An Investigation of Superoxide Dismutase Lys-143, Ile-143, and Glu-143 Mutants: Cu_2Co_2SOD Derivatives

Lucia Banci,[†] Ivano Bertini,*[†] Claudio Luchinat,[‡] and Robert A. Hallewell[§]

Contribution from the Department of Chemistry, University of Florence, Florence, Italy, Institute of Agricultural Chemistry, University of Bologna, Bologna, Italy, and Chiron Corporation, Emeryville, California 94608. *Received March 23, 1987*

Abstract: The Cu_2Co_2 derivatives of recombinant human superoxide dismutase from yeast in which Arg-143 has been substituted with Ile, Lys, and Glu residues have been prepared and characterized through electronic spectroscopy. The ^1H NMR spectra have been measured and compared with those of the wild type derivative. The structure of the four derivatives appears very similar. The same holds for the four azide derivatives in which a histidine, possibly His-46, is replaced by the anion. On the other hand the affinity of N_3^- for the proteins is 154, 63, 16, and 6 M^{-1} for wild type, Lys-143, Ile-143, and Glu-143 derivatives, respectively. Despite the similarity of the chromophores, the affinity of anions is shown to be controlled by the charge at the 143 position. Since the change in affinity of anions strikingly parallels the SOD activity over a two orders of magnitude range, it may mean that the thermodynamic affinity of substrate for the enzyme is also modulated by the same effects and that the mechanism of O_2^- oxidation implies the presence of O_2^- inside the cavity.

Copper–zinc superoxide dismutase (SOD) is a dimer of identical subunits each containing one copper and one zinc atom. In the crystal structure the zinc ion is not accessible to solvent, whereas the copper ions is.[1] Copper is essential for catalysis and acts cyclically as an electron acceptor to produce dioxygen and then as an electron donor to produce hydrogen peroxide during successive encounters with O_2^-.[2]

When cobalt(II) substitutes zinc(II) in the native SOD, a derivative is obtained with full enzymatic activity[3,4] and with peculiar spectroscopic properties[5] which permit a thorough investigation of the system under various conditions. In particular, the ^1H NMR spectra show the signals of each proton of the histidines bound to either cobalt or copper.[6] Such signals have been tentatively assigned both for the bovine enzyme[7] and for recombinant human SOD from yeast[8] which has been recently obtained.[9]

The cloning and expression of human SOD[9] has made it possible to investigate the role of specific residues at the active center of the enzyme by analyzing the effects of substituting other amino acid residues at these positions with site-directed mutagenesis. There is an Arg residue (Arg-143 in human SOD) in the active site cavity immediately adjacent to the copper ion. It has been suggested that Arg-143 functions both to stabilize an intermediate Cu^{2+}–O_2^-–$^+$Arg along the catalytic pathway[10,11] and to provide a center of positive charge for electrostatic attraction of O_2^- toward copper(II).[1,12-16]

We now report on an investigation of Cu_2Co_2SOD in which Arg-143 has been substituted with Lys (SOD-Lys-143), Ile (SOD-Ile-143), and Glu (SOD-Glu-143) through site-directed mutagenesis.[17] The diffuse positive charge with high pK_a of an Arg is thus replaced by a simple ammonium group with lower pK_a, with a hydrophobic residue, and finally with a negative charge. The specific activities of the new derivatives are 43 and 11% that of the wild type for SOD-Lys-143 and SOD-Ile-143, respectively.[17] The SOD-Glu-143 is essentially inactive (activity 2–4%).[18] The latter mutant has been obtained on a derivative in which Cys-6 and Cys-111 have been substituted with alanine and serine respectively and therefore is labeled SOD-AS. The latter two substitutions are far from the active site and have no effect on the enzyme activity.[19] Electronic and EPR spectra, as well as ^1H NMR spectra of the Cu_2Co_2SOD-AS derivative, are also essentially the same as those of the wild type.[20] We have investigated the cobalt(II)-substituted derivatives of the mutants at the 143 position through ^1H NMR spectroscopy since the ^1H NMR spectra are extremely sensitive to geometrical changes of the residues around the metal ions.[6-8] We have also studied the binding mode of N_3^-, which is known to be an inhibitor of the enzyme,[21] in order to understand how the affinity of an anion can be modulated by the change of a group in the enzymatic cavity. This latter information may be relevant also with respect to the interaction of the negative anionic substrate.

Experimental Section

HSOD Lys-143, HSOD Ile-143, and HSOD AS Glu-143 were expressed in yeast and purified to homogeneity as previously reported.[9,17] HSOD AS Glu-143 (HSOD Glu-143 hereafter) has been obtained in

[†] University of Florence.
[‡] University of Bologna.
[§] Chiron Corporation.

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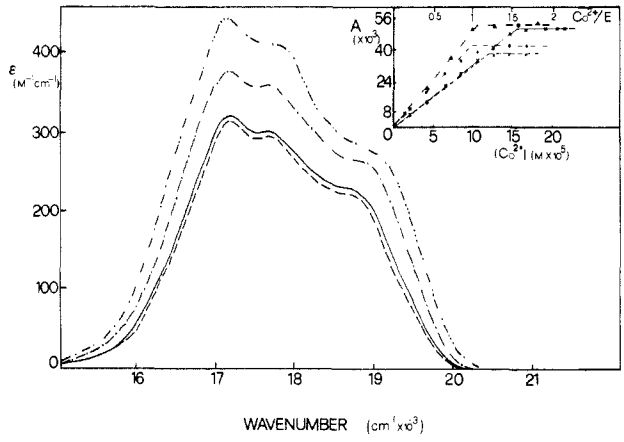

Figure 1. Electronic spectra of E_2Co_2HSOD WT (—),[8] E_2Co_xHSOD Lys-143 (-·-), E_2Co_xHSOD Ile-143 (---), and E_2Co_xHSOD Glu-143 (-···-). Molar absorbances are per cobalt ion. Conditions: pH 5, 5×10^{-2} M acetate buffer. The inset shows the increase in absorbance with increasing amounts of Co^{2+}. Saturation is apparently reached when 1.6 (WT), 1.0 (Lys), 1.2 (Ile), or 1.1 (Glu) mol of cobalt are added to 1 mol of dimeric protein, yielding molar absorbances of 320 (WT), 380 (Lys), 316 (Ile), or 436 (Glu) M^{-1} cm^{-1} per cobalt(II).

similar fashion.[17-19] Demetalation of these mutants with EDTA at pH 3.6 according to established procedures[22] is a slow process as compared with the wild-type enzyme. By monitoring the copper absorbance in the visible region copper depletion requires about 4 days for Glu-143, 8 days for Lys-143, and about 20 days for Ile-143, whereas the wild type completely releases copper in less than 2 days.[22] Zinc content was always less than 15%, as judged from atomic absorption spectroscopy. 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,[22] cobalt(II) first and then copper(II) were added at pH 5.6 following the metal incorporation spectrophotometrically.[6] Cobalt(II) uptake was instantaneous. Complete copper uptake was achieved in a few hours, as judged from the characteristic change of the cobalt absorption bands.[3a,6]

Protein concentration was determined by Lowry's method.[23]

Electronic spectra were recorded on a Cary 17D spectrophotometer, and ^1H NMR spectra on Bruker CXP 300 or Bruker MSL 200 instruments. Experimental conditions for spectroscopic measurements have been reported elsewhere.[6,7]

Results

Metal Substitution. As mentioned in the Experimental Section, copper removal is kinetically slow with respect to the native derivative, and the rate is much slower for SOD–Ile-143. Apparently a charge in the cavity is important in the metal removal mechanism. The final content of zinc, checked through atomic absorption, corresponded to less than 15% of the site occupancy. Addition of cobalt gives rise to an E_2Co_x derivative with spectral properties similar to those of the native derivative (Figure 1). However, only between 1.0 and 1.6 mol of cobalt are taken up by 1 mol of protein for the three mutants and the native protein under the present conditions. The degree of cobalt incorporation is irrelevant for the present research, since the ^1H NMR spectra typical of the sytem only reflect fully remetalized subunits,[6,7] and this behavior has not been further investigated. However, the stoichiometry of cobalt uptake by apoSOD may not be straightforward. Depending on experimental conditions, partial incorporation has been observed in both the zinc[3b] and copper[24] sites; furthermore, a marked break in electronic absorption upon addition of the first equivalent of cobalt has been recently reported for the yeast isoenzyme.[25] The spectra of the $Co_{0.5}E_2SOD$'s are reported in Figure 1, with the aim of showing that they are all

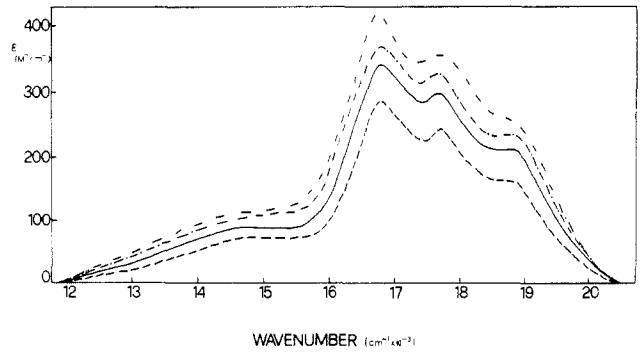

Figure 2. Electronic spectra of Cu_2Co_2HSOD WT (—),[8] Cu_2Co_xHSOD Lys-143 (-·-), Cu_2Co_xHSOD Ile-143 (---), and Cu_2Co_xHSOD Glu-143 (-···-). Conditions as in Figure 1. Molar absorbances are per cobalt ion.

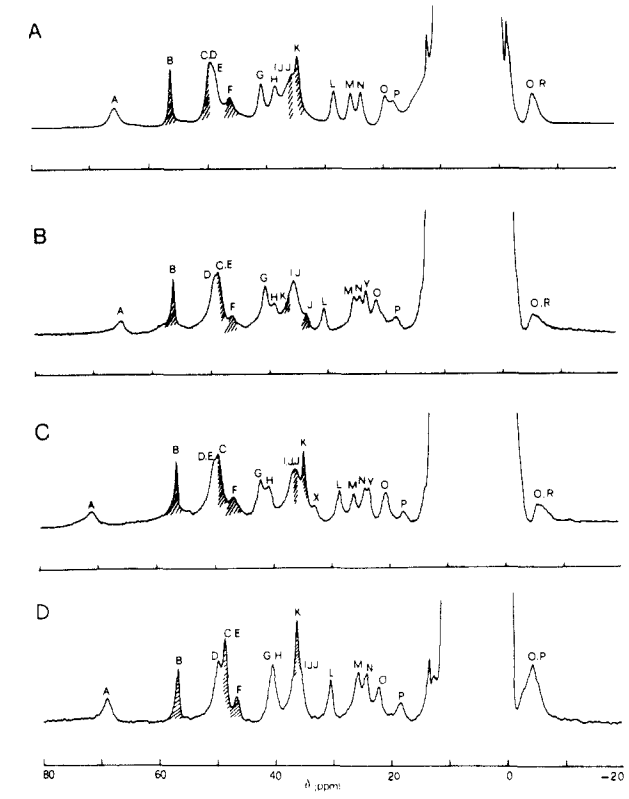

Figure 3. 30 °C 300-MHz ^1H NMR spectra of Cu_2Co_2HSOD WT (A),[8] Cu_2Co_xHSOD Lys-143 (B), and Cu_2Co_xHSOD Ile-143 (C) and 30 °C 200-MHz ^1H NMR spectrum of Cu_2Co_xHSOD Glu-143 (D). The shadowed signals disappear or reduce their intensity in D_2O. Conditions as in Figure 1.

very similar, although SOD-Lys-143 displays a slightly higher and SOD-Glu-143 a sensibly higher intensity. The differences are real, as appears from the dependence of the absorbance as a function of the cobalt concentration (see inset of Figure 1). Copper incorporation by the mutants is complete within a few hours. The electronic spectra of the derivatives with 2 equiv of copper (Figure 2) are similar to each other. Besides the differences in the cobalt spectrum, the major difference among the derivatives is the position of the absorption due to copper(II), which shows a blue shift in the Lys derivative with respect to Ile, Glu, and wild type. Such a shift is the same as in the Cu_2Zn_2SOD's.[17,26]

The ^1H NMR Spectra. The ^1H NMR spectra of human Cu_2Co_2SOD wild type previously obtained,[8] Cu_2Co_xSOD-Lys-143, Cu_2Co_xSOD-Ile-143, and Cu_2Co_xSOD-Glu-143 are shown in Figure 3. Only when the active site contains a Cu–Co pair do the ^1H NMR signals of the coordinated histidine protons appear

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Table I. NMR Parameters of the Signals of the Wild Type, Lys-143, Ile-143, and Glu-143 Mutants Cu_2Co_2SOD Derivatives at 300 MHz and 30 °C

		δ, ppm (T_1,[b] ms)			
signal	proton	wild type	Lys-143	Ile-143	Glu-143[a]
A	His-63 Hδ2	66.0 (1.0)	65.8 (1.2)	70.9 (c)	68.8 (1.6)
B	His-120 Hδ1	56.4 (6.2)	56.8 (4.5)	56.5 (5.0)	56.6 (8.5)
C	His-48 Hδ1	50 } (2.7)	49.8 } (2.4)	49.3 (3.2)	48.5 (4.3)
D	His-80 Hδ2	49.4 }	49.8 }	49.3 (c)	49.7 (3.9)
		[49.0]		[50.0]	
E	His-71 Hσ2	48.8 (c)	49.0 (c)	50.0 (c)	48.5 (4.3)
			[49.8]	[49.3]	
F	His-80 Hε2	46.2 (c)	46.8 (3.9)	46.8 (3.6)	46.5 (3.3)
G	His-48 Hε1	40.9 (3.0)	40.9 (2.5)	42.1 (2.2)	40.4 (3.3)
H	His-120 Hε1	38.5 (1.4)	39.9 (1.3)	40.7 (1.3)	40.4 (c)
I	His-80 Hε1	36.3 (c)	35.5 (1.7)	36.1 (2.4)	35.5 (2.1)
J′	His-71 Hε1	36 (c)	34.0 (c)	36.1 (3.3)	35.5 (c)
J	His-71 Hε2	35.6 (c)	35.5 (3.0)	36.1 (3.3)	35.5 (c)
K	His-46 Hδ2	34.6 (3.6)	37 (c)	34.6 (8.8)	36.2 (5.0)
L	His-46 Hδ2	28.3 (3.6)	30.7 (3.8)	28.4 (3.0)	30.3 (3.9)
M	His-48 Hδ2	25.5 (2.4)	25.6 (2.8)	25.9 (1.8)	25.7 (2.8)
N	His-120 Hδ2	23.6 (2.3)	24.6 (2.9)	24.1 (2.1)	24.2 (2.9)
O	His-46 Hε1	19.6 (2.0)	21.8 (2.0)	20.4 (1.7)	22.1 (2.3)
P	His-46 Hβ1	18.3 (1.5)	18.5 (c)	17.5 (c)	18.4 (1.5)
Q		−5.6 } (2.4)	−5.0 } (c)	−5.5 } (c)	−4.5 (c)
R		−5.6 }	−5.0 }	−5.5 }	−5.4 (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.

isotropically shifted.[6,7] The spectra indicate that the geometry of the active site remains essentially unaltered. The shift values for the individual signals in the four derivatives are collected in Table I. Signal A, which is assigned to a proton of the bridging histidine, experiences a larger shift for the SOD-Ile-143 and SOD-Glu-143 derivatives. Signals D, E, F and I, J, J′, which are assigned to ring protons of histidines bound to cobalt, are essentially in the same position. The same holds for signals B, N and C, M, which have been assigned to protons of copper-bound histidines 120 and 48, respectively. Signal H of histidine 120 and signal G of histidine 48 experience some small variation in shift, the former when passing from wild type to modified, the latter when passing from the SOD-Ile-143 to the other derivatives. Some differences are noted in signals K, L, O, which are assigned to His-46, as far as SOD-Lys-143 and SOD-Glu-143 are concerned. Signal K in the SOD-Lys-143 derivative looks different than in the other derivatives and is assigned through titration with N_3^- and through comparison between the spectra in H_2O and D_2O. Signal K disappears in D_2O, consistent with its assignment to an NH proton. Finally two more signals, labeled X and Y, are observed in the Ile derivative. Signal X has a fractional intensity. The latter signals decrease in intensity upon addition of azide (see below). Such signals are not present in the bovine and yeast derivatives but they have been observed in some preparations of the wild type human Cu_2Co_2SOD.[8] In a sample obtained from human erythrocyte SOD no such signals were observed. Their assignment is still unclear. In the Lys derivative signal Y is again observed; signal J′, which resembles X, actually behaves as the wild type J′ upon N_3^- addition (see below), and therefore is assigned to a histidine NH.

The T_1 values of the signals are also reported in Table I. Again, there are noticeable similarities between the wild type and the mutant derivatives and probably most of the deviations are within experimental error if an allowance of 15% is given for the average values. The largest difference is observed for signal K, assigned as the NH proton of His-46, which experiences a sizably longer T_1 in the SOD-Glu-143 derivative, possibly as a result of a difference in the hydrogen bond strength to Asp-122.[1,12]

Titration with N_3^-. Titration with N_3^- causes major changes in the ^1H NMR spectra of Cu_2Co_2SOD and is of great help in the assignment since the signals of protons of histidines bound to cobalt essentially do not move, whereas the signals of protons

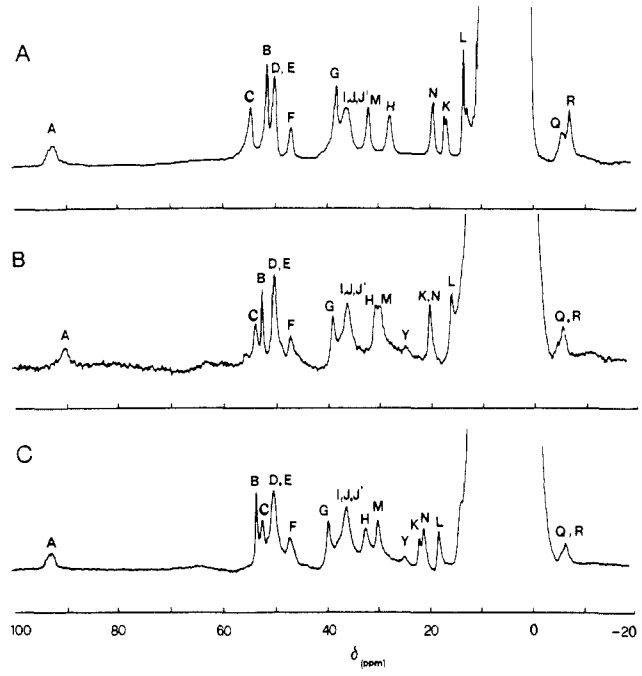

Figure 4. 30 °C 300-MHz ^1H NMR spectra of the N_3^- derivatives of Cu_2Co_2HSOD WT (A, 0.31 M N_3^-),[8] Cu_2Co_2HSOD Lys-143 (B, 0.34 M N_3^-), and Cu_2Co_2HSOD Ile-143 (C, 0.48 M N_3^-). Conditions as in Figure 1. The ^1H NMR spectrum of the fully formed N_3^- adduct of Glu-143 cannot be obtained due to the low affinity constant. The calculated shift values are reported in Table II.

of one histidine, which we have assigned to His-46,[7] move toward the diamagnetic position. The signals of the protons of the other two histidines bound to copper change their positions as in the wild type derivative.

The spectra of the derivatives with N_3^- are reported in Figure 4 and the shift dependence on N_3^- concentration in Figure 5. It is apparent that the behavior of N_3^- is essentially the same for the three mutant enzymes and for the wild type one. One histidine, possibly His-46, is removed upon N_3^- binding. The affinity of N_3^-, estimated through best fitting of the data in Figure 5 to a single binding constant, drops from 154 ± 8 (3σ) M^{-1} in the wild type derivative to 63 ± 3 (3σ) M^{-1} in SOD-Lys-143, to 16 ± 1 (3σ) M^{-1} in SOD-Ile-143, and to 6 ± 1 (3σ) M^{-1} in SOD-Glu-143 (i.e., 100, 41, 9, and 4%). This closely parallels the enzymatic activity of the four derivatives (100, 43, 11, and 2–4%)[17,18] and is suggestive of a decrease in the affinity of O_2^- for the active cavity.

The spectrum of N_3^- adduct of the SOD-Glu-143 derivative could not be obtained experimentally due to the low affinity constant. Even the spectra of the other N_3^- adducts shown in Figure 4 are not the limit spectra, especially for the SOD-Ile-143 derivative. If we compare the calculated limit spectra of the four N_3^- derivatives (Table II), it appears that they are indeed similar. However, signal A for SOD-Lys-143 and SOD-Glu-143 has a sensibly smaller shift than in the other derivatives. Furthermore all the shifts of histidine 46 (K, L, O, P) are larger in the mutants than in the wild type. Also, signal X disappears, and signal Y quenches its intensity without variation in shift (Figure 5).

Discussion

Analysis of the Spectral Data. The structural changes in the Cu-liganding residues among the derivatives studied here are minor, although SOD-Lys-143 appears to have a blue shift in the copper electronic transition and SOD-Lys-143 and SOD-Glu-143 an higher intensity in cobalt transitions. It seems that Lys-143 favors a more tetragonal environment around copper(II) and a slightly more tetrahedral environment around cobalt(II). The effect on cobalt is more marked in the SOD-Glu-143 derivative. The same blue shift in the copper band is observed in the zinc-containing SOD-Lys-143 derivative and a more axial EPR spectrum is also observed.[17,26] The ^1H NMR spectra of the

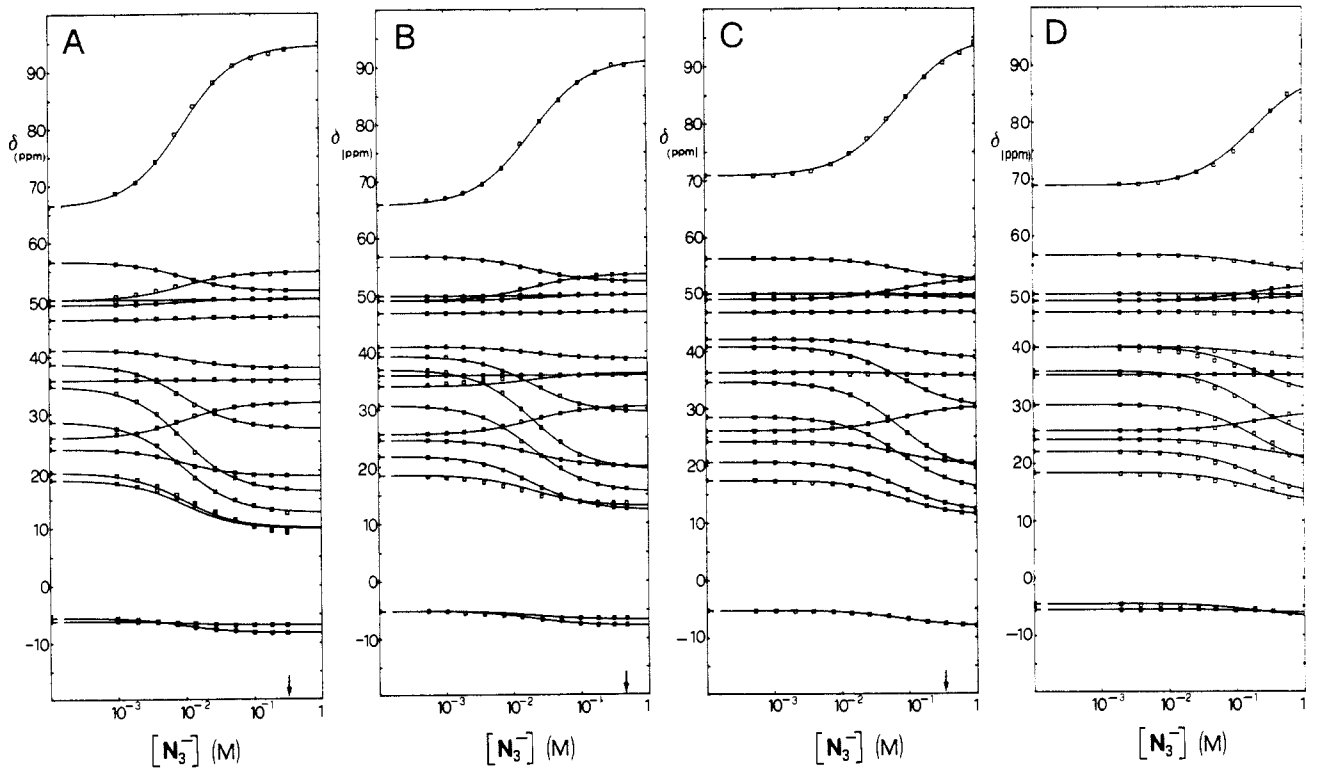

Figure 5. 30 °C 300-MHz ^{1}H NMR titrations of Cu_2Co_2HSOD WT (A),[8] Cu_2Co_xHSOD Lys-143 (B), Cu_2Co_xHSOD Ile-143 (C), and Cu_2Co_xHSOD Glu-143 (D) (at 200 MHz) with increasing amounts of sodium azide. The arrows indicate the N_3^- concentrations at which the spectra of Figure 4 were taken. The affinity constants (K = 154 ± 8 for WT, 63 ± 3 for Lys, 16 ± 1 for Ile, and 6 ± 1 M^{-1} for Glu) were obtained from a nonlinear best-fit analysis of all the data. The best fit curves through the data points are also shown.

Table II. Calculated Limit δ Values of the Wild Type, Lys-143, Ile-143, and Glu-143 Mutants Cu_2Co_2SOD-N_3^- Derivatives at 300 MHz and 30 °C

		δ, ppm			
signal	proton	wild type	Lys-143	Ile-143	Glu-143[a]
A	His-63 Hδ2	94.4	91.5	95.3	89.1
B	His-120 Hδ1	51.4	52.2	52.4	53.4
C	His-48 Hδ1	54.8	53.7	52.8	51.6
D	His-80 Hδ2	49.9	50.0	49.8	49.5
E	His-71 Hδ2	50.1	50.1	49.3	49.3
F	His-80 Hε2	46.9	47.0	46.8	46.2
G	His-48 Hε1	37.9	38.7	38.7	38.0
H	His-120 Hε1	27.2	29.2	29.9	31.7
I	His-80 Hε1	35.8	35.9	35.6	35.5
J′	His-71 Hε1	35.8	36.3	35.6	35.5
J	His-71 Hε2	35.8	35.9	35.6	35.5
K	His-46 Hε2	16.3	19.2	18.7	23.4
L	His-46 Hδ2	12.6	15.3	15.4	18.9
M	His-48 Hδ2	31.9	30.6	30.5	29.1
N	His-120 Hδ2	18.9	19.8	19.9	20.6
O	His-46 Hε1	9.9	12	12	14.0
P	His-46 Hβ1	9.8	13	11	12.8
Q		-6.0	-5.0	-8.1	-7.0
R		-7.5	-5.5	-8.1	-6.2

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

Cu_2Co_2SOD derivatives have shown that the protons of histidine 46 of SOD-Lys-143 and SOD-Glu-143 derivatives experience larger isotropic shifts than the corresponding His-46 protons in the other derivatives. In any case the isotropic shifts of the signals of histidine 46 (K, L, O, P) are the smallest with respect to the other histidines. This may mean that histidine 46 has a longer copper–nitrogen distance. As in the wild type derivative, azide displaces this histidine from coordination as shown by the decrease of the above shifts. The calculated limit isotropic shifts for the histidine 46 signals are significantly larger for the mutants as compared to the wild type. This may mean that the overall copper–proton distances are somewhat shorter in the former case, though we feel that the histidine is experiencing the same movement as in wild type, i.e. rotation along the C_β–C_γ bond which brings copper outside the histidine plane.

Implication for the Enzymatic Mechanism. The affinity of azide decreases from the wild type to SOD-Lys-143, SOD-Ile-143 and SOD-Glu-143 mutants. It appears that the positive charge and its shape at the entrance of the cavity determine the affinity of the anions. This is the first time in which the affinity of an anion could be followed as it depends on the nature of a single group in the cavity, systematically varied.

The affinity of azide had been shown to vary upon chemical modification of Arg-143.[13] In this case the affinity of N_3^- decreases from ≃150 to 24 M^{-1},[28] possibly as a result of the steric hindrance of the cavity by the bulk phenylglyoxal group. In the case of lysine modification the activity decreases to 10% of the unmodified enzyme and this decrease was related to the increase in the negative charge of the protein.[29] It should be noted that some variation of azide affinity is also observed on passing from the native enzyme to the zinc-deprived derivative,[30] but it is minor and can be accounted for on the basis of the smaller ligand strain in the former derivative. It is striking that the affinity of the arginine-containing wild type enzyme drops by two orders of magnitude in the Glu-143 mutant, the metal coordination polyhedra before and after adduct formation being the same as those observed for the wild type and the other mutants.

These results may also be relevant in the understanding of the different behavior of metalloenzymes toward anions, provided that metal ion has a solute-accessible coordination position. Carbonic anhydrase has a free histidinium group in the cavity which accounts for the larger affinity of anions for zinc at low pH;[31] carboxypeptidase A does not bind anions probably due to a COO^-

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residue[32,33] (Glu-270) in the cavity. Liver alcohol dehydrogenase has an anion binding site, about 15 Å from the metal, whose occupancy may account for the low affinity of a second anion for the metal.[34,35] Even transferrin, which requires carbonate to bind the metal, does not allow any other anion to bind to the metal ion.[36]

The decrease in the affinity of N_3^- among the SOD mutants here described strictly parallels the decrease in the superoxide dismutase activity. We speculate that the activity of the SOD enzyme depends on the affinity of the superoxide anion for the active cavity, i.e., that the decrease in activity is essentially due to an increase in K_m rather than to a decrease in k_{cat}. Only recently, have reliable values for k_{cat} and K_m been obtained:[37] measurements of this type are difficult to perform owing to the difficulties in saturating the enzyme with O_2^-. A test of our hypothesis through K_m measurements on the mutants would be even more difficult to perform if their K_m values are indeed larger.

Our results support the view that superoxide does enter the active cavity and that the O_2^-–Cu^{II} electron transfer is not a far away outer-sphere process. This does not necessarily mean that O_2^- must bind copper(II) in the same fashion as azide does, i.e., moving in the equatorial plane and displacing His-46. The substrate may pass its electron to copper(II) at an earlier stage, either through the semicoordinated water molecule or by removing it and weakly interacting with copper(II) in the axial coordination position.

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Registry No. Arg, 74-79-3; Ile, 73-32-5; Lys, 56-87-1; Glu, 56-86-0; His, 71-00-1; N_3^-, 14343-69-2; Cu, 7440-50-8.

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Temperature Dependence and Electronic Transition Energies in the Magnetic Circular Dichroism Spectrum of Horseradish Peroxidase Compound I†

William R. Browett, Zbigniew Gasyna, and Martin J. Stillman*

Contribution from the Department of Chemistry, University of Western Ontario, London, Ontario, Canada N6A 5B7. *Received April 7, 1987*

Abstract: Optical absorption and magnetic circular dichroism (MCD) spectra of horseradish peroxidase (HRP) compound I and HRP compound II have been recorded between 1.6 and 120 K. Above 30 K, the MCD spectrum of HRP compound I is dominated by temperature-independent transitions of the paramagnetic porphyrin π-cation-radical species. Below 30 K, temperature-dependent bands intensify until they dominate the MCD spectrum below 15 K and finally saturate below 2 K. This temperature dependence in the MCD spectrum is attributed to the effects of the $S = {}^3/_2$ Kramers' doublets ground state of the coupled Fe(IV) heme π-cation-radical species. Zero-field splitting in the $S = {}^3/_2$ ground state, which is calculated to be 15 ± 5 cm^{-1}, results in a nonlinear temperature dependence in the MCD spectrum. In contrast, the temperature dependence in the MCD spectrum of HRP compound II is much less pronounced, and the intensity reaches a maximum between 25 and 30 K. This unique temperature dependence is associated with zero-field splitting that is estimated to be 32 ± 5 cm^{-1}, for the Fe(IV), $S = 1$, ground state of the heme. Deconvolution calculations for both the MCD and the absorption spectra of the HRP and catalase compound I species are reported for the first time and provide meaningful data for the energies, intensities, and polarizations of the optical transitions. Little, if any, excited-state degeneracy is associated with the optical transitions in the experimental spectra of HRP or catalase compound I. The energies and intensities of the temperature-independent bands of HRP compound I are directly compared with those obtained in recent theoretical calculations that are based on Gouterman's four-orbital model and porphyrin π-cation-radical species. The results of the fitting procedure reveal that a single set of electronic transitions can account for the complete MCD and absorption spectral envelopes of *both* HRP and catalase compound I. These results indicate that the ground states in *both* the HRP and catalase compound I species should be considered to be admixtures of the $^2A_{1u}$ and $^2A_{2u}$ configurations, rather than an equilibrium mixture of configurations.

Horseradish peroxidase (HRP) (EC 1.11.1.7) catalyzes the oxidation of various substrates in the presence of H_2O_2 by forming two sequential intermediates, HRP compound I and HRP compound II.[1] The compound I and II intermediates, which are two and one oxidation equivalents above the native ferric heme, respectively, have been extensively studied.[1-5] Much of the previous work on this enzyme was undertaken in order to understand the role that these unusual heme electronic structures play in the

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP I}$$

$$\text{HRP I} + \text{AH}_2 \rightarrow \text{HRP II} + \text{AH}$$

$$\text{HRP II} + \text{AH}_2 \rightarrow \text{HRP} + \text{AH}$$

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