A Multinuclear Ligand NMR Investigation of Cyanide, Cyanate, and Thiocyanate Binding to Zinc and Cobalt Carbonic Anhydrase

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Received December 2, 1991

Relaxation rate measurements on ^{13}C-enriched NCO^-, NCS^-, and CN^- as well as on ^{15}N-enriched NCS^- have been performed in presence of cobalt(II)-substituted carbonic anhydrase (CA). It is shown on firm basis that NCO^- and NCS^- do bind cobalt although with different geometries. The data of the NCS^- case are consistent with the available X-ray data. No information could be obtained on CN^- because of slow exchange between its free and enzyme-bound forms. ^{13}C relaxation measurements have been performed on $^{13}C^{15}N^-$ in the presence of $^{67}ZnCA$. The large quadrupolar moment of $^{67}Zn(II)$ induces relaxation on ^{13}C, thus showing that there is direct coordination. NMR spectroscopy provides strong important pieces of information on the present debate on the way of binding of anions to carbonic anhydrase.

Introduction

Carbonic anhydrase (CA) is a zinc enzyme of MW 30 000 which catalyzes the reversible hydration of CO_2 to bicarbonate with an extremely high catalytic turnover.[1] Many anions are known to inhibit the enzyme, and it has been proposed that in most of these cases inhibition takes place by a direct binding of the ligand to the metal ion.[2-4]

CA possesses a cavity 15 Å deep, at the bottom of which there is the zinc ion coordinated to three histidyl residues, His 94, His 96, and His 119, and to an apical water molecule, with an approximately tetrahedral geometry (see Figure 1, numbering according to the human II isoenzyme).[5,6] When inhibitors bind the zinc ion they may displace the water molecule or bind to a further coordination site.[7] In the latter case the water molecule is expected to change position in order to provide more room for the fifth ligand.

Unfortunately, the zinc ion is diamagnetic, does not present d–d electronic transitions, and has little nuclear magnetic moment as ^{67}Zn. Therefore the spectroscopic study of the native enzyme has been historically limited. However, the zinc can be removed by using appropriate chelating agents, furnishing an inactive apoenzyme.[8] Several divalent cations can be added to this enzyme, like Cd(II), Co(II), Mn(II), and Cu(II), but only the Co(II) derivative provides a derivative whose activity is sizable with respect to that of the native enzyme.[9] The cobalt(II) in the enzyme is high spin ($S = {}^3/_2$) and suitable for electronic absorption studies[10-13] and for ligand NMR studies based on the nuclear relaxation rates enhancement due to the interaction between the resonant nuclei and the paramagnetic center.[13,14] For this reason, studies on Co(II)-substituted CA have been of interest to shed light on the enzyme structure and catalytic mechanism.

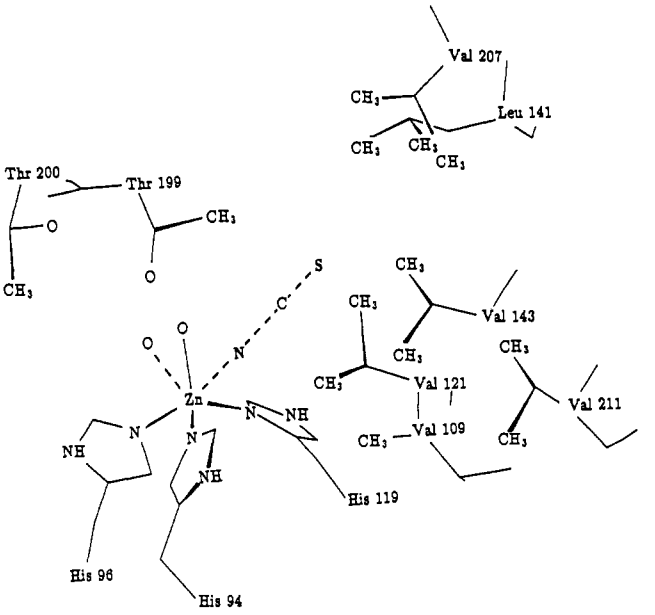

Figure 1. Schematic representation of the active site of carbonic anhydrase[6] and of the carbonic anhydrase–NCS^- adduct.[15] The dashed lines show the orientation of the bound NCS^- and of the tilted solvent molecule in the adduct.

By means of electronic and ^1H NMR spectra of Co(II) BCA II (where B stands for bovine), it had been suggested that the inhibitors NCO^- and CN^- substitute the water molecule whereas NCS^- coordinates to a fifth coordination binding site.[7,14] Eventually, the X-ray structure confirmed that the NCS^- derivative is five-coordinated,[15] with nitrogen as the donor atom and with sulfur on a hydrophobic pocket of the cavity (see Figure 1). X-ray structures showed that also sulfonamides bind the metal

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0020-1669/92/1331-3975$03.00/0

substituting the coordinated water molecule,[16] as previously suggested on a spectroscopic basis.[7]

Recent crystallographic studies on human Zn(II) HCA II whose crystals had been soaked in CN^-- and NCO^--containing solutions showed that the ligands are located between the NH of Thr199 and zinc with the closest atom of the exogenous ligand at 3.3 and 3.2 Å, respectively, from the zinc ion.[17] These extraordinary results prompted us to undertake a further and decisive spectroscopic investigation of the interaction of NCO^- and NCS^- with the enzyme. The first approach has been that of investigating the properties of the ^{13}C nuclei of the ligands in the presence of the cobalt-substituted enzyme. Paramagnetic heteronuclear relaxation measurements of ligands and substrates interacting with metal-substituted CA provide a unique method of elucidating structural details in solution.[18-20] If the ligands exchange rapidly between their free and enzyme-bound forms, the NMR signal can be observed and its nuclear relaxation rate contains information on the nucleus–cobalt distance.[21,22] Studies on ^{15}N and ^{13}C of NCS^- have also been performed for comparative purposes. In the case of cyanide, the ligand exchange has been found to be slow and no ^{13}C signal could be observed. However, the signal has been observed for the diamagnetic ^{67}Zn derivative. ^{67}Zn has $I = {}^5/_2$ and a large quadrupolar moment ($Q = 0.15 \times 10^{-24}$ cm^2) which is responsible for large relaxation rates. If cyanide coordinates to ^{67}Zn, it is possible that the large zinc relaxation rate induces relaxation on the coordinated ^{13}C nucleus. The comparison between the ^{13}C cyanide relaxation rate in the presence of $^{67}ZnCA$ and $^{64}ZnCA$, ^{64}Zn having no magnetic moment, may reveal, and indeed does, direct ^{13}C–Zn interactions. In both cobalt and ^{67}Zn cases, analogous studies have been performed on model complexes in order to test the quantitative meaning of the results.

Experimental Section

Bovine carbonic anhydrase II (BCA II) was purchased from Sigma Chemical Co. and used without further purification. All reagents used were of analytical grade. $K^{13}C^{15}N$ was purchased from ISOTEC Inc., while $K^{13}CN$, $KN^{13}CO$, $Na^{15}NCS$, and $NaN^{13}CS$ were purchased from Prochem BCO Ltd. ^{64}ZnO and ^{67}ZnO were purchased from Oak Ridge National Laboratory.

Zinc(II) ions were removed from carbonic anhydrase by dialysis against solutions of pyridine-2,6-dicarboxylic acid (5×10^{-2} M) in phosphate buffer (2×10^{-1} M) at pH 6.9.[23] The cobalt(II) addition was performed by titration of the apoenzyme with a $CoSO_4$ solution followed spectrophotometrically. The zinc oxides were dissolved in hydrochloric acid and the ^{64}Zn(II) and ^{67}Zn(II) cations were added to the apoenzyme as chloride solutions. In all cases enzyme concentrations were determined from the absorbance at 280 nm, using an ϵ of 57 000 cm^{-1} M^{-1}.[24] The electronic spectra were recorded on a Cary 17 spectrophotometer. Protein solutions were kept in 15 mM Hepes buffer at pH 7.5, except when indicated. The anion solutions were also in Hepes buffer at pH 7.5. The ^{13}C NMR measurements on ^{64}Zn and ^{67}Zn–$^{13}C^{15}N$ were made on 7.4 mM solutions of the enzyme in 50 mM Hepes at pH 8.3. The cobalt(II) and zinc(II) complexes of 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane (tetramethylcyclam, TMC) were prepared according to the procedure reported in the literature[25] and used as chloride salts. Viscosity values for the water–glycerol mixtures were taken from standard tables,[26] and the correlational time τ_r of these solutions was calculated by using the Stokes–Einstein equation $\tau_r = 4\pi\eta r^3/3kT$, where η is the solvent viscosity, r is the solute molecular radius, k is the Boltzmann constant, and T is the absolute temperature.

^{13}C NMR measurements were performed on Bruker MSL 200, ACP 300, and AMX 600 instruments at 50.33, 75.47, and 150.90 MHz. ^{15}N measurements were performed on an ACP 300 Bruker spectrometer at 30.42 MHz. FIDs were multiplied by an exponential function with line broadenings between 2 and 3 Hz. Longitudinal relaxation times T_1 were measured by the inversion recovery method[27] and the data fitted by using an appropriate nonlinear least-squares fitting program. Transverse relaxation times T_2 were calculated from the line width at half-height by the relation $T_2^{-1} = \pi\Delta\nu$.

Table I. Effect of the Addition of Substoichiometric Amounts of Co(II) BCA II on the Nuclear Relaxation Times of NCS^-, NCO^-, and CN^-

	T_1 (s)[a]	T_2 (ms)	$\Delta\nu$ (Hz)	T_{1M}^{-1} (s^{-1})
$N^{13}CS^-$	7.12	22.2	14.3	
$N^{13}CS^-$ + Co(II) BCA II[b]				
300 K	0.12	2.2	146.9	819
305 K	0.14	1.5	215.5	700
$^{15}NCS^-$	7.33	24.5	13.0	
$^{15}NCS^-$ + Co(II) BCA II[c]				
300 K	0.33	11.7	27.0	2894
305 K	0.35	11.0	28.0	2721
$N^{13}CO^-$	8.66	9.8	32.4	
$N^{13}CO^-$ + Co(II) BCA II[b]				
300 K	0.014	2.4	133.5	7130
305 K	0.012	1.9	170.7	8313
$^{13}CN^-$	32.0	5.6	57.0	
$^{13}CN^-$ + Co(II) BCA II[b]				
300 K	35.0	5.9	54.0	
305 K	33.0	6.1	52.0	

[a] Measured with an error of ±10%. [b] Anion concentration 15 mM; enzyme concentration 0.15 mM. [c] Anion concentration 100 mM; enzyme concentration 0.1 mM.

Results and Discussion

Ligand Binding to Co(II) BCA II. Co(II) BCA II was added in small quantities to buffered solutions (pH 7.5) of the following isotopically enriched anions: $N^{13}CS^-$, $^{15}NCS^-$, $N^{13}CO^-$, and $^{13}CN^-$, and the effect on T_1 and T_2 of the ligand nuclei was measured. The experimental T_1 values are the sum of a paramagnetic and a diamagnetic contribution:

$$T_1^{-1} = T_{1p}^{-1} + T_{1d}^{-1} \quad (1)$$

The latter has been determined through measurements on the native enzyme. On its turn, T_{1p}^{-1} is given by

$$T_{1p}^{-1} = f(\tau_M + T_{1M})^{-1} \quad (2)$$

where f is the molar fraction of bound ligand, τ_M is the exchange time, and T_{1M} is the relaxation time of the investigated nucleus in the compound.

T_{2p} is defined in a way analogous to T_{1p}; however, it depends on T_{2M}, τ_M, and the difference in shift between free and bound ligand.[21]

The addition of 1% of the stoichiometric quantity of Co(II) BCA II to a 15 mM solution of $N^{13}CS^-$ induced a drastic decrease of T_1 of the carbon nucleus, as well as a substantial broadening of the signal (Table I). The line width increased with increasing temperature, in a behavior which clearly indicates a semislow exchange between free and bound forms.[21] On the other hand, the T_{1p}^{-1} values decreased with increasing temperature, indicating fast exchange conditions. This behavior is not unexpected, since T_{2p}^{-1} usually experiences a strong contribution from the chemical shift difference between free and bound ligand.[21] The further

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Table II. Effect of the Addition of Substoichiometric Amounts of Co(II) TMC on the Nuclear Relaxation Times of NCS^-, NCO^-, and CN^-

	T_1 (s)[a]	T_2 (ms)	$\Delta\nu$ (Hz)	T_{1M}^{-1} (s^{-1})[b]
$N^{13}CS^-$	8.00	21.2	15.0	
$N^{13}CS^-$ + Co(II) TMC[c]				
298 K	0.27	1.6	200.0	365
303 K	0.29	1.8	180.0	
$N^{13}CO^-$	10.30	9.6	33.0	
$N^{13}CO^-$ + Co(II) TMC[d]				
298 K	0.14	0.6	500.0	364
303 K	0.14	0.7	470.0	
$^{13}CN^-$	9.07	18.7	17.0	
$^{13}CN^-$ + Co(II) TMC[c]				
298 K	8.60	14.5	22.0	

[a] Measured with an error of ±10%. [b] Calculated by using the association constants for the ligands reported in Ref 36. [c] Anion concentration 50 mM; enzyme concentration 0.5 mM. [d] Anion concentration 25 mM; enzyme concentration 0.5 mM.

addition of 9% Co(II) BCA II produced such a large effect on the line width that the carbon signal could not be detected any longer.

On the other hand, a similar experiment was performed on a 0.1 M solution of ^{15}N-enriched thiocyanate, but with the addition of $^1/_{1000}$th of the stoichiometric quantity of Co(II) BCA II. A behavior of the nuclear relaxation rates analogous to that in the case of ^{13}C was observed upon the addition of the enzyme, i.e., a decrease of T_1 and a line broadening. The temperature dependence of T_{1p}^{-1} and T_{2p}^{-1} was analogous to that observed for ^{13}C (Table I). It should be noted that the effect on the nitrogen signal is larger than that on the ^{13}C resonance, as will be discussed later.

When Co(II) BCA II is added in a 1:100 ratio to a 15 mM solution of ^{13}C-enriched cyanate, a larger effect than that observed on $N^{13}CS^-$ is observed for the carbon signal. In this case, T_{1p}^{-1} increases with increasing temperature, indicating a quasi-slow exchange also for T_{1p}, consistent with a stronger paramagnetic effect. Therefore, the T_{1p}^{-1} value was taken as a lower limit for calculating T_{1M}^{-1} (see eq 2 with τ_M not negligible).

A 1% addition of Co(II) BCA II to a 15 mM solution of $^{13}CN^-$ showed that the ligand is under slow exchange, because the line width is almost unaffected by the addition of the enzyme, and the same is found for fT_1^{-1}.

In order to have a calibration of these measurements, we performed also a series of similar experiments upon the Co(II) complex with TMC. The substoichiometric addition of the complex Co(II) TMC to solutions of the anions gave meaningful results. The ^{13}C cyanide resonance did not broaden upon the addition of the complex (like in Co(II) BCA II), and its T_1 did not change, consistent with slow-exchange conditions. On the other hand, both NCO^- and NCS^- ^{13}C signals were significantly broadened, and their T_1 diminished a considerable amount, as shown in Table II. In this case it should be pointed out that the calculated T_{1M}^{-1} values for the ^{13}C signal of NCO^- and NCS^- are similar.

Theoretical Approach and Interpretation of the Data. The nuclear longitudinal relaxation rate enhancements are due to the coupling between the resonating nuclei and the unpaired electrons. Such coupling can be dipolar and contact in origin. We analyze here the experimental results by assuming that the coupling is entirely dipolar in nature, because our interest is focused on the determination of whether anions are bound or not. In this way, if there is no binding, the estimate of the distance between the metal and the resonating nucleus is not biased. If there is binding, the estimated distance is always shorter than the actual one, because T_1^{-1} is larger as other contributions due to the chemical bond are operative. Such contributions are contact in origin and dipolar for that part of unpaired electrons which delocalize onto the ligand.[21,28] The equation for the electron–nucleus dipolar coupling is[29,30]

$$T_{1M}^{-1} = \frac{2}{15}\left[\frac{\mu_0}{4\pi}\right]^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left[\frac{7\tau_c}{1+\omega_S^2\tau_c^2} + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \quad (3)$$

where μ_0 is the permeability of vacuum, r is the distance between the observed nucleus and the cobalt(II) ion, γ_N is the nuclear magnetogyric ratio, g_e is the electron g factor, μ_B is the Bohr magneton, S is the spin quantum number, τ_c is the correlation time, and ω_I and ω_S are the nuclear and electronic Larmor frequencies.

This equation holds in the absence of zero field splitting. Its effect, however, has been estimated to affect the distance by ±10%.[31]

For the NCS^- derivative, an estimate of τ_s is available from water proton relaxation measurements, which is 5.6×10^{-12} s.[32] This provides a metal–carbon distance of 2.32 Å through eq 3. This value indicates that NCS^- is bound, in agreement with previous spectroscopic studies[7,14] and X-ray determination.[15] Note that the paramagnetic effect on T_{1p}^{-1} extrapolated to the fully bound ligand (T_{1M}^{-1}) was more than 3 times larger for the ^{15}N signal than in the ^{13}C resonance, despite the smaller magnetogyric ratio of ^{15}N (see eq 3). This is expected, however, in the case of metal coordination through the nitrogen atom: a closer location of the nitrogen atom with respect to the carbon should be reflected in a larger paramagnetic effect, since it depends on r^{-6}. Through eq 3, a metal–nitrogen distance of 1.38 Å is found. For both nuclei the estimated distance is shorter than expected, for the reasons explained above.

By using the T_{1M}^{-1} for ^{13}C of NCO^-, which is only a lower limit and yet much larger than that in the NCS^- case, and the same τ_s value, an unreasonably short distance (1.62 Å) is obtained. Independent measurements[32] on this derivative had indicated a τ_s of 3.1×10^{-11} s. The difference between the NCS^- and NCO^- derivatives had been accounted for on the basis of the different coordination number and on the availability of low-lying excited states in five-coordinated complexes which would provide efficient electron relaxation pathways.[33,34] With such a value of τ_s a cobalt–carbon distance of 2.15 Å is estimated, which is similar to that obtained for thiocyanate. Therefore, we consider this experiment as proof of direct binding of cyanate to the metal ion.

In order to avoid possible artifacts, we performed a series of similar experiments upon Co(II) TMC (see Experimental Section). This is a square-pyramidal complex in which four nitrogen atoms are coordinated to Co(II) and a water molecule is in the apical position.[25,35,36] The geometry of this ligand is such to prevent the approach of a sixth coordinating molecule (see Figure 2). Therefore, the adducts with CN^-, NCO^-, and NCS^- will be all pentacoordinated, as it was already demonstrated.[36]

Like in Co(II) BCA II, CN^- was shown to be in slow exchange while NCS^- and NCO^- are in a fast exchange. In this case, the calculated ^{13}C T_{1M}^{-1} values are almost the same for NCS^- and NCO^- (365 and 364 s^{-1}, respectively) and similar to that of Co(II) BCA II–NCS^-, as expected since all adducts are pentaco-

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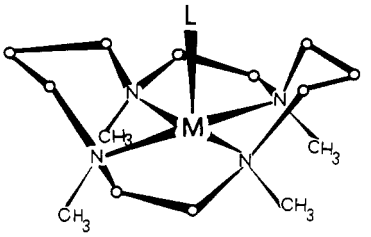

Figure 2. Preferred conformation of the metal–TMC complexes, where M (metal) may be in this case zinc(II) or cobalt(II) and L (ligand) = H_2O, CN^-, NCS^-, or NCO^-.

ordinated. Taking the τ_s value of 2.6×10^{-12} s measured for Co(II) TMC (which is again similar to that of Co(II) BCA II-NCS^-),[35] a Co–C distance of 2.22 Å is calculated, in agreement with the values obtained for Co(II) BCA II. This is the final proof of the soundness of the present analysis.

Cyanide Binding to Zn(II) BCA II. The slow exchange of cyanide did not allow us to obtain information on the interaction of the ligand with cobalt carbonic anhydrase. We therefore decided to study the diamagnetic Zn–^{13}CN derivative. Studies on this system are available[37] which showed that the ^{13}C signal of cyanide experienced a downfield shift of 29.1 ppm with respect to free cyanide at pH 7.5, and in a position close to that of $Zn(CN)_4^{2-}$. This was taken as indicative of direct binding of cyanide. The final proof could come from an experiment which is sensitive to the nuclear–nuclear J coupling. So, we have used ^{67}Zn as an NMR probe. The large quadrupolar moment of this nucleus provides an efficient relaxation mechanism for the zinc nucleus according to the following equation:[38]

$$\frac{1}{T_{1q}} = \frac{3\pi^2(2I+3)}{10I^2(2I-1)}\frac{e^2q_{zz}Q}{h}\left[1+\frac{\eta^2}{3}\right]\tau_r \qquad (4)$$

where e is the electron charge, q_{zz} is the biggest component of the electric field gradient at the nucleus, Q is the nuclear quadrupole moment, η is the asymmetry parameter, I is the nuclear spin, and τ_r is the rotational correlation time.

The ^{67}Zn nucleus has a low sensitivity;[39,40] furthermore, with the τ_r of CA, which is estimated around 10^{-8} s, we expect a very short nuclear relaxation of the order of 10^{-7} s for a large range of q_{zz} values. This value, together with the low sensitivity of the nucleus, does not allow detection of the signal and measurement of relaxation times.

The ^{13}C nucleus may relax through J coupling with ^{67}Zn.[38,41,42] The ^{13}C transverse nuclear relaxation rate, in the motional narrowing limit, is expected to be[38]

$$T_2^{-1} = (8/3)\pi^2J^2S(S+1)T_{1q} \qquad (5)$$

where J is the ^{13}C–^{67}Zn scalar coupling constant, S is the nuclear spin of ^{13}C, and T_{1q} is the longitudinal relaxation time of ^{67}Zn as given by eq 4.

Therefore, owing to the short nuclear relaxation times of ^{67}Zn in the protein, only a small line broadening is expected from the ^{67}Zn–^{13}C interaction. As a final device, we have thus used $^{13}C^{15}N$-enriched cyanide. In fact, ^{14}N is also a quadrupolar nucleus, and in order to perform reliable measurements we tried to avoid ^{13}C relaxation pathways which could come from the nuclear relaxation of ^{14}N. Indeed, experiments with $^{13}C^{14}N^-$ were unsuccessful.

Figure 3 shows that the ^{13}C line width in the presence of ^{67}Zn CA at 330 K is sizably larger (74 Hz) than the line width measured with the magnetically inactive ^{64}Zn CA (40 Hz). At lower temperature, with a larger rotational correlation time τ_r, a shorter ^{67}Zn T_1 value is expected (see eq 4), which has a smaller line-broadening effect on the ^{13}C line (see eq 5). Indeed, no appreciable difference in line width is observed at 295 K (Figure 4).

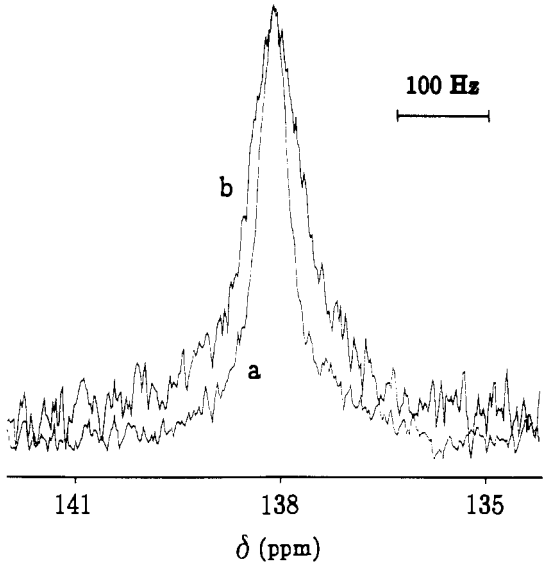

Figure 3. Cyanide resonance in the ^{13}C NMR spectra at 75.42 MHz of solutions of (a) ^{64}Zn(II) BCA II–$^{13}C^{15}N$ and (b) ^{67}Zn(II) BCA II–$^{13}C^{15}N$, both performed at 330 K. The protein concentration is 7.4 mM, in buffer Hepes 50 mM at pH 8.3.

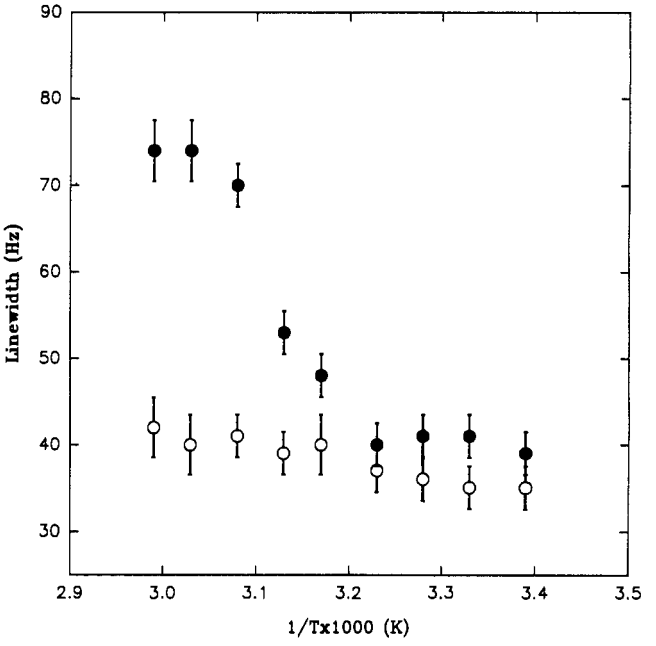

Figure 4. Temperature dependence for the ^{13}C line width of the ^{64}Zn(II) BCA II–$^{13}C^{15}N$ (○) and ^{67}Zn(II) BCA II–$^{13}C^{15}N$ (●) adducts under the same conditions as specified in Figure 3.

This experiment has proven that cyanide indeed interacts with zinc, though we do not have any semiquantitative estimate. For this reason, an analogous experiment has been performed on ^{67}Zn TMC and ^{64}Zn TMC and the rotational correlation time of the complexes has been varied by using water–glycerol solutions. The effect on the ^{13}C line width of $^{13}C^{15}N$ cyanide as a function of τ_r (as calculated from the Stokes–Einstein equation) is shown in Figure 5. It appears that for τ_r values of the order of the protein at 295 K (1.7×10^{-8} s) the ^{13}C line broadening is small and comparable to that found in CA. The increase in line width observed in CA solutions at shorter τ_r values (8.4×10^{-9} s, estimated at 330 K) is also observed in the model system, although to a smaller extent (Figure 5). We can thus conclude that cyanide

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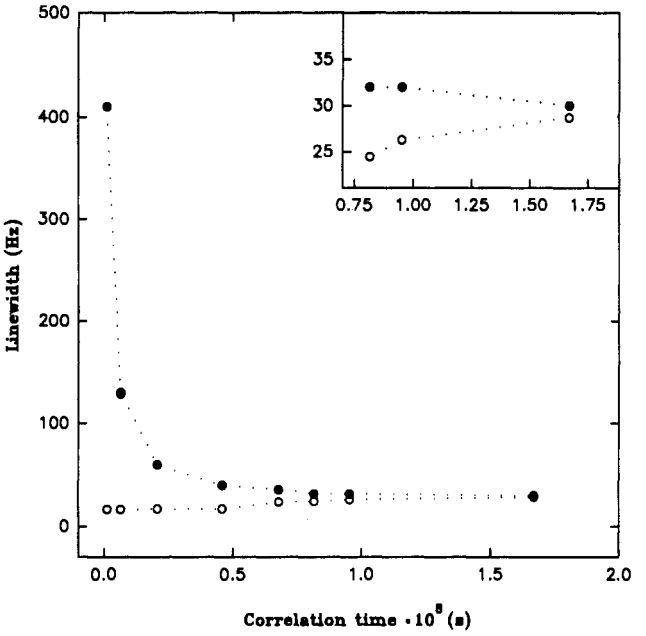

Figure 5. Dependence of the line width of the cyanide ^{13}C resonance versus correlation time of $^{64}Zn(II)$ TMC–$^{13}C^{15}N$ (○) and $^{67}Zn(II)$ TMC–$^{13}C^{15}N$ (●) in water–glycerol mixtures at 50.42 MHz. The complex concentrations were 15 mM, in 50 mM Hepes buffer at pH 8.3. An expanded region is shown on the upper right corner.

is bound to zinc in the enzyme, though we do not have a direct estimate of ^{67}Zn T_1 and of J.

Conclusions

The present data indicate that CN^- and NCO^- do bind the metal ion in BCA II in solution. The strict similarity between the bovine and the human isoenzyme II, demonstrated by a large variety of experimental evidence,[3,4,43] suggests that the difference with respect to the recent X-ray data is not due to the different isoenzyme studied.

One of the major items on the anion binding in CA is what are the driving forces for the observed variety of coordinations.[14] It seems that, while the ligand nitrogen binds to zinc, the soft sulfur atom of NCS^- interacts with the hydrophobic residues which are displayed in Figure 1.[7,14] The orientation of anions toward this hydrophobic site causes the coordinated water to be retained, although tilted from the original position.[15]

When the X-ray structure of the sulfonamide adduct[16] was solved, it appeared that the NH group could substitute the hydroxide coordinated to zinc. This moiety is bridging zinc and the oxygen of Thr 199. From a thermodynamic point of view, this behavior fits with the general scheme of ligand binding in CA, according to which anions would only bind the low-pH aquo form. Indeed, anions of weak acids could be viewed as binding as undissociated molecules to the high-pH hydroxo form of the enzyme. In this picture, an ionized sulfonamide should substitute the coordinated hydroxide.

The problem arises for anions which, unlike thiocyanate, would rather remove water than bind to the fifth coordination position in the hydrophobic pocket. The present results strongly point out the formation of pseudotetrahedral adducts with removal of water for both NCO^- and CN^-. Of course, in this case, the favorable hydrogen bond net involving Thr 199 present in both the native hydroxo form and in the sulfonamide derivative is lost. The X-ray picture, on the other hand, preserves this bond at the expense of the coordinative bond to the zinc ion. The point is thus to reconcile the X-ray data with the spectroscopic data. We believe that what we have found is a faithful picture of the behavior of the enzyme in solution. We also believe that the X-ray pictures may correspond to other free energy minima accessible to the system. The possibility of choosing one minimum or another must be strictly dependent on the experimental conditions. From this point of view the X-ray data are extremely instructive about the possible interaction regions for small molecules in the cavity, including the elusive CO_2 substrate.

CA may thus turn out to be one of the few examples of a remarkable difference between solution and solid-state behavior. The difference here is not a more or less small displacement of amino acid side chains but is the selection of one or another markedly different binding sites for the inhibitor molecules.[44]

With this in mind, X-ray data on the human isoenzyme I, where the low pH form is stable up to more than one pH unit higher than the human II and bovine enzyme, would be highly desirable. Spectroscopy has already accumulated overwhelming evidence also for this isoenzyme that anion binding is strictly analogous to that observed in isoenzymes II.[3,4,43]

Acknowledgment. Thanks are expressed to Prof. A. Liljas for kindly sharing with us his results (prior to acceptance), as well as to Prof. S. Lindskog and Prof. H. B. Gray for helpful discussions. R.P. is grateful to Bruker Spectrospin Italiana srl for a research fellowship. A.J.V. thanks the International Centre for Genetic Engineering and Biotechnology (UNIDO) for a fellowship, as well as the Fundación Antorchas for further support.

Registry No. CA, 9001-03-0; Zn, 7440-66-6; CN^-, 57-12-5; NCO^-, 661-20-1; NCS^-, 302-04-5.

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