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Role of Arg-143 in Human Cu_2Zn_2SOD Studied through Anion Binding

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Introduction

At the entrance of the active cavity of superoxide dismutase (SOD hereafter, HSOD, human; BSOD, bovine) there is a positive residue, Arg-143, which seems fundamental for the enzymatic activity. It has been suggested that Arg-143 functions both to stabilize an intermediate Cu^{2+}–O_2^-–Arg^+ along the catalytic pathway[1,2] and to provide a center of positive charge for electrostatic attraction of O_2^- toward copper(II).[3-7]

The affinity of N_3^- was shown to be controlled by the charge at position 143, in the case of the Cu_2Co_2– derivative of recombinant human SOD from yeast in which Arg-143 has been substituted with Lys, Ile, and Glu.[8] Since the change in affinity of anions strikingly parallels the SOD activity[8,9] over a 2 order of magnitude range, it was suggested that the thermodynamic affinity of N_3^- for the enzyme is also modulated by the same effects. It was also proposed that phosphate binds at the Arg-143 site.[10]

We have studied the interaction between the Ile-143 derivative and phosphate through ^{31}P NMR spectroscopy. For the copper–cobalt-substituted species the interactions with CN^- and NCO^- have been investigated through ^{1}H NMR spectroscopy. These results are compared with the previously obtained data for the WT, in order to better elucidate the catalytic role of Arg-143.

Experimental Section

HSOD Ile-143 was expressed in yeast and purified to homogeneity as previously reported.[9,11] The Cu_2Co_2– derivatives for both HSOD WT and Ile-143 were obtained as previously described.[12]

EPR spectra at room temperature were recorded on a Bruker ER200 spectrometer operating at 9.6 GHz (X band), and ^{1}H NMR spectra on a MSL200 Bruker instrument operating at 200.13 MHz. ^{31}P NMR measurements were performed at 36.4 MHz on a Bruker CXP90 spectrometer. Longitudinal relaxation times, T_1, were measured with the inversion recovery method.[13]

Results

Interaction with CN^-. The ^{1}H NMR spectra of Cu_2Co_2HSOD WT and Ile-143 were previously obtained.[8,14] The ^{1}H NMR spectra of a Cu_2Co_2HSOD Ile-143 solution in 20 mM Hepes buffer at pH 7 were recorded at increasing amounts of CN^-. Analogous to the case of Cu_2Co_2BSOD WT,[15,16] upon addition of CN^-, the ^{1}H NMR signals somewhat broaden and decrease in intensity and simultaneously another set of broad signals appears (Figure 1B). Finally, the spectrum was obtained with only the signals of the second species (Figure 1C). This behavior is typical of quasi-slow-exchange conditions. Owing to the slow-exchange conditions with respect to the NMR time scale, the affinity constant has been estimated from the intensity of the signals of the free and ligated species. The affinity constant is 175 ± 35 (3σ) M^{-1} (Table I).

In the case of the Cu_2Co_2HSOD WT derivative, under the same experimental and slow-exchange conditions, an affinity constant of 1700 ● 340 (3σ) M^{-1} was obtained.

The ^{1}H NMR spectra of the CN^- adduct for both WT and Ile-143 are essentially identical and identical with the spectrum of the adduct of the bovine isoenzyme.[15,16]

Interaction with NCO^-. From the investigation of Cu_2Co_2BSOD with NCO^-, we know[12] that the latter binds copper(II), exchanges rapidly with the free ligand on the NMR time scale, and changes the ^{1}H NMR spectrum. The ^{1}H NMR spectra of a solution of Cu_2Co_2HSOD Ile-143 at increasing amounts of NCO^- do not show

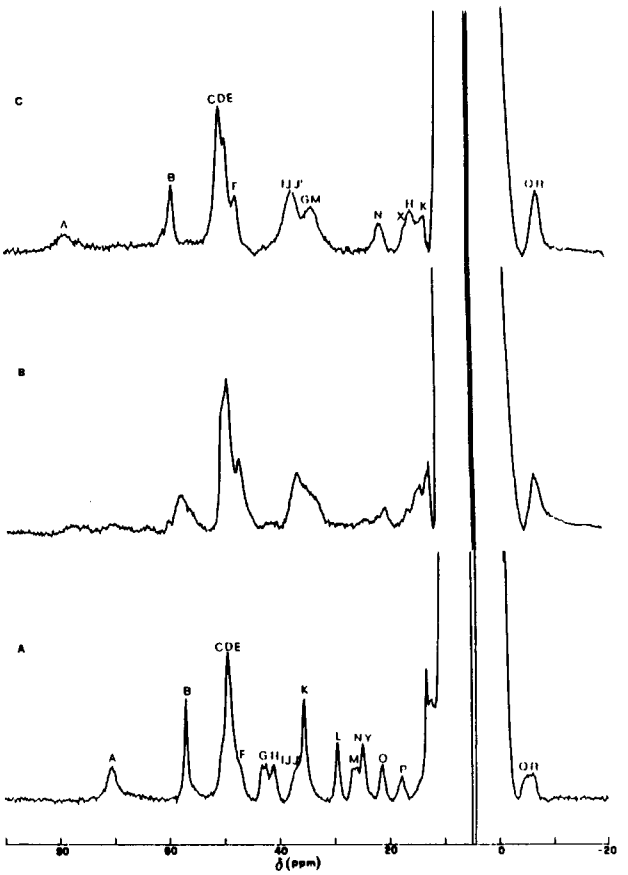

Figure 1. 200-MHz, 300 K ^{1}H NMR spectra: (A) Cu_2Co_2HSOD Ile-143; (B) intermediate point of the titration with CN^-; (C) Cu_2Co_2HSOD Ile-143–CN^- adduct. All the spectra were recorded at pH 7 in Hepes buffer (20 mM).

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Table I. Affinity Constants and $\Delta G°$ Values of Different Anions for HSOD WT and Ile-143 Derivatives at pH 7.0

	K, M^{-1} ($\Delta G°$, kJ mol^{-1})			
	CN^-	N_3^-	NCO^-	P_i
WT	1700 ± 340 (−18.6)	94 ± 8 (−11.3)[a]	25 ± 2 (−8.0)[c]	34 ± 3 (−8.8)[d]
Ile-143	175 ● 35 (−12.9)	16 ± 1 (−6.9)[b]	$\leq 2 \times 10^{-2}$ (2.3)	0

[a] From ref 24. [b] From ref 8. [c] Value obtained for the bovine isoenzyme at pH 7.0. [d] From ref 10.

any spectral variation. The maximum NCO^- concentration used was 0.9 M. By taking the smallest measurable chemical shift difference equal to 0.2 ppm and assuming that the difference in shift between free and bound species is equal to that experienced by the bovine enzyme, we obtained an upper limit for the affinity constant of 2×10^{-2} M^{-1}.

Interaction with the Phosphate Anion. Paramagnetic effects on the ^{31}P NMR parameters of the phosphate anion caused by copper(II) ions in the native Cu_2Zn_2BSOD have been used to monitor the interaction of this anion with the protein.[10,17-19] From T_1 measurements, it was found that the affinity constants for phosphate binding to the native protein are 20 ± 4 and 34 ± 3 M^{-1} at pH 8.0 and 7.0, respectively, and that the copper(II)–phosphate distance is 5.3 Å.[10] Thus, it was suggested that the site of phosphate binding is Arg-143, which is known from X-ray data to be located approximately 5 Å from the copper center.[3]

We have repeated these measurements on the Ile-143 mutant. We have measured T_1 values of the ^{31}P NMR signal of a 0.26 mM Cu_2Zn_2HSOD Ile-143 sample at pH 7 at increasing amounts of phosphate. These values are comparable with those found with phosphate in absence of protein, under the same experimental conditions. The same results are obtained at a higher protein concentration (1.28 mM).

Interaction of the WT Enzyme with CN^- in the Presence of Phosphate. The EPR spectra of a Cu_2Zn_2HSOD WT solution 0.26 mM at pH 7 in presence of 0.11 M phosphate were measured with increasing amounts of CN^-. In the absence of CN^-, the EPR spectrum is rhombic and identical with that of Cu_2Zn_2HSOD[20] without phosphate. This confirms that phosphate binding does not perturb the copper(II) coordination geometry. The ^{31}P NMR T_1^{-1} data have a sizable paramagnetic enhancement, nearly identical with that found for the bovine enzyme under the same experimental conditions.[10] The EPR spectrum of the CN^- adduct is essentially identical with that of the Cu_2Zn_2HSOD–CN^- adduct in the absence of phosphate. ^{31}P NMR T_1 values are typical of free phosphate. The EPR spectra of the cyanide adduct are not modified even at higher phosphate concentration (0.72 M).

Discussion

The present results show that Arg-143 really plays a key role in the binding of anions and presumably in the catalytic cycle. In the absence of Arg-143, phosphate and NCO^- do not bind within the active cavity. The affinity constants of CN^- and N_3^- are decreased by a factor of 10 (Table I). It is difficult to factorize the entropic and the enthalpic contribution in order to account for the experimental data. It is reasonable to believe that there is an electrostatic stabilization of anions inside the cavity by the positively charged group and a hydrogen bond between the pseudohalide and the guanidyl group. The presence of two possible binding sites for anions, i.e. Arg-143 and the metal ion, may, at least partially, account for the different binding behaviors of NCS^- and N_3^- toward SOD found by Dooley.[21]

Regarding the phosphate ion, there has been a controversy as to whether it had an ionic strength effect on the activity[22] or had a specific inhibitory binding site.[7,10,19] The present results are in favor of the latter hypothesis though ionic strength effects show up through specific interactions. Finally, it is shown that when CN^- binds copper(II) and probably interacts via a hydrogen bond with Arg-143, the affinity of phosphate drops below detection.

The activity of the Ile-143 derivative is 1 order of magnitude lower than that of the WT.[9] It seems that the same mechanisms which reduce the affinities of anions also reduce the activity. This picture is also consistent with recent theoretical calculations.[23]

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Registry No. SOD, 9054-89-1; Arg, 74-79-3; Ile, 73-32-5; CN^-, 57-12-5; NCO^-, 661-20-1; P_i, 14265-44-2.

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