is related to changes in the electrostatic potential due to the deprotonation process.

Key words: Superoxide dismutase – NMR spectroscopy – Metal substitution – Superoxide

Introduction

Human copper zinc superoxide dismutase (HSOD hereafter) catalyses the dismutation of the superoxide anion O_2^- (McCord and Fridovich 1969; Fee 1981; Fridovich 1987; Valentine and Pantoliano 1981). The activity profile versus pH, besides a small indent around pH 6–7 (Bertini et al. 1989), is pH-independent up to pH 9.5 and then shows a marked decrease at high pH values (Bertini et al. 1989; Klug et al. 1972; Argese et al. 1984). A rough estimate of the pK_a is around 10.5. Water ^1H T_1^{-1} (Terenzi et al. 1974; Boden et al. 1979) and ^{17}O T_2^{-1} (Bertini et al. 1981 b) NMR measurements at various pH values indicate an increase in the coupling between these nuclei and the unpaired electron at high pH. The rough estimate of the pK_a is around 11.3. Around this value the enzyme starts to decompose, thus preventing a deeper investigation of the system at pH values above the observed pK_a's.

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The *Ile*-137 mutant shows an activity profile versus pH with the more significant pK_a more than one unit lower than that of the WT (Bertini et al. 1989). We have therefore tried to characterize the high pH form of this derivative in order to learn also about the corresponding WT derivative. At the position 137 there is a Thr whose OH group is interacting either with a glutamic acid (Tainer et al. 1983) or with a water molecule weakly coordinated to the copper ion (Tainer et al. 1982). We show that the *Ile*-137 mutant is suitable for the spectroscopic investigations at pH values above the pK_a at about 9.7. Electronic, CD, and EPR spectra of the copper zinc derivative as well as the ^1H NMR spectra of the copper cobalt derivative have been measured as a function of pH. Solvent ^{17}O and ^1H NMR measurements have also been performed. We have then compared the results with those available in the literature or obtained during this research on the HSOD WT at the highest possible pH values.

Experimental procedures

Materials

HSOD WT and the mutant HSOD *Ile*-137 were expressed in yeast and purified to homogeneity as previously reported (Bertini et al. 1989; Hallewell et al. 1985; Hallewell et al. 1987). The Cu_2Co_2-derivatives were prepared using the previously reported methodology (Pantoliano et al. 1979; Fee 1973; Bertini et al. 1985 b). Both the samples for ^1H NMR and ^{17}O NMR measurements were dissolved in Hepes buffer 20 m*M* at pH 7.0. The pH of the solutions was then increased with NaOH. Reduced enzyme was obtained by addition of sodium dithionite and checked through the disappearance of the d–d transition in the electronic spectrum.

Methods

The electronic absorption spectra were obtained with a Cary 17D spectrophotometer. The CD spectra were

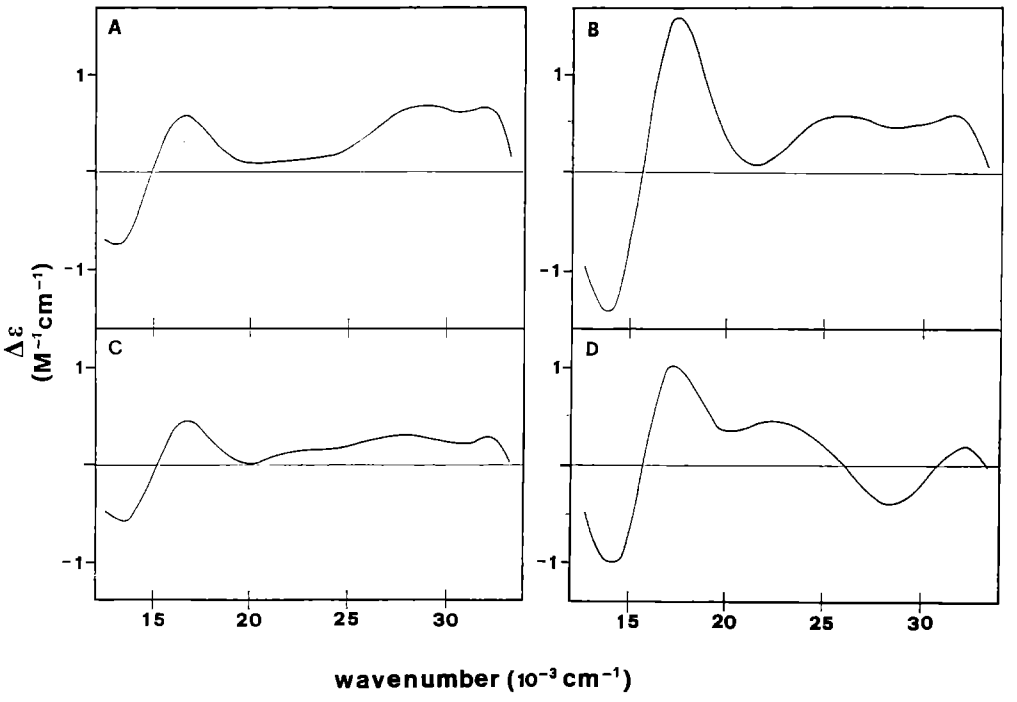

Fig. 1 A–D. CD spectra in 20 m*M* Hepes buffer of Cu_2Zn_2-HSOD. WT at pH 7.5 (**A**), *Ile*-137 at pH 7.5 (**B**), WT at pH 11.5 (**C**), and *Ile*-137 at pH 10.9 (**D**)

recorded on a Jasco J 500 C spectropolarimeter, using 1 cm path length cells. Ellipticity is expressed as $\Delta\varepsilon$ in units of $M^{-1}\,cm^{-1}$. EPR spectra at room temperature were recorded on a Bruker ER 200 operating at 9.8 GHz (X-band). ^1H NMR spectra were recorded at 200.13 MHz on a Bruker MSL 200 instrument using a super WEFT pulse sequence (Inubushi and Becker 1983) in order to suppress solvent and bulk protein signals. ^{17}O NMR measurements were performed with a Bruker CXP 90 spectrometer operating at 12.2 MHz. T_1 values were calculated from a best fitting treatment of the peak heights obtained through the inversion recovery method (Vold et al. 1968). T_2 values were obtained from experimental linewidths, appropriately reduced for the line broadening introduced by exponential weighting the free induction decay, through the relationship $T_2 = (\pi \Delta\nu)^{-1}$.

Results

The Cu_2Zn_2SOD derivatives

The electronic transitions have been measured through CD spectroscopy because of the enhanced resolution with respect to the usual absorption spectroscopy. In fact, the electronic absorption spectra of the WT derivative show a single, unresolved, absorption around 14 700 cm^{-1}, whereas the CD spectra show two well resolved transitions with opposite chirality in the same region (Pantoliano et al. 1982; Banci et al. 1988). Furthermore, the charge transfer bands are clearly apparent in the CD spectra (Pantoliano et al. 1982; Banci et al. 1988). In Fig. 1 b the CD spectra for the WT and Ile-137 mutant are reported at pH 7.5 and at the highest pH attainable before decomposition: that is pH 11.5 for WT and 10.9 for the *Ile*-137 derivative. Gaussian lineshape analysis of the spectra at neutral pH (Banci et al. 1990) provided d–d transition energies at 13 300 and 16 700 cm^{-1} for the WT and 14 300 and 17 500 cm^{-1} for the *Ile*-137 mutant. The differences between the two proteins have been accounted for by a small variation in the coordination around copper, the CuN_4 moiety being more planar in the *Ile*-137 mutant (Bertini et al. 1989; Banci et al. 1989a). In the higher energy region, transitions are observed at 22 400, 29 600 and 33 000 cm^{-1} for the WT and at 25 200, 28 500, 32 400 cm^{-1} for the mutant. As the pH is increased, the CD spectra of both WT and *Ile*-137 mutant do not show any appreciable variation in the d–d transition energies, besides a decrease in the intensity of the transitions; however in the case of the *Ile*-137 mutant the transition at 28 500 cm^{-1} changes sign and increases in intensity. Such spectral variation is of sigmoidal type with a pK_a of 9.6 $\pm$0.3 (3 σ).

The EPR spectra do not show any appreciable variation with pH for either species. The spectrum of the WT at pH values from 5.5 up to 10.9 is rhombic ($g_z = 2.28$, $g_x = 2.02$, $g_x = 2.08$, $A_{\|} = 142 \times 10^{-4}\,cm^{-1}$) (Banci et al. 1988; Briggs and Fee 1978; Rotilio et al. 1971, 1972) while *Ile*-137 mutant shows an axial spectrum ($g_{\|} = 2.27$, $g_{\perp} = 2.06$, $A_{\|} = 162 \times 10^{-4}\,cm^{-1}$) over the same pH range (Bertini et al. 1989).

The ^{17}O T_1^{-1} and T_2^{-1} of $H_2{}^{17}O$ enriched solutions of the native and reduced form of the *Ile*-137 mutant have been measured as a function of pH and temperature. The paramagnetic contribution to the nuclear relaxation rates was obtained by subtracting the corresponding values measured on the reduced protein. No sizeable enhancement outside the experimental error was observed for either T_1^{-1} or T_2^{-1} over the pH range 7 to 11. In addition, measurements on the *Ile*-137 mutant at various pH values as a function of temperature from 3° up to 30°C do not reveal any paramagnetic effect. These data have to be

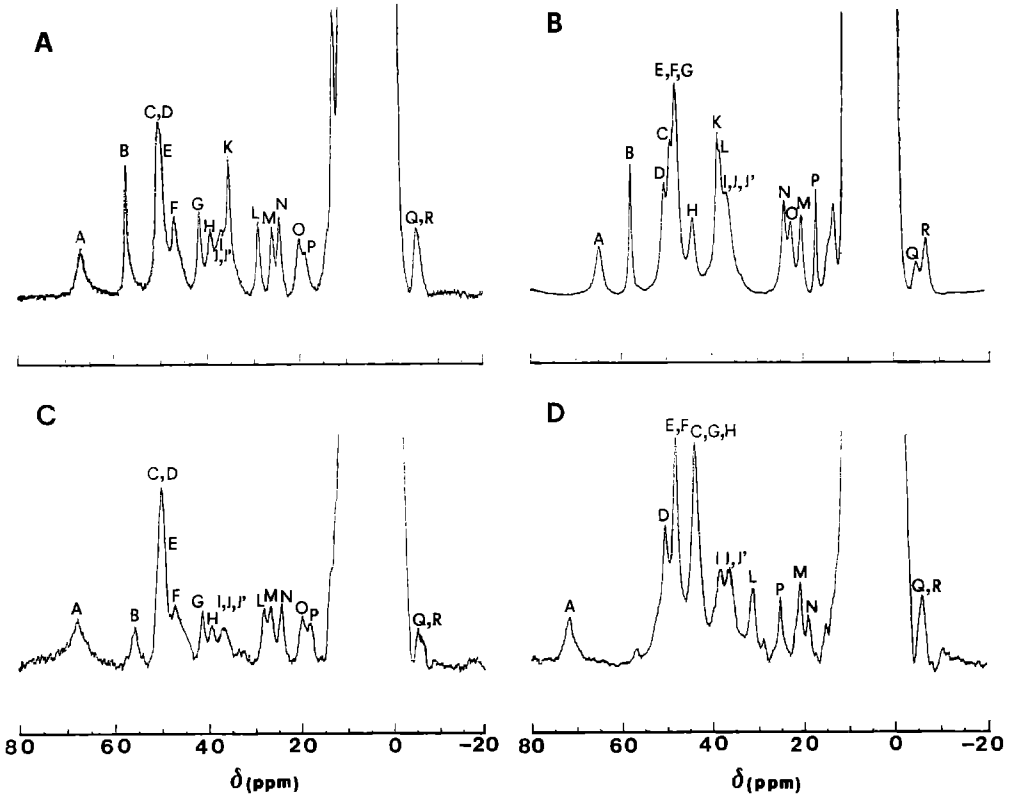

Fig. 2 A–D. 300 K 200 MHz ^{1}H NMR spectra in 20 m*M* Hepes buffer of Cu_2Co_2HSOD. WT at pH 7.5 (**A**), *Ile*-137 at pH 7.5 (**B**), WT at pH 10.9 (**C**), and *Ile*-137 at pH 10.9 (**D**)

reconciled with those obtained from water ^{1}H NMR T_1^{-1} data at various frequencies which show an increase of five times of paramagnetic effect on T_1^{-1} from pH 6 to pH 11 with a sigmoidal profile (Banci et al. 1989 a). The pK_a of this effect is about 9.6. It is possible that ^{17}O is interacting with copper(II) in very slow exchange with the solvent. This would set the upper limit for the exchange rate at about 10^{-6} s^{-1}, if the contact contribution is assumed to be the same as was estimated in the case of the native enzyme. In the case of the native enzyme an increase in both ^{1}H and ^{17}O relaxation rates is obtained with increasing pH though, if the behavior is of sigmoidal type, only the low part of the sigmoid is observed. The pK_a can be estimated to be somewhat higher than 11 (Terenzi et al. 1974; Boden et al. 1979; Bertini et al. 1981 b).

The Cu_2Co_2SOD derivatives

Zinc(II) can be substituted by cobalt(II) without perturbing the structure of the active site (Desideri et al. 1981) and without affecting the enzymatic activity (Beem et al. 1974). This substitution allows the detection of the ^{1}H NMR spectrum of the active site with sharp and well resolved signals for all the protons of the residues bound to both cobalt and copper (Bertini et al. 1985 b, 1989) because of the magnetic coupling between the two metal ions (Bertini and Luchinat 1986; Bertini et al. 1988; Banci et al. 1987). The extent of the coupling has been independently measured (Morgenstern-Badarau et al. 1986).

The assignment of the ^{1}H NMR spectra (Fig. 2 A, B) at low pH for both WT (Banci et al. 1987; Ming et al. 1988; Banci et al. 1989 b) and *Ile*-137 (Bertini et al. 1989) mutant is available and the differences were related to structural variation in the arrangement of the histidines around the copper ion. A slightly stronger binding of *His*-48 was proposed in the case of the *Ile*-137 mutant (Banci et al. 1989 a). A semi-coordinated water molecule was detected in the case of the native enzyme (Bertini et al. 1985 a) which is not present in the mutant (Banci et al. 1989 a) probably because of the presence of the large hydrophobic group. As the pH is increased, the ^{1}H NMR spectrum of the Cu_2Co_2HSOD WT derivative (Fig. 2C) shows only small variations up to pH 11, besides the signals due to the exchangeable protons. Some of them disappear as the pH is raised above 8.5 because the NH protons exchange with the bulk water is fast on the NMR time scale. As previously reported for the bovine isoenzyme (Bertini et al. 1985 b), the hyperfine shifts become pH-dependent above pH 9. The effect is, however, small; the two most affected signals (Fig. 3 A) are A (*His*-63 Hδ2) and L (*His*-48 Hδ2): between pH 9 and pH 11 their shifts change from 66.8 to 67.6 ppm and from 28.8 to 28.0 ppm respectively. Therefore, on increasing the pH, the pattern is similar to that observed upon addition of anions, although the variations in shift are small and the titration cannot be followed in full because after pH 11 oxidation of cobalt(II) starts to occur, preventing the detection of any hyperfine shifted NMR signal.

The NMR signals of the Cu_2Co_2SOD *Ile*-137 derivative significantly change their shift values with pH in a sigmoidal fashion (Fig. 3 B). At pH 10.9 the final adduct is formed (Fig. 2 D), the pK_a for its formation being 9.9 ± 0.1 (3σ). The signals of the two non-exchangeable ring protons of *His*-48 (O and L) experience a decrease of the

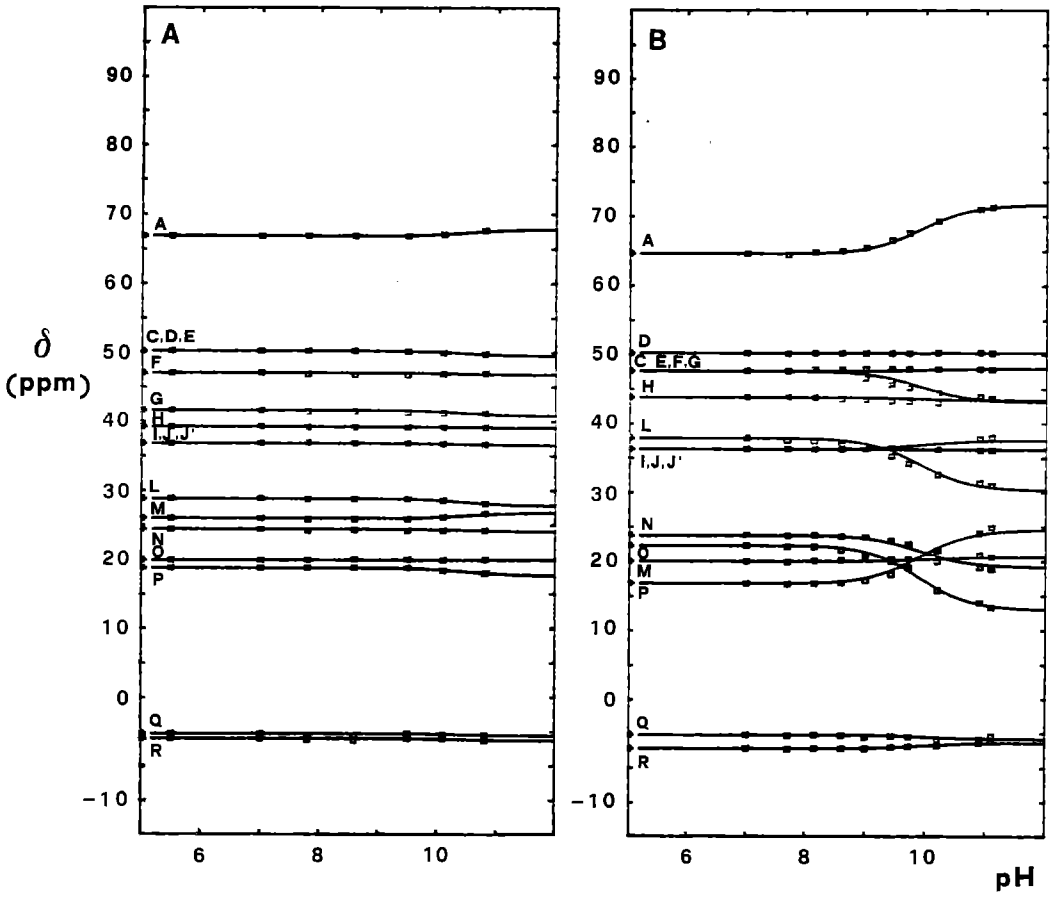

Fig. 3 A, B. Chemical shift dependence with pH of ^{1}HNMR signals. Cu_2Co_2-HSOD WT (**A**) and *Ile*-137 mutant (**B**). The conditions are the same as in Fig. 2

shift of 9 and 7 ppm respectively while the signals of *His*-120 and of the residues of the cobalt domain experience only small variations. This behavior is similar to the effect of some anions such as NCS^- (Bertini et al. 1985b) and F^- (Banci et al. 1989c). In contrast, the signals of *His*-46 experience a behavior different compared with that in the presence of anions: signal C (Hε2) goes upfield, whereas in the presence of anions it experiences a downfield shift, and signals G (Hδ2) and M (Hε1) experience only small variations compared with those in the presence of strong binding anions, although in the same direction. The signals P and R, assigned to protons of the β-CH_2 group of *His*-46 (Banci et al. 1989b), have a smaller and a larger hyperfine shift respectively compared to the WT a neutral pH. As the pH is raised, the shift of P increases while that of R decreases; the different behavior of the shift for the two protons of the same β-CH_2 group could be explained with the large mobility of this group alredy observed in the native protein from X-ray data analysis (Tainer et al. 1983; Getzoff et al. 1983) and upon anion binding to copper (Banci et al. 1990b).

The ^{1}H NMR spectrum of the high pH of Cu_2Co_2 *Ile*-137 clearly shows that all the histidines are still bound at the metal ion. Significantly, this spectrum is more similar to that of the WT derivative at neutral pH than it is that at neutral pH.

SOD binds N_3^- at the copper site (Rotilio et al. 1971; Fee and Gaber 1972; Bertini et al. 1981a). Through ^{1}H NMR spectroscopy on the copper-cobalt derivative, the adducts of both WT and *Ile*-137 were shown to be very similar, at neutral pH. In the case of the WT derivative, the affinity constant of azide was shown to be pH dependent;[1] it decreases as the pH is increased. The *Ile*-137 derivative experiences the same behavior in azide binding (see Table 1). This result shows that the high pH form of *Ile*-137 still has some affinity for azide. The ^{1}H NMR spectra of the N_3^--adducts of the two derivatives are essentially equal both at low and high pH.

Discussion

The relatively low pK_a of the *Ile*-137 mutant has allowed us to characterize the high pH species through spectroscopic techniques.

The CD measurements on the copper-zinc derivative and the ^{1}H NMR spectra on the copper-cobalt derivative reveal a pK_a around 9.7. The increase of water ^{1}H T_1^{-1} values follows the same pattern. The activity profile seems to decrease with the same pK_a (Banci et al. 1989a). It is reasonable to assume that the same acidic group or groups are responsible for the variations so far observed. In the WT the pK_a's are about 7 and 10.6. The high and low pH species have the same EPR spectra and the same CD spectra in the d–d region within the sensitivities of the techniques. This means that the coordination geometry is little affected by the dissociation of the titrable

[1] Banci et al. (submitted for publication)

Table 1. Affinity constants[a] of N_3^- at various pH values for the Cu_2Co_2SOD-derivatives

pH	WT (M^{-1})[b]	*Ile*-137 (M^{-1})[b]
5.5	138 (±5)	175 (±8)
7.5	94 (±5)	140 (±2)
10.8	47 (±3)	46 (±3)

[a] The values are obtained through a least-square best fitting to the single equilibrium $SOD + N_3^- \rightleftharpoons SOD \cdot N_3^-$.
[b] In parenthesis the 3 σ value is reported

group. Sizeable changes are observed in the charge transfer transition region of the CD spectra. Such changes are, however, difficult to interpret.

In agreement with this, the ^1H NMR spectra on the Cu_2Co_2SOD derivative indicate that no histidine is removed from coordination as the pH is increased. However, the signals of *His*-48 are shifted upfield towards the values of Cu_2Co_3HSOD WT. We have previously proposed that the position of the signals of *His*-48 is a sensitive tool to monitor small structural changes, larger hyperfine shifts indicating more regular planar CuN_4 chromophore with a better bound *His*-48 (Banci et al. 1989a). Often small hyperfine shifts are accompanied by large values of water proton T_1^{-1}. The high pH species of HSOD *Ile*-137 does indeed have a large water ^1H T_1^{-1} value (Banci et al. 1989a). The paramagnetic contribution, i.e. the experimental T_1^{-1} value subtracted from the corresponding T_1^{-1} value of the reduced protein and divided by the molar fraction of the bound water, is five times larger than that of the low pH species but 1.5 times larger than that of WT at neutral pH. If a single water molecule sensed the copper ion, this would correspond to a 7% shorter copper-water hydrogen distance with respect to the WT at neutral pH. The data can be explained with a water molecule becoming closer to copper as a group with a pK_a about 9.7 is deprotonated. This would not affect the energies of the *d* levels within the sensitivity of the electronic and EPR spectroscopies. The ^1H NMR spectra reveal some minor changes within the coordination polyhedron, consistent with a water molecule approaching the copper ion. The ^{17}O NMR spectra do not reveal such an effect probably on account of slow exchange. It appears therefore that the high pH species of the HSOD *Ile*-137 derivatives is quite similar to the low pH species of the WT.

Above the pK_a of 9.7, the further negative charge causes a substantial decrease in the affinity of N_3^-. It may be proposed that both the pK_a's of 10.6 or 11.3 in the WT enzyme correspond to the pK_a of 9.7 in the mutant. The difference could be ascribed to a more hydrophobic cavity due to the aliphatic part of the *Ile* residue. The ^1H NMR spectra indicate that *His*-48 tends to be less tightly bound as the pH is raised since signal L ($H\delta 2$) and signal O ($H\varepsilon 1$) shift upfield. Since anions such as NCS^- (Bertini et al. 1985b) and F^- (Banci et al. 1989c) have a similar effect and they do bind the metal ion, though loosely, it was suggested that OH^- could bind at high pH (Bertini et al. 1985b). The spectroscopic effects of increasing pH on the WT derivative are similar in the pH range available before denaturation. The water ^1H T_1^{-1} (Terenzi et al. 1974; Boden et al. 1979) and ^{17}O T_2^{-1} (Bertini et al. 1981b) values increase with pH. We cannot quantify these effects; however, in the light of the conclusions on the *Ile*-137 mutant, we can consider the possibility that H_2O or OH^- interact with the metal ion somewhat more strongly than at low pH. At high pH, the hydrophilicity of the cavity increases and water may bind a little more tightly, thus accounting for the increase in the relaxation parameters of water nuclei. It should be noted hat the relaxation rates of the water nuclei and the ^1H NMR shifts of the copper-cobalt derivatives are much more sensitive to slight structural changes than the electronic or EPR spectra.

The major effects of the groups responsible for the above pK_a's are the increased hydrophobicity of the cavity, the reduction of N_3^- affinity and the disappearence of the enzymatic activity. Such a group or groups should be inside or nearby the access of the cavity and reasonable candidates are lysine residues. The present behavior shows how dramatic is the effect of a charge on the activity of the enzyme. This is more striking if it is also considered that no other major variation occurs within the active cavity. Finally, it appears that the enzymatic catalysis is much more affected by the electrostatic potential than the anion affinity.

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