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Cyanide and Azide Behave in a Similar Fashion *Versus* Cuprozinc-Superoxide Dismutase*

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The ^{1}H NMR spectra of the cyanide adduct of Cu_2Co_2-superoxide dismutase have been remeasured at pH 7.5. The exchange rate of CN^- is slow on the NMR time scale. The correlation with the spectrum of the unligated enzyme has been established through saturation-transfer techniques of the system in which 50% of the cyanide adduct is formed and through comparison with the spectrum of a Cu_2Co_2-superoxide dismutase-CN^- sample in which the histidines have been deuterium labeled at the position ϵ_1. The similarities between the spectra of the CN^- and N_3^- derivatives are stressed, in particular with respect to the removal from copper coordination of the same histidine, assigned as His-46.

Cuprozinc-superoxide dismutase is a dimeric enzyme, each subunit containing a zinc(II) ion and a copper(II) ion bridged through a histidine ligand (1, 2). The zinc ion can be substituted by cobalt(II) without loss of activity (3, 4), and in the resulting derivative (Cu_2Co_2-superoxide dismutase hereafter) the two paramagnetic ions are antiferromagnetically coupled (5). As a result of such coupling the ^{1}H NMR spectra show sharp signals of the protons of the histidines bound to both cobalt and copper (6, 7). The assignment of the spectrum was proposed on the basis of 1) H_2O/D_2O experiments which show the signals of the histidine ring NH protons (7); 2) the analysis of T_1 and T_2 which led to the identification of the protons of the copper and cobalt domains (7)[1] and 3) ^{1}H nuclear Overhauser effect experiments and selective deuteriation[2] which led to the labeling of the sets of signals belonging to each histidine. We have, furthermore, investigated the behavior of the various anions (CN^-, N_3^-, NCO^-, NCS^-) (6, 7, 10)[1] *versus* the modified enzyme and we have shown that N_3^- binds copper causing a dramatic lengthening of the copper-N (His-46) distance in such a way that almost no isotropic shift is observed for the His-46 protons (7).[1] It is possible to follow the signals during the titration with N_3^- because the free-bound exchange rate for N_3^- is fast on the NMR time scale. In the case of CN^-, owing to the larger affinity constant (11), the exchange is slow on the NMR time scale and it is not therefore possible to follow the signals during the titration (10). Therefore the resulting spectrum may not be simply related to the spectra of the parent Cu,Co derivative. Nevertheless, with the aid of saturation-transfer techniques we proposed that both CN^- and N_3^- behave in a similar fashion at pH 5.5 (10). Simultaneously a paper appeared (12) which reported the ^{1}H NMR spectra of Cu_2Co_2-superoxide dismutase-CN at pH 7 claiming that CN^- and N_3^- behave in different ways as far as the coordination to copper was concerned. The claim was based on ill-resolved spectra measured at 80 MHz. Since CN^- has the same shape as the natural substrate O_2^-, the issue is very important. We have remeasured all the spectra at 200 MHz at pH 7, extended the saturation transfer experiments, and finally we prepared a sample in which the human superoxide dismutase expressed in *Escherichia coli* is deuterium labeled at the histidine positions ϵ_1.[2] Human su-

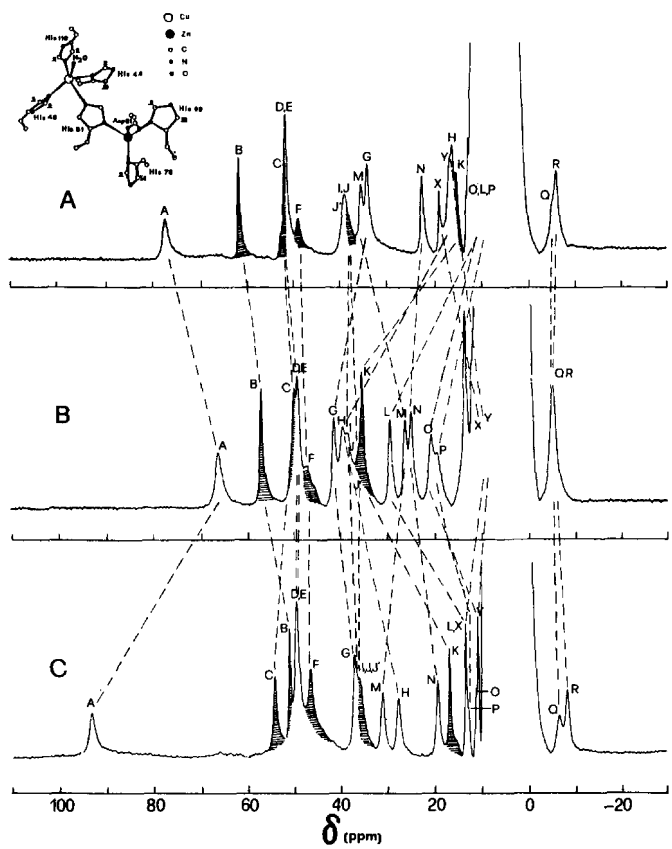

FIG. 1. **200 MHz ^{1}H NMR spectra of Cu_2Co_2-superoxide dismutase (*B*) and its cyanide (*A*) and azide (*C*) derivatives at 303 K.** The samples contain about 1 mM enzyme in 50 mM Hepes buffer at pH 7.5. The *shaded signals* disappear when the spectra are recorded in D_2O. The *dashed lines* connect signals due to the same proton in the three derivatives. *x* and *y* indicate protons which are unresolved in the diamagnetic region of the spectrum of Cu_2Co_2-superoxide dismutase (*B*) and move downfield upon addition of cyanide or azide.

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[1] L. Banci, I. Bertini, C. Luchinat, and M. S. Viezzoli, submitted for publication.

[2] L. Banci, I. Bertini, C. Luchinat, M. Piccioli, A. Scozzafava, and P. Turans, submitted for publication.

TABLE I

200 MHz ^1H NMR parameters for Cu_2Co_2-superoxide dismutase and its azide and cyanide adducts at 303 K

Signal	Assignment	Shift (ppm) (T_1, ms)		
		Cu_2Co_2-superoxide dismutase[a,b]	Cu_2Co_2-superoxide dismutase + N_3^-[c]	Cu_2Co_2-superoxide dismutase + CN^-[d]
A	His-61 Hδ2[a]	66.2 (1.1)	95.1 (<1)	76.6 (1.6)
B	His-118 Hδ1[a]	56.5 (4.1)	51.2 (5.8)	60.2 (4.9)
C	His-44 Hε2[a]	50.3 (–)	55.0 (1.4)	51.1 (2.6)
D	His-78 Hδ2 (His-69 Hδ2)[b]	9.4 (3.1)	49.7 (1.9)	50.1 (3.5)
E	His-69 Hδ2 (His-78 Hδ2)[b]	48.8 (3.1)	50.1 (1.9)	50.1 (3.5)
F	His-78 Hε2 (His-69 Hε2)[b]	46.7 (–)	46.9 (1.8)	47.2 (2.6)
G	His-44 Hδ2[a]	40.6 (2.8)	37.3 (2.9)	32.1 (4.0)
H	His-118 Hε1[a]	39.0 (1.7)	27.3 (2.3)	14.2 (2.9)
I	Asp-81 Hβ1 (Asp-81 Hβ2)[b]	37.4 (1.4)	36.3 (–)	37.3 (1.8)[e]
J′	Asp-81 Hβ2 (Asp-81 Hβ1)[b]	35.6 (1.6)	35.7 (–)	37.3 (1.8)[e]
J	His-69 Hε2 (His-78 Hε2)[b]	35.4 (–)	35.7 (–)	37.3 (1.8)[e]
K	His-46 Hδ1[a]	34.5 (4.5)	15.9 (19.1)	13.0 (–)
L	His-46 Hδ2[a]	28.4 (4.2)	12.5 (10.9)	9.1 (–)
M	His-44 Hε1[a]	25.3 (2.5)	31.4 (2.3)	33.7 (1.8)
N	His-118 Hδ2[a]	24.1 (2.5)	18.9 (2.9)	20.4 (3.2)
O	His-46 Hε1[a]	19.6 (2.4)	10.5 (4.5)	<11 (–)
P	His-44 Hβ1[a]	18.7 (1.2)	11.6 (8.7)	<11 (–)
x			13.1 (–)	16.8 (–)
y			10.4 (–)	14.4 (–)
Q	His-44 Hβ2-(–)[a]	–6.2 (2.2)	–9.2 (–)	–8.7 (–)
R	(–)-(His-44 Hβ2)[a]	–6.2 (2.2)	–7.4 (–)	–8.7 (–)

[a] Footnote 1.
[b] Ref. 7 and Footnote 2.
[c] Ref. 6.
[d] Ref. 10 and present work.
[e] Individual T_1 values cannot be resolved.

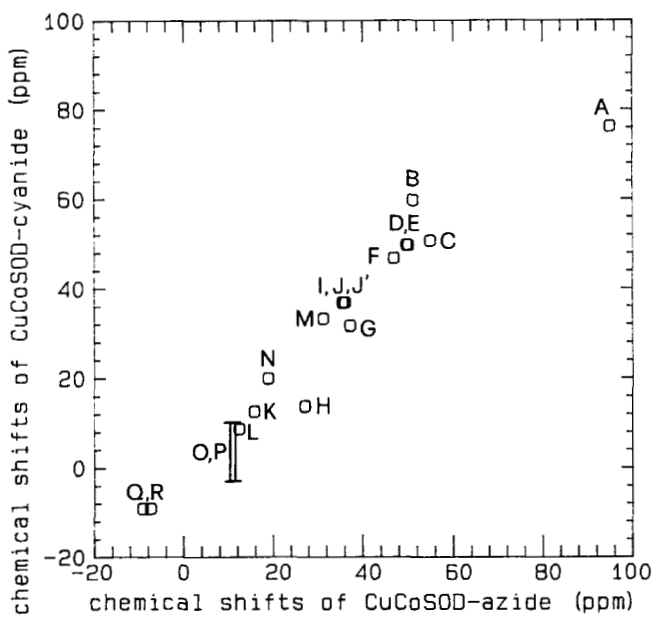

FIG. 2. **Correlation between the shift values of the cyanide and azide derivatives of Cu_2Co_2-superoxide dismutase.** Experimental conditions are as described in the legend to Fig. 1. The *O* and *P* signals in the CN^- adduct lies in the diamagnetic envelope.

peroxide dismutase has a ^1H NMR spectrum nearly identical to that of the bovine isoenzyme (13). With this experiment we have reached a complete assignment of the spectrum of the CN^- derivative which nicely compares with that of N_3^-.

MATERIALS AND METHODS

Chemicals and Solutions Preparation—Bovine liver superoxide dismutase was purchased from Diagnostic Data Inc., Mountain View, CA. The Cu_2Co_2-superoxide dismutase derivative was prepared using previously reported methodology (14, 15). Cu_2Co_2-superoxide dismutase solutions were prepared in 50 mM Hepes[3] buffer at pH 7.5. In order to remove the exchangeable protons the apoprotein was lyophilized, dissolved in D_2O, lyophilized again, and then dissolved in 1 ml of D_2O buffered with 50 mM acetate at pH 5.5 prior to reconstitution with the appropriate metal ions. Human superoxide dismutase was detained from *E. coli* and checked by comparing the spectra of the Cu/Co derivative with those of the human erytrocyte superoxide dismutase (13). The spectra were absolutely identical.[2] Samples of human superoxide dismutase with histidines deuterium labeled at position ϵ_1 were obtained as described elsewhere.[2]

NMR Measurements—The ^1H NMR spectra were obtained on a Bruker MSL 200 spectrometer by using the modified-DEFT pulse sequence (90-τ-180-τ-90-AQ) (9) in order to suppress H_2O and bulk protein signals. Irradiation of selected signals for saturation transfer experiments was performed during the τ interval following the 180° pulse of the modified-DEFT sequence.

RESULTS AND DISCUSSION

The spectrum of the CN^- derivative of Cu_2Co_2-superoxide dismutase at 200 MHz and pH 7 is shown in Fig. 1*A* together with the spectra of the unligated (1*B*) and N_3^- (1*C*) derivatives at the same pH. The assignment of the signals for the unligated enzyme (Table I) has been previously proposed (7).[1] The correlation between the signals of the unligated enzyme and the N_3^- adduct can be easily obtained because the exchange rate of N_3^- is fast on the NMR time scale and at any stage of the titration the signal position depends on the molar fraction of bound enzyme. In the case of cyanide, the assignment is again a difficult task. The assignment of signals C, F, J, and K as histidine ring NH protons has been obtained by comparing the spectra obtained in H_2O and D_2O. The assignment of signals B, M, L, and N has been independently established by saturation transfer techniques on the 50% bound system. Signals M and H have been shown to be ϵ_1 protons because they are not present in the deuterium-labeled

[3] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEFT, driven equilibrium Fourier transform.

protein. Therefore it has been possible to connect (*dashed lines* in Fig. 1) the resonances of the unligated enzyme to those of the cyanide complex. The ϵ_1 signals of the histidines coordinated to cobalt are not observed in any derivative because they are too broad.[2] The ϵ_1 signal of His-46 (signal O) is presumably below the envelope of the diamagnetic part of the protein. Signal P, assigned as H-β_1 of His-44, is also lacking in the cyanide adduct and therefore is likely to be below the diamagnetic region of the spectrum. The shift and T_1 values for the isotropically shifted proton signals of the unliganded enzyme and of the two anion derivatives are reported in Table I.

It is apparent that signals K, L, and O of His-46 move towards the diamagnetic position upon cyanide binding as they do upon azide binding. The movement is more pronounced in the case of cyanide. This is consistent with the general trend according to which the larger the affinity constant of the anion the larger the shift variation of the signals of the detaching histidine (6).

Small differences between azide and cyanide spectra are evident in the downfield region: signals B and C do not cross upon binding of cyanide and signals G and H, assigned to H-ϵ_2 of His-44 and H-ϵ_1 of His-118, respectively, experience larger upfield shift variations. Since the technique is very sensitive and the observed shifts are a fine balance of several contributions, due to both spin delocalization mechanisms and dipolar effects, these minor variations in the shifts are not easily related to structural changes. On the other hand, the T_1 values, which are related to the metal-proton distances, have similar patterns consistently with the proposed similar overall structures for the azide and cyanide complexes.

In Fig. 2 the shifts of the resonances of cyanide and azide adducts are plotted in such a way as to pinpoint the strong correlation between the two sets of signals. The deviations of the shifts for the two anion derivatives from the diagonal line gives an idea of the different behavior of the two adducts. Only signal H seems to be significantly off diagonal. Most importantly, and contrary to the conclusions in the report (12) which stimulated this research, in the cyanide derivative the resonances of His-46 undergo the changes towards the diamagnetic position expected if this residue does not feel any paramagnetic contribution any longer, as in the N_3^- case.

The new 200 MHz data at pH 7.5 presented here confirm our previous interpretation of data taken at pH 5.5, that both azide and cyanide cause the detachment of one and the same histidine as a ligand to the copper ion in Cu_2Co_2-superoxide dismutase. The present conclusions are consistent with a recent extended x-ray absorption fine structure study performed on the cyanide and azide adducts of the enzyme, suggesting that the anions bind in an equatorial position pushing away a coordinated histidine (8).

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