for the less stable carbene. In fact, the stabilization of carbenes by solvent can be significant. The heats of solvation of methylene with CH_3CN and acetone to form nitrile and carbonyl ylides, respectively, are estimated to be exothermic by 45–50 kcal/mol.[6c] In an attempt to examine potential differences in solvation energies, $\Delta H_{(1\rightarrow 2)}$ was measured for the carbenes in solvents of different polarities. However, the difference in solvation enthalpies between heptane and CH_3CN for each carbene is small based on their similar $\Delta H_{(1\rightarrow 2)}$ values.

In conclusion, we have used PAC to determine the heats of formation and reaction with CH_3OH of several singlet carbenes in solution. The stabilization of the carbenes by substitution can be related to their reaction rates and exothermicities. Further studies will examine substituent and temperature effects on the energetics and dynamics of carbene reactions.

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Registry No. 1a, 87282-19-7; **1b**, 4460-46-2; **1c**, 4222-25-7; **1d**, 4222-27-9; CH_3OH, 67-56-1; CH_3CN, 75-05-8.

An Investigation of a Human Erythrocyte SOD Modified at Position 137

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Abstract: A human copper–zinc superoxide dismutase mutant in which the active site residue Thr-137 is substituted with Ile has been characterized by activity measurements, electronic, CD, and EPR spectroscopies, and ^1H NMR spectroscopy of its cobalt-substituted derivative. The mutant displays gross changes in the metal coordination sphere. The chromophore is considerably more tetragonal than in the WT and its coordination may be consistent with no apical water. The formation of a more regular square plane results from a more regular binding of the four histidines. Despite these sizable alterations, the mutant has essentially the same enzymatic activity as the WT at physiological pH. It follows that the detailed stereochemistry about copper is not relevant for activity. Consistent with this result, we find that the affinity of anions is similar to that of the WT. The mutant, however, shows a 0.8 unit lower pK_a for the high pH decrease of enzymatic activity. The possible candidates for the group responsible for such behavior are considered.

Site directed mutagenesis is increasingly proving its power as a tool in the investigation of enzyme mechanisms.[1,2] Since its successful cloning and expression in yeast[3] and *E. coli*,[4] we can now include erythrocyte Cu/Zn human superoxide dismutase (SOD) as well. The great advantage of site directed mutagenesis is the possibility of directing specific substitutions anywhere within a protein at enzyme active sites and, accordingly, permitting mechanistic hypotheses to be tested.

SOD catalyzes the dismutation of superoxide with very high efficiency;[5] human and bovine isoenzymes typically show k_{obs} of the order of 10^9 M^{-1} s^{-1}. Recent measurements, under saturating amounts of O_2^-, yielded k_{cal} and K_M values of $\approx 1 \times 10^6$ s^{-1} and $\approx 3 \times 10^{-3}$ M, respectively, for the bovine isoenzyme.[6]

SOD is a dimeric protein of MW 32000, each monomer containing one copper and one zinc ion bridged by a histidinato residue. The copper ion is the catalytic center. The active site structure as it appears from X-ray studies[7] on the strictly similar bovine isoenzyme is shown in Figure 1. Besides the metal ligands, a particularly important residue present at the entrance of the active site cavity of all known natural isoenzymes is an arginine, which in the human isoenzyme is Arg-143.[8] This residue has been often discussed as playing a role in the catalysis.[9,10] It is possible that it attracts O_2^-,[11] as well as other anions,[12] into the active site. Modification of Arg with phenylglyoxal produces a strong decrease in the enzyme activity[13] and in anion affinity.[14] Recently, studies on derivatives substituted by mutagenesis with Ile, Lys, and Glu at position 143 have also shown a sharp decrease in both activity,[15,16] and anion affinity.[12] Yet, the metal geometry

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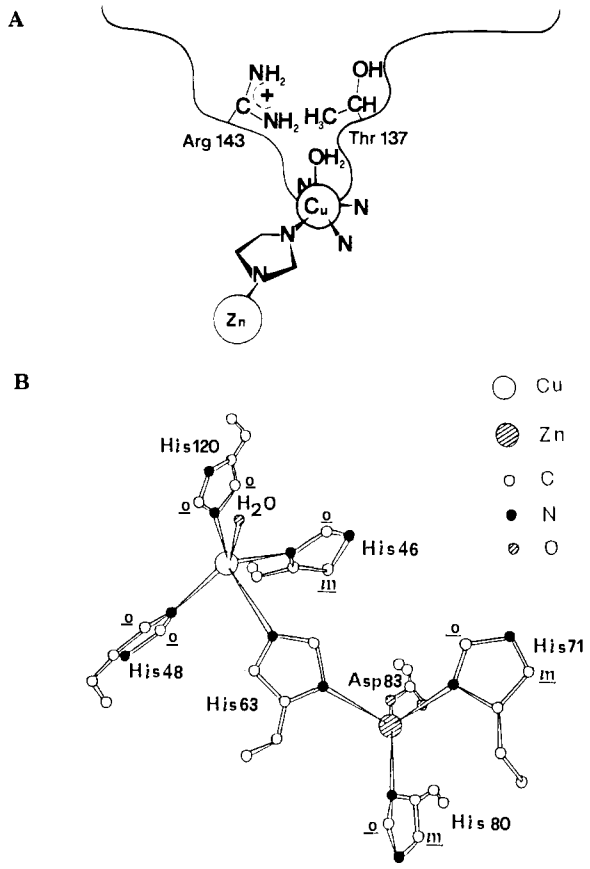

Figure 1. Schematic drawing of the superoxide dismutase active cavity (A) and of the copper–zinc site (B).

in the active site remains virtually unchanged.[12] The latter information has been obtained by exploiting the magnetic properties of derivatives substituted with cobalt in place of zinc. Magnetic coupling[17] between cobalt(II) and the native copper(II) ions has permitted the detection of ^{1}H NMR signals arising from the metal ligand protons, which are distinct from all the other proton signals of the protein.[12] The NMR parameters of the ligand proton signals in paramagnetic metal complexes are extremely sensitive to even slight alterations of the metal coordination geometry.[18]

We report here the characterization of a new derivative of human SOD in which Thr-137, also located inside the active cavity, has been substituted by Ile residue (i.e., SOD-Ile-137). This residue has not been invoked as being directly involved in any crucial mechanistic step. However, it is located in proximity of the copper ion and, together with Arg-143, forms a bottleneck at the entrance of the active site. The hydroxyl group may participate in relevant H-bonding interactions. If so, its substitution with a hydrophobic residue may significantly change the enzyme's structure and activity. We have studied this mutant by kinetic measurements and electronic, CD, and EPR spectroscopy. Finally, we have substituted the zinc ion with cobalt in order to investigate the ^{1}H NMR spectra and possibly detect subtle structural differences.

Experimental Section

Preparation of the Mutant. The mutant human SOD gene containing the Thr-137 to Ile-137 change was constructed with use of M13 site-directed mutagenesis as described previously.[15] A yeast expression plasmid containing human SOD Ile-137 was constructed, the yeast strain 2150-2-3 transformed, and colonies selected on plates lacking leucine as described previously.[3] This strain produced approximately 30% of total cell protein as mutant human SOD Ile-137 protein, which was purified to greater than 95% homogeneity.[3,15,16]

Kinetic Measurements. Pulse radiolysis experiments were carried out using the 2 MeV Van der Graaf accelerator at Brookhaven National Laboratory, New York, as described previously.[19] Dosimetry was accomplished with KNCS, assuming $G((NCS)_2^-) = 6.13$ and $\epsilon_{472\ nm} = 7950$ M^{-1} cm^{-1}. Superoxide radicals were generated in O_2-saturated aqueous solutions containing formate according to a well-established mechanism[20] and the concentration of O_2^- was determined from its absorbance at 245 nm ($\epsilon = 2350$ M^{-1} cm^{-1} at pH 8[21]).

Solutions were prepared with water that, after distillation, had been passed through a Millipore ultrapurification system. The pH was adjusted by the addition of either NaOH (GFS Chemical Co., 99.999%) or H_2SO_4 (Aristar, BDH Chemical Ltd.). All solutions contained 0.01 M formate (Sigma Chemical Co.) and 500 μM phosphate (Ultrex, JT Baker Chemical Co.), which are the buffer for the SOD solutions. The addition of 50 μM diethylenetriaminepentaacetate (DTPA, Sigma Chem. Co.) did not alter the observed rate of dismutation of O_2^-. Solutions in which ionic strength studies were carried out contained either phosphate or NaCl in 1 mM phosphate buffer (JT Baker Chemical Co.) both adjusted to pH 8.0.

Metal Substitution. Demetalation of SOD-Ile-137 was performed with EDTA at pH 3.9, according to established procedures;[22] the rate of metal loss was similar to that of the wild type enzyme. After extensive dialysis against 0.1 M NaCl in 5×10^{-2} M acetate buffer at pH 3.9 and then against acetate buffer alone, cobalt(II) first and then copper(II) were added at pH 5.5. Metal incorporation was followed spectrophotometrically.[23] Protein concentration was determined by Lowry's method.[24] Cobalt(II) uptake, in the 2:1 cobalt-to -protein ratio, was instantaneous. The electronic spectra of the E_2Co_2-SOD-Ile-137 (E = empty) are very similar to those of the wild-type isoenzyme, with a molar absorbance value of 360 M^{-1} cm^{-1} at 16 950 cm^{-1} per cobalt ion. Complete copper uptake was achieved in a few hours, as judged from the characteristic change of the cobalt absorption bands.[23]

Spectroscopic Measurements. Electronic spectra were recorded on a Cary 17D spectrophotometer; circular dichroism (CD) spectra were recorded on a JASCO J500C. EPR spectra at room temperature were recorded on a Bruker ER200 operating at 9.6 GHz (X-band) and ^{1}H NMR spectra on Bruker CXP 300 or Bruker MSL 200 instruments. Experimental conditions for spectroscopic measurements have been reported elsewhere.[23,25]

Results

Kinetic Measurements. The activity of the SOD-Ile-137 mutant as a function of pH is reported in Figure 2A, together with that of the wild type (WT) enzyme. The pH profiles are similar, but not identical. In particular, the Ile-137 mutant, besides showing a small decrease in k_{obs} in the low pH range, has a pK_a for the main ionization of 9.6 ± 0.2 versus 10.4 ± 0.1 of the WT. This effect is obviously not a direct one, since a Thr → Ile subtitution does not involve alteration of an ionizing acid–base group. However, the lack of the hydroxyl proton in the hydrogen bonding network in the active site apparently alters the pK_a of a catalytically important acid–base group. If so, the latter is likely proximal to the active site. Parts B and C of Figure 2 show the activity of WT and SOD-Ile-137 mutant enzyme with ionic strength values, using either sodium chloride or phosphate buffer. The latter has been studied in view of its controversial effects.[11,26] Both wild type and mutant enzymes show a parallel decrease in activity with increasing ionic strength, both with sodium chloride and phosphate buffer. It appears that phosphate does not inhibit more than NaCl does.[11] The mutation does not substantially alter the response of the enzyme.

Electronic and EPR Spectra. The electronic spectra of the SOD-Ile-137 show an absorption in the d–d transitions region at

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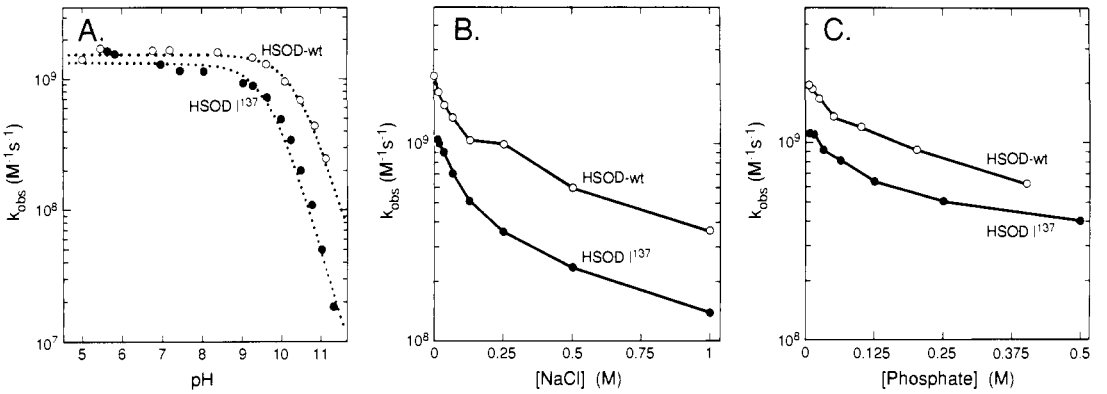

Figure 2. (A) pH dependence of specific activity of Ile-137 SOD (●) compared with that of WT (○). The dotted lines represent best fit values assuming a single pk_a. Best fit parameters are the following: pk_a = 9.6 ± 0.2 and 10.4 ± 0.1, and k_{obs} (low pH limit) = 1.4 (±0.3) × 10^9 and 1.6 (±0.2) × 10^9 M^{-1} s^{-1} for mutant and WT, respectively. Deviations are ±3σ. (B) Decrease in activity of Ile-137 SOD (●) and WT (○) as a function of NaCl concentration at pH 8.0. (C) Decrease in activity of Ile-137 SOD (●) and WT (○) as a function of phosphate ($[H_2PO_4^-]$ + $[HPO_4^{2-}]$) concentration at pH 8.0. All the solutions are in 0.01 M formate and 500 μM phosphate buffer. The discrepancy in the activity values at pH 8 between parts A and B and C is within experimental error.

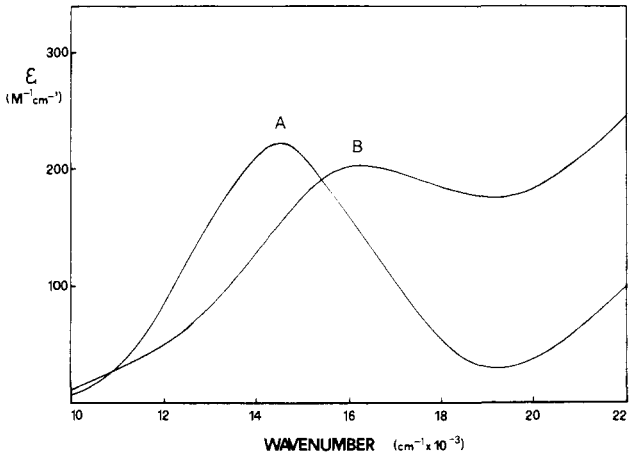

Figure 3. Electronic spectra of WT (A) and SOD-Ile-137 (B) at pH 5.5.

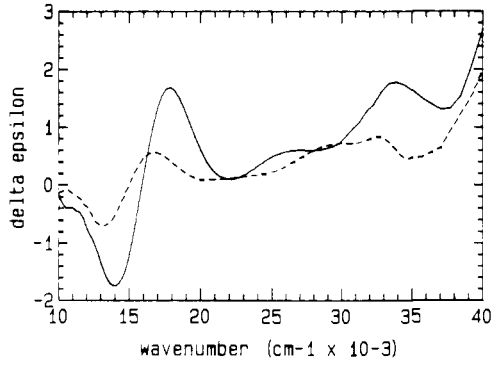

Figure 4. CD spectra of SOD-Ile-137 (solid line) and WT (dashed line) at pH 5.5 in H_2O. The protein concentration was 5.9 × 10^{-4} and 6.7 × 10^{-4} M for the SOD-Ile-137 and WT, respectively. The energies of the CD bands were evaluated through Gaussian analysis of the spectra. SOD-Ile-137: 14.3, 17.5, 25.2, 28.5, 33.7, 41.0, 42.8 × 10^3 cm^{-1}. WT: 13.4, 16.5, 25.3, 29.3, 33.0, 40.4, 42.9 × 10^3 cm^{-1}.

$\simeq$16 × 10^3 cm^{-1} (Figure 3) which appears as a shoulder of a stronger absorption and which is more than 10^3 cm^{-1} higher in energy with respect to WT-SOD. The CD spectra of SOD's are quite informative[22] and in the present case show two absorptions at 14.3 and 17.5 × 10^3 cm^{-1}, again blue-shifted with respect to those at 13.4 and 16.5 × 10^3 cm^{-1} observed for the WT (Figure 4). The near-ultraviolet absorptions may be consistent with three imidazolato to copper charge transfer bands. The hypsochromic shift of the d–d transitions may be consistent with a more square planar structure of the mutagenized derivative with respect to a five-coordinate structure (four + 1 semicoordinated). The EPR

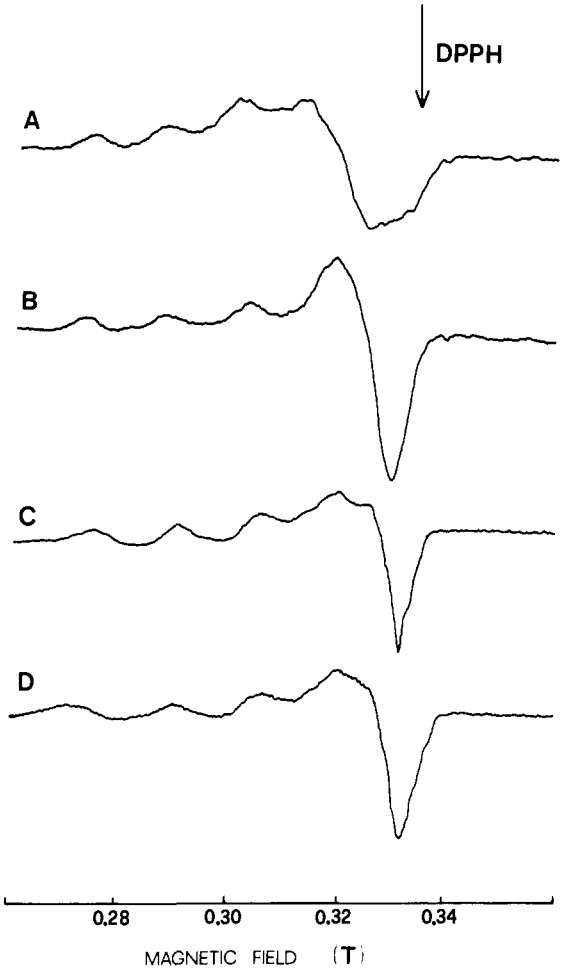

Figure 5. Room temperature 9.6-GHz EPR spectra of (A) WT, (B) SOD-Ile-137, (C) WT + 1.33 M N_3^-, and (D) SOD-Ile-137 + 1.23 M N_3^-. Conditions: pH 7.5, 50 mM Hepes buffer. The enzyme concentrations are about 1 mM for all the samples.

spectra of SOD-Ile-137 are essentially axial with $g_\parallel$ = 2.27, $g_\perp$ = 2.06, and $A_\parallel$ = 166 × 10^{-4} cm^{-1} (Figure 5B). These values are typical of a tetracoordinated chromophore.[27] The WT protein is rhombic with $g_\parallel$ = 2.28 and $A_\parallel$ = 148 × 10^{-4} cm^{-1} (Figure 5A). The change in $A_\parallel$ is a measure of the geometrical variation from a distorted square pyramid to a planar coordination polyhedron.[28]

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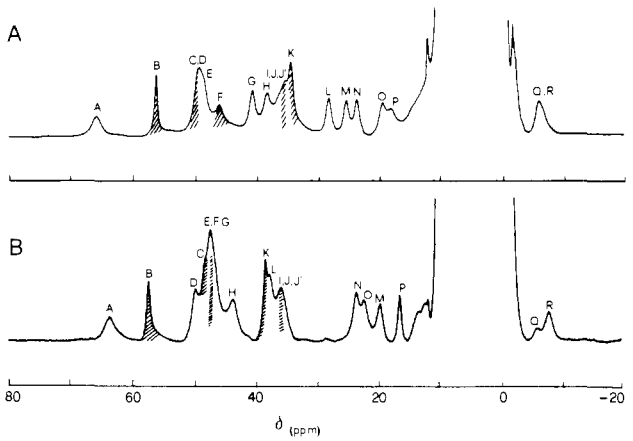

Figure 6. 303 K ^{1}H NMR spectra of (A) Cu_2Co_2WT, pH 5.5, 50 mM acetate buffer, at 300 MHz, and (B) Cu_2Co_2Ile-137, pH 5.5, 50 mM acetate buffer, at 200 MHz. Dashed signals disappear in D_2O.

Table I. NMR Parameters of the Signals of the Wild Type and of the Ile-137 Mutant Cu_2Co_2SOD Derivatives at 300 MHz and 303 K

signal	tentative assignment[d]	wild type δ, ppm (T_1, ms)[b]	Ile-137[a] δ, ppm (T_1, ms)[b]
A	*His-63 Hδ2	66.0 (1.0)	63.3 (1.7)
B	His-120 Hδ1	56.4 (6.2)	57.3 (10.3)
C	His-46 Hε2	50 } (2.7)	48.4 (3.3)
D	His-80 Hδ2	49.4 }	49.6 (3.2)
E	His-71 Hδ2	48.8 (c)	47.3 }
F	His-80 Hε2	46.2 (c)	47.3 } (3.2)
G	*His-46 Hδ2	40.9 (3.0)	47.3 }
H	*His-120 Hε1	38.5 (1.4)	43.8 (1.7)
I	His-80 Hε1	36.3 (c)	36 }
J′	His-71 Hε1	36 (c)	36 } (1.5)
J	His-71 Hε2	35.6 (c)	36 }
K	*His-48 Hδ1	34.6 (3.6)	38.6 (5.8)
L	His-48 Hδ2	28.3 (3.6)	38.0 (3.1)
M	*His-46 Hε1	25.5 (2.4)	20.1 (2.5)
N	His-120 Hδ2	23.6 (2.3)	23.7 (2.6)
O	*His-48 Hε1	19.6 (2.0)	22.5 (2.3)
P	His-46 Hβ1	18.3 (1.5)	16.8 (26)
Q		−5.6 } (2.4)	−6.5 (c)
R		−5.6 }	−8.1 (c)

[a] The data on this derivative have been obtained at 200 MHz. [b] Estimated error is ±15%. [c] Not measured because the signal is under a complex envelope. [d] Protons experiencing differences in shift larger than 2.5 ppm are marked with an asterisk.

Azide binds copper in SOD-Ile-137, as it does with all SOD's.[29-31] In the present case a charge transfer band appears at 375 nm in the presence of N_3^-. For N_3^- concentrations above 10^{-1} M, another absorption appears at 325 nm which could be due to weak binding of a second azide or to a change in the tertiary structure due to salt effects. By following the variation of the intensity of the electronic spectra in the 400–300 nm region with the ligand concentration, we estimate two affinity constants (54 ± 15 and 2 ± 1 M^{-1}) for N_3^- toward SOD-Ile-137. The EPR spectra of the mutant azide derivative are essentially identical with those of the WT derivative, with an axial type of spectrum and $A_{\parallel} = 182 \times 10^{-4}$ cm^{-1} (Figure 5C,D).

The Cu_2Co_2SOD-Ile-137 Derivative. Substitution of zinc with cobalt provides a derivative structurally very similar to the native system.[32] Furthermore, the new derivative can be studied by ^{1}H NMR spectroscopy to yield meaningful structural information.[23]

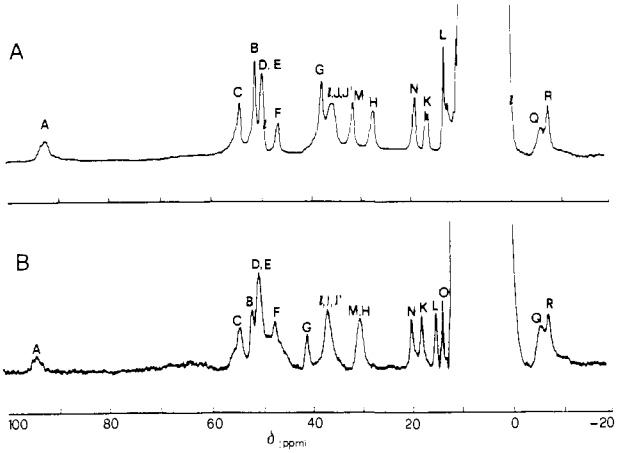

Figure 7. 303 K ^{1}H NMR spectra of Cu_2Co_2 WT at 300 MHZ (A) and Cu_2Co_2 Ile-137 at 200 MHz (B) derivatives in 50 mM acetate buffer, pH 5.5, in the presence of 3.1×10^{-1} M and 1.85×10^{-1} M N_3^-, respectively. As judged from the affinity constants the N_3^- adducts are 98% and 97% formed under these conditions.

The ^{1}H NMR spectrum is shown in Figure 6, together with that of the WT. The resonances of the latter have been tentatively assigned[31,33] by H_2O/D_2O exchange in order to reveal the exchangeable NH protons of imidazole rings, by measurement of the longitudinal relaxation times, and by titration with N_3^-. The comparison of the T_1 values with the metal–proton distances from X-ray has led to the assignment of Table I. However, the large ligand-centered dipolar relaxation contribution which may change from proton to proton may make the criterion based on the relation between metal–proton distance and T_1 less safe. Even allowing nuclear relaxation to be a weaker criterion, the assignment results substantially correct from the following: (i) the shift behavior of the signals upon titration with anions; (ii) the H_2O/D_2O exchange as far as the histidine NH signals are concerned; (iii) the magnetic field dependence of the line width which permits discrimination between the cobalt and the copper domain. By allowing the T_1–distance relationship to fail, the uncertainty may remain of the labeling of His-46 and 48 ring protons. Recent ^{1}H NOE experiments have led us to the assignment reported in Table I. From Figure 6 and Table I it is evident that some sizable changes have occurred upon substitution of Thr-137. In particular, signals G and M become less equivalent with regard to isotropic shifts, and another ortho-like proton assigned to His-120 (signal H) experiences a downfield shift with respect to the WT derivative. The major changes, however, are observed in the signals K, L, O, assigned to His-48, where the NH (signal K) and the ortho-like Hδ2 (signal L) protons are further shifted downfield by more than 10 ppm. This also holds for the other ortho-like proton (signal O), though to a lesser extent. On the other hand, one of the β-CH_2 protons of His-46 (signal P) experiences a smaller shift. It appears that His-48 is more firmly bound to copper in a more symmetrical environment. The smaller shift of signal P might be ascribed to the resulting rotation of the β-CH_2 moiety around the histidine C_β–C_γ bond, which would increase the copper–proton distance. The latter distance is, indeed, very short (2.7 Å) in bovine SOD[7] and, presumably, in the human-WT isoenzyme. The much longer T_1 value of this signal is consistent with this hypothesis. Minor but probably significant changes occur also in the proton signals of His-71 and His-80 (particularly signals E and F) bound to cobalt. Clearly, the structural alterations around cobalt(II) are minor; indeed, as mentioned in the Experimental Section, the electronic spectra of E_2Co_2SOD-Ile-137 show no appreciable difference from the WT analogue.

Addition of azide causes major variations in the NMR spectra because the anion binds the metal ion in a fast exchange regime. The shift of each signal can be followed from the position of the

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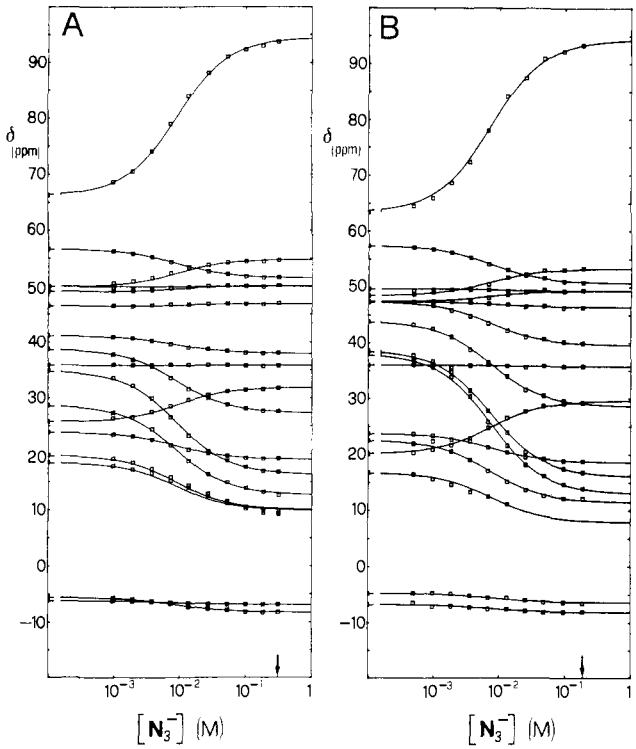

Figure 8. 303 K ^{1}H NMR titration of Cu_2Co_2WT at 300 MHz (A) and Cu_2Co_2Ile-137 at 200 MHz (B) with sodium azide in 50 mM acetate buffer, pH 5.5. The lines are simultaneous best fit curves to a single binding constant of 154 ± 8 M^{-1} for WT and 175 ± 8 M^{-1} for SOD-Ile-137. The arrows indicate the N_3^- concentration relative to the spectra in Figure 7. Titrations in 50 mM Hepes buffer at pH 7.5 give the same affinity constants for azide binding.

pure enzyme to that of the fully inhibited derivative as a function of N_3^- concentration, as previously demonstrated for several isoenzymes of SOD[23,31] and some of its mutants.[12] It appears (Figures 7 and 8) that the spectrum of the azide adduct is very similar in both mutant and WT derivatives. In particular, one of the histidines, His-48 in our assignment, no longer senses the unpaired electron, as shown by the shifts of its signals which move toward the diamagnetic position; the signals of the protons of histidines coordinated to copper undergo the same typical variation in shift as observed in the WT derivative. At variance with the WT, signal E, belonging to the cobalt-coordinated His-71, moves slightly upfield upon azide binding.

The affinity constant can be accurately measured by following the shift variation for each signal. A value of 175 ± 8 M^{-1} is obtained which compares with 154 ± 8 M^{-1} obtained for the WT derivative.[31]

Discussion

The Ile-137 mutant shows essentially the same activity as the wild type at low pH but, owing to the decrease of almost one unit in the average pK_a for inactivation, is as much as five times less active at high pH. This is somewhat surprising because Thr-137 does not have an obvious role in the catalysis and because its substitution with an isoleucine leaves the overall charge of the cavity unaltered. The effect must then be an indirect one, possibly related to some important hydrogen bonding interaction involving the Thr hydroxyl in the WT which is lacking in Ile 137. Among possible candidates for important titratable acid–base groups are a coordinated water, Arg-143, and one or more of the surface lysines, especially Lys-136 which is next to Thr-137 and also near the entrance of the cavity.[7,34] Direct ionization of the coordinated water has been shown not to occur;[35-37] rather, at high pH a hydroxide ion adds to the copper coordination sphere,[37] with a pK_a > 11. This binding is accompanied by spectroscopic and NMRD changes that are easily monitored; and the pH profiles for the changes do not match with the pH–rate profile, at least for the bovine and porcine enzymes.[38] A possible candidate for the pK_a of the activity profile is Arg-143; however, recent activity data on the Ile-143 mutant[15,16] give the same activity profile, indicating that Arg-143 is not responsible for the drop in activity at high pH. No direct interaction between Thr-137 and the ϵ-NH_3^+ of any lysine is possible.[7,34] If Lys-136 were the one responsible for the observed activity-linked pK_a, the alteration caused by the Thr-137 → Ile-137 substitution might be induced by a change in the structure of the electrostatic channel loop to which both Ile-137 and Lys-136 are attached.

Table II. Calculated Limit δ Values of the Wild Type and the Ile-137 Mutant Cu_2Co_2SOD-N_3^- Derivatives at 300 MHz and 303 K

signal	tentative assignment	wild type δ, ppm	Ile-143[a] δ, ppm
A	His-63 Hδ2	94.4	94.6
B	His-120 Hδ1	51.4	50.5
C	His-46 Hε2	54.8	53.3
D	His-80 Hδ2	49.9	49.2
E	His-71 Hδ2	50.1	49.3
F	His-80 Hε2	46.9	46.3
G	His-46 Hδ2	37.9	39.4
H	His-120 Hε1	27.2	28.3
I	His-80 Hε1	35.8	35.7
J′	His-71 Hε1	35.8	35.7
J	His-71 Hε2	35.8	35.7
K	His-48 Hδ1	16.3	15.7
L	His-48 Hδ2	12.6	12.7
M	His-46 Hε1	31.9	29.7
N	His-120 Hδ2	18.9	18.4
O	His-48 Hε1	9.9	11
P	His-46 Hβ1	9.8	
Q		−6.0	−6.5
R		−7.5	−8.2

[a] The azide titration on this derivative has been performed at 200 MHz.

Ionic strength effects are very similar in WT and Ile-137. This is not surprising, regardless of which positive residues are most sensitive to the shielding of charges induced by salts in solution, since the difference in pK_a between the SOD-Ile-137 mutant and the wild type protein is unimportant as long as the residues are fully protonated at the pH of the experiment.

When Thr-137 is substituted by Ile-137, the latter residue can point either inside or out of the crevice; the crevice itself is essentially hydrophilic. Our spectroscopic data are consistent with the view that the Ile-137 residue points into the crevice and disrupts the water structure. Indeed, water ^{1}H Nuclear Magnetic Relaxation Dispersion (NMRD) measurements have shown that SOD-Ile-137 has not fast-exchanging water interacting with copper.[39] As a consequence of the possible removal of the fifth donor atom, the CuN_4 chromophore may undergo some changes and looks more planar. The axialization in the EPR spectrum, the increase in $A_{\parallel}$, and the hypsochromic shift of the d–d transitions are consistent with the removal of the fifth donor atom. On the other hand, there is no evidence from the X-ray structure that the 137 residue can interact with any of the coordinated histidines.

One major result of the present study is that the electronic structure and coordination geometry of the metal ion has virtually no influence on the enzymatic activity. It should be recalled that all spectroscopic data on both native and cobalt-substituted mutants have been taken at pH values where the activity is essentially that of the WT enzyme.

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The affinity of azide for copper does not change significantly between the cobalt derivatives of the WT and of the SOD-Ile-137 mutant, being 154 and 175 M^{-1}, respectively. This may not hold for the zinc-copper derivatives if one should judge from the formation of the azide to copper charge transfer band, since values of 54 ± 15 M^{-1} for the mutant versus 93 ± 31 M^{-1} for the WT are obtained. The discrepancy revealed between the values obtained through NMR and electronic spectroscopy may arise not from intrinsic differences between the native and cobalt-substituted enzymes but from the different experimental conditions at which the titrations were performed, i.e., large differences in enzyme concentrations or changes in molar absorbance due to salt effects.

In the NMR experiment, the affinity values are obtained by simultaneous fitting of the azide dependence of the isotropic shifts of as many as 17 signals, and the results are very reliable. Accepting the latter values for our discussion, we may conclude that changing the residue 137 does not have an effect upon either the enthalpy or entropy contributions toward binding, despite the difference in the water structure inside the cavity and the copper coordination geometry.

In the study of another series of mutants[12] we have proposed that the charge inside the cavity determines the affinity of azide and of substrate (superoxide) for the protein, thus determining the enzymatic affinity. A close correlation between azide affinity and activity was found. In the present case, activity and anion affinity again appear to be closely interlinked.

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Registry No. SOD, 9054-89-1; L-Thr, 72-19-5; O_2^-, 11062-77-4; Cu, 7440-50-8; phosphate, 14265-44-2.

Mechanism and Products of Electrochemical Oxidation of 5,7-Dihydroxytryptamine

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Abstract: It has been postulated that the mechanisms of electrochemical and autoxidation of the chemical neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) are different. The former has been proposed to be a $2e^-,2H^+$ reaction leading to the corresponding *p*-quinone imine, while the latter has been proposed to be a $1e^-,1H^+$ reaction yielding a radical intermediate. The fundamental mechanism by which 5,7-DHT is oxidized is important because it is widely believed that the neurodegenerative effect of this compound is manifested by a reactive oxidation product formed in the central nervous system. The electrochemical oxidation of 5,7-DHT at low pH has been investigated. Contrary to earlier postulations, the initial step is a $1e^-,1H^+$ oxidation of 5,7-DHT to a radical intermediate. When electrolyses are performed at low applied potentials ($E_{p/2}$ for the first voltammetric oxidation peak of 5,7-DHT), several simple dimers are formed as ultimate reaction products. In view of the fact that the major dimer formed is 4,4′-bi-5,7-dihydroxytryptamine, it may be concluded that the predominant form of the radical intermediate is that in which the unpaired electron is located at the C(4) position. With increasingly positive applied potential the latter dimer is oxidized ($2e^-,2H^+$) to 4,4′-bi-[5-hydroxy-3-(2-aminoethyl)indolyliden-7-one]. However, at potentials greater than or equal to E_p for the first voltammetric oxidation peak, the primary radical is electrochemically oxidized ($1e^-,1H^+$) to the corresponding *p*-quinone imine. Nucleophilic attack by water yields 4,5,7-trihydroxytryptamine, which is rapidly further oxidized ($2e^-,2H^+$) to 5-hydroxytryptamine-4,7-dione.

5,7-Dihydroxytryptamine (5,7-DHT) is widely used for the selective chemical denervation of 5-hydroxytryptamine- (serotonin-) containing neurons.[3-10] The selectivity of 5,7-DHT is probably derived from its high-affinity uptake by the membrane pump of serotonergic neurons. It is rather generally believed that the neurodegenerative properties of 5,7-DHT result from an inherent chemical property, namely, ease of oxidation. Autoxidation of 5,7-DHT in vivo has been proposed to give reactive quinone imine species (**1**, **2**) which can alkylate nucleophiles such as neuronal membrane proteins as illustrated in Scheme I.[11] This reaction would, presumably, modify the neuronal membrane and, hence, denervate the neuron. The neurotoxicity of 5,7-DHT has also been speculated to be caused by interactions of intermediates **1** and **2** with the electron transport chain[10] or by the formation of cytotoxic reduced oxygen species ($O_2^{\cdot-}$, $HO^{\cdot}$, H_2O_2) as illustrated in Scheme I.[8,9,12,13,15]

Recently, Sinhababu and Borchardt[16] attempted to elucidate the mechanisms of both the autoxidation and electrochemical oxidation of 5,7-DHT. Autoxidation was found to be first order in terms of both O_2 and 5,7-DHT. This observation is consistent with a mechanism involving the incorporation of O_2 into the

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