

Cadmium-113 NMR of carbonic anhydrases: Effect of pH, bicarbonate, and cyanide

(zinc hydroxide model/inner sphere/rapid exchange)

NALLE B.-H. JONSSON, LENA A. E. TIBELL, JEFFREY L. EVELHOCH, STUART J. BELL, AND JAMES L. SUDMEIER

Department of Chemistry, University of California, Riverside, California 92521

Communicated by Max Tishler, March 18, 1980

ABSTRACT ^{113}Cd -Substituted human and bovine erythrocyte carbonic anhydrases have been studied by ^{113}Cd NMR as a function of pH and bicarbonate concentration. Plots of chemical shift versus pH give sigmoidal titration curves in the pH range of the study, 6.9 to 10.5. The pK_a values vary from 9.2 to 9.7, which correlates well with available activity profiles for the Cd-enzymes. Because the samples contain no buffers and no anions other than hydroxide, the results point to the existence of high and low pH forms of the enzymes in rapid exchange and differing in inner sphere coordination. When bicarbonate is added to the samples, upfield shifts are produced which eventually level off. Only a single CN^- binds to the metal for all three enzymes. These observations are best explained by a rapid exchange among three species in which the open coordination site of the metal ion is occupied by hydroxide, water, or bicarbonate, as in the scheme: $\text{E}-\text{OH}^- \rightleftharpoons \text{E}-\text{H}_2\text{O} \rightleftharpoons \text{E}-\text{HCO}_3^-$.

Carbonic anhydrases (carbonate hydro-lyase, EC 4.2.1.1) are zinc metalloenzymes that are found in animals, plants, and certain bacteria and catalyze the reversible hydration of carbon dioxide ($\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$), the hydrolysis of certain esters, and various other reactions (1). Human carbonic anhydrase B (hCAaseB) and C (hCAaseC) and bovine carbonic anhydrase (bCAase) from human and bovine erythrocytes are monomeric enzymes of molecular weight $\approx 29,000$, each molecule containing a single equivalent of firmly bound Zn(II) which is required for catalytic activity. The Zn can be replaced by various divalent metal cations, including Co(II) and Cd(II) (2). The activity appears to be controlled by a single titratable group with a pK_a value around 7; the high pH form of the enzyme is required in the hydration reaction and the low pH form is required in the dehydration. A parallel between the spectral changes of Co(II)-enzymes and their activities versus pH has been taken as support for a mechanism in which the low and high pH forms are zinc-coordinated water and hydroxide ion, respectively (3, 4). However, the identity of the catalytic group remains in dispute, and alternative mechanisms have been proposed (5).

In this report we present a systematic ^{113}Cd NMR study of hCAaseB, hCAaseC, and bCAase as a function of pH and bicarbonate concentration. Such ^{113}Cd NMR studies of metallo-proteins have been shown to provide information regarding the coordination sphere of the metal ion (6-10).

MATERIAL AND METHODS

Enzymes. hCAaseB, hCAaseC, and bCAase were prepared by affinity chromatography according to the Khalifah *et al.* (11) modification of the method of Osborne and Tashian (12), followed by chromatography on DEAE-cellulose (Whatman

DE-23). Enzyme concentrations were estimated spectrophotometrically by using the following extinction coefficients ($A_{280}^{1\%}$) and molecular weights: 18.7 cm^{-1} , 16.3 cm^{-1} , and 19.0 cm^{-1} and 29,300, 28,850, and 29,500 for hCAaseC, hCAaseB, and bCAase, respectively (1, 13, 14). The metal-free enzymes were prepared by dialysis against 75 mM pyridine-2,4-dicarboxylic acid/0.2 M phosphate buffer (Aldrich) according to Hunt *et al.* (15). The chelator was removed by dialysis under an inert atmosphere against six changes of CO_2 -free deionized water. The ^{113}Cd -substituted enzymes were prepared by addition of stoichiometric $^{113}\text{Cd}^{2+}$ (as $^{113}\text{CdSO}_4$) to the apoenzymes at pH 6.5 followed by exhaustive dialysis under an inert atmosphere against freshly boiled CO_2 -free deionized water. The samples were finally concentrated by pressure dialysis (Amicon, model 403) under an inert atmosphere. In order to exclude atmospheric CO_2 , exposure to air was kept minimal during the NMR titrations. The pH, measured before and after acquisition of the NMR spectra, underwent negligible change.

Solutions. All reagents were analytical grade. The $^{113}\text{CdSO}_4$ solution was prepared by addition of a slight excess of concentrated H_2SO_4 to ^{113}CdO (Oak Ridge National Laboratory). The excess acid was neutralized by careful addition of dilute CO_2 -free NaOH (50% solution, Mallinckrodt AR). $^{13}\text{CN}^-$ solutions were made by dissolving Na^{13}CN (90% enriched, Merck, Sharp & Dohme Isotopes) in CO_2 -free deionized water.

NMR Spectra. The NMR spectra were obtained at ≈ 19.97 MHz by using a modified multinuclear Bruker WH90D-18 (18-inch 2.11-T magnet). The probe uses an insert designed for 15-mm (outside diameter) sample tubes (containing 5-ml samples) and an external $^2\text{H}_2\text{O}$ lock. An Avanteq wideband preamplifier, Locus image-reject mixer, and quarter-wave line made of semirigid coaxial cable are used. The spectrometer uses quadrature phase detection for all nuclei. Normally, we use: sweep width, 15 kHz; acquisition time, 0.27 sec (4000 data in real spectrum); exponential multiplication, to produce line broadening of 10 Hz; and a pulse flip angle, 30° . The 90° pulse was 12.5 μsec for 25 W of pulse power. No proton decoupling was used. Chemical shifts are reported versus external 0.20 M CdSO_4 .

RESULTS AND DISCUSSION

Typical ^{113}Cd NMR spectra are shown in Fig. 1 A and B. The ^{113}Cd chemical shifts varied continuously with pH from about 215 ppm at low pH to about 275 ppm at high pH (Fig. 2). Linewidths are in the range 40-240 Hz. Great care was taken at every stage in the sample preparation and handling to exclude atmospheric CO_2 , bicarbonate, sulfate, and other anions. Stepwise addition of bicarbonate was carried out for all three enzymes (Table 1). Without exception, bicarbonate produced

Abbreviations: hCAase, human carbonic anhydrase; bCAase, bovine carbonic anhydrase.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

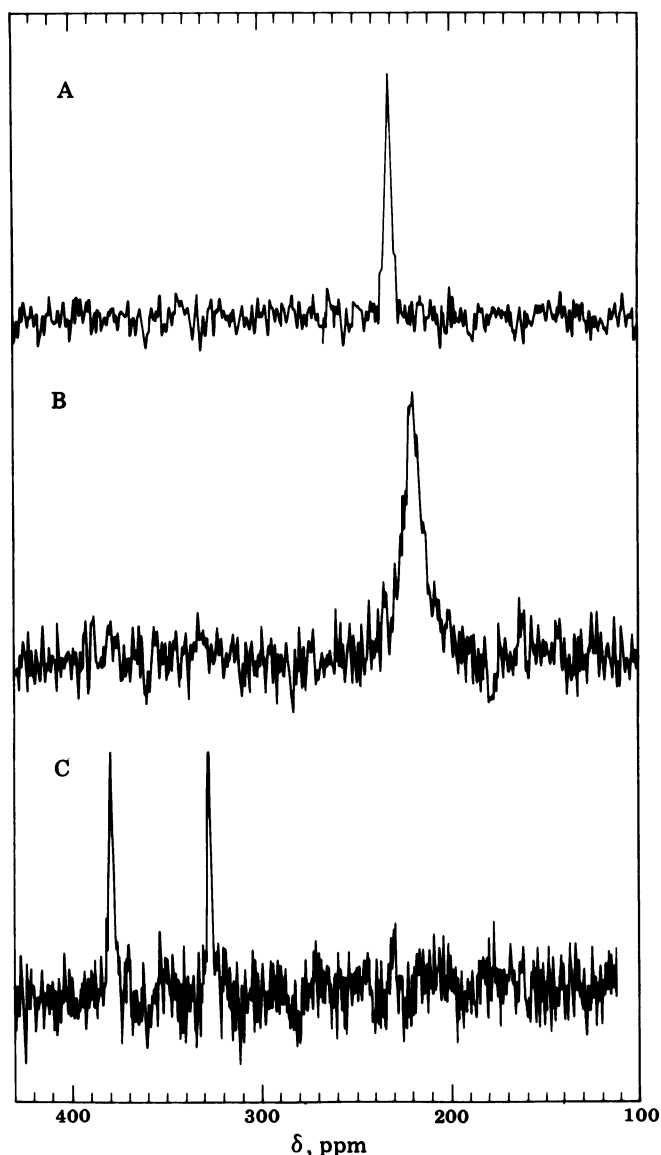
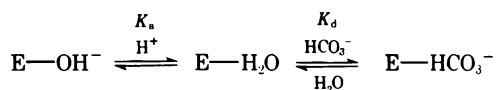


FIG. 1. NMR spectra of ^{113}Cd -substituted enzymes in CO_2 -free water. (A) hCAaseC; 3.4 mM; 6-hr data collection. (B) hCAaseB; 7.0 mM; 10-hr data collection. (C) hCAaseC (3.0 mM) and 50 mM Na^{13}CN ; pH, 9.55; 14-hr data collection.

a continuous upfield shift, leveling off as the enzyme became saturated. These observations are consistent with a model involving rapid exchange among three inner-sphere complexes of the enzyme-bound ^{113}Cd ion. The enzyme provides three imidazole ligands and the exchanging ligands are assumed to occupy an "open" coordination site giving the complexes: (i) a high pH form, (ii) a low pH form, and (iii) an enzyme-bicarbonate complex. The simplest interpretation of the identities of the exchanging ligand in the high and low pH forms are hydroxide and water, respectively, as proposed (1, 3, 4, 16, 17) for the Zn(II)-carbonic anhydrases. These ideas are summarized in Scheme I.



Scheme I

The constants in Scheme I are defined as: $K_a = [\text{E}-\text{OH}^-] \times [\text{H}^+] / [\text{E}-\text{H}_2\text{O}]$; $K_d = [\text{E}-\text{H}_2\text{O}] \times [\text{HCO}_3^-] / [\text{E}-\text{HCO}_3^-]$.

Results obtained from a nonlinear least squares analysis of

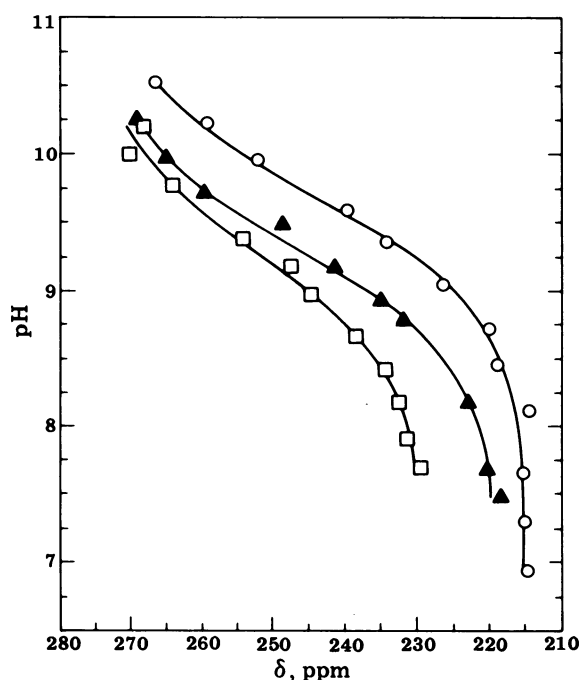


FIG. 2. ^{113}Cd chemical shift of 7.0 mM hCAaseB (O), 3.4 mM hCAaseC (□), and 4.5 mM bCAase (▲) in CO_2 -free water plotted versus pH. The enzyme samples were exhaustively dialyzed, and the pH was increased by addition of carbonate-free NaOH solution. The lines represent the nonlinear least squares fit to the data, based on Scheme I.

the ^{113}Cd chemical shifts based on Scheme I are given in Table 2. The top five variables in Table 2 were derived with the assumption that negligible bicarbonate (<0.5 mM) was present during the pH titrations. That assumption is supported by an experiment at pH 6.5 in which a sample of hCAaseC undergoing several stages of successive dilution with CO_2 -free water showed no change in ^{113}Cd chemical shift.

It has been shown that values, obtained by optical spectra and proton relaxation measurements, for the pK_a of the equilibrium between high and low pH forms of Co(II)-bCAase decrease with decreasing SO_4^{2-} concentration (18, 19). In our studies, SO_4^{2-} was present at less than a few percent of the enzyme

Table 1. HCO_3^- titration of ^{113}Cd (II)-substituted carbonic anhydrases

Enzyme	E_0 , mM	HCO_3^- , mM	Shift, ppm
^{113}Cd -hCAaseB pH = 8.3	8.65	0	223.1
	8.57	8.9	187.5
	6.59	30.3	175.9
	6.59	158.9	168.6
	6.59	785.3	164.7
^{113}Cd -hCAaseC pH = 8.1	3.39	0	231.7
	3.24	6.6	226.5
	3.16	32.3	219.9
	2.80	146.3	218.6
	2.80	399.9	216.3
^{113}Cd -bCAase pH = 8.3	4.49	0	224.1
	4.45	8.9	214.6
	4.36	26.1	210.8
	4.09	81.5	209.5
	4.09	433.0	208.5
^{113}Cd -bCAase pH = 9.7	4.5	0	262.3
	4.4	88	217.0
	3.8	381	207.0

Table 2. Effect of pH, bicarbonate, and cyanide on ^{113}Cd -substituted carbonic anhydrases

	hCAaseC	bCAase	hCAaseB
δ_{OH^-} , ppm*	275 \pm 2	274 \pm 3	274 \pm 2
$\delta_{\text{H}_2\text{O}}$, ppm*	229 \pm 2	219 \pm 2	215 \pm 3
$\delta_{\text{HCO}_3^-}$, ppm*	216 \pm 2	208 \pm 5	164 \pm 2
K_d , mM*	10 \pm 1	4.3 \pm 0.2	3.6 \pm 0.5
pK_a^*	9.2 \pm 0.1	9.3 \pm 0.1	9.7 \pm 0.1
$\delta_{^{13}\text{CN}^-}$, ppm	354 \pm 1	354 \pm 1	411 \pm 1
$J_{^{113}\text{Cd}-^{13}\text{C}}$, Hz	1040 \pm 10	1040 \pm 10	1060 \pm 10

Data are shown as mean \pm SD.

* Obtained from nonlinear least squares analysis of the data based on the three-site exchange model in Scheme I.

concentration. Nevertheless, we observed sigmoidal titration curves (Fig. 2) with pK_a values (Table 2) in reasonable agreement with results from esterase activity measurements performed in SO_4^{2-} buffers containing (20, 21).

All three enzymes were titrated with bicarbonate at low pH (≈ 8) (Table 1). The data in Table 1 were fitted by nonlinear least squares to an equation derived from Scheme I, yielding the values of $\delta_{\text{HCO}_3^-}$ and K_d shown in Table 2. The binding of HCO_3^- can also be displayed by means of a linear plot (Fig. 3). bCAase was also titrated at pH 9.75, yielding the same K_d value as at low pH, which supports the idea (in Scheme I) that bicarbonate binds only to the low pH enzyme form.

Table 2 also reports the chemical shifts and ^{113}Cd - ^{13}C spin-coupling constants for the $^{13}\text{CN}^-$ complexes. The ^{113}Cd NMR signal was a doublet and showed no difference whether we added 1 equivalent of $^{13}\text{CN}^-$ or as much as 16 equivalents (Fig. 1C). Nor was there any change in the ^{113}Cd spectra of the $^{13}\text{CN}^-$ complex when the pH was varied in the range 8 to 10. This suggests that under these conditions there is only one site available for stable CN^- coordination (22–26).

The high-activity enzymes, hCAaseC and bCAase, gave identical ^{113}Cd chemical shifts (354 ppm) for the $^{13}\text{CN}^-$ complex, whereas the low-activity enzyme, hCAaseB, gave a shift of 411 ppm. In addition, the observed shifts for the bicarbonate complexes with hCAaseC and bCAase were similar (216 and 208), whereas the corresponding shift for hCAaseB was 164 ppm. The spin-coupling constants for the three enzymes are virtually identical, indicating identical geometries of Cd–CN coordination. It is conceivable that the magnetic environment of the ^{113}Cd ion in the $^{13}\text{CN}^-$ and HCO_3^- complexes is affected by the same factors that determine the enzyme activity. Indeed, comparison of the metal-binding sites from the 2.0-Å x-ray crystallographic structures of hCAaseB and hCAaseC reveals significant differences in ligand geometry, particularly regarding the twist angles of His-93 (27). Weighing against this, however, is the observation of essentially the same shift for the high pH form of all three enzymes and similar shifts for the low pH forms.

Our results deviate from those of Armitage *et al.* (8, 9) in two respects: we observed ^{113}Cd NMR signals for hCAaseB at pH 8.9 and below (which they did not),* and we found no hCAaseB resonance as high upfield as 146 ppm. Earlier, we reported a hCAaseB resonance at pH 9.7 at 228 ppm. The reported shift is easily understood as a result of HCO_3^- contamination of the highly buffered solution after exposure to CO_2 in the air.

* The ^{113}Cd linewidths, particularly for hCAaseB, are dependent on bicarbonate concentrations because of insufficiently rapid exchange between the free and complexed forms (unpublished data). Therefore, at certain bicarbonate concentrations it is possible for the resonances to broaden out below the limits of detection.

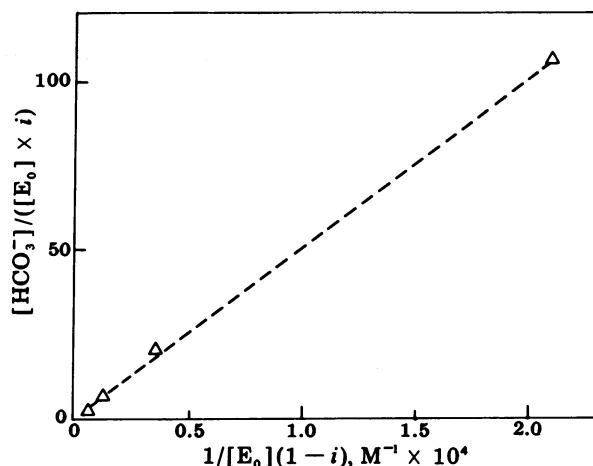


FIG. 3. Bicarbonate titration of bCAase at pH 8.3 (data from Table 1) plotted according to: $i/([E_0] \times [\text{HCO}_3^-]) = K_d/([\text{HCO}_3^-] \times (1 - i)) + 1$ in which $i = (\delta_0 - \delta_i)/(\delta_0 - \delta_\infty)$, δ_0 = chemical shift when $[\text{HCO}_3^-] = 0$, δ_i = chemical shift when $[\text{HCO}_3^-] > 0$, and δ_∞ = chemical shift when $[\text{HCO}_3^-] = \infty$.

This research was supported by National Science Foundation Grant CHE-7811548, U.S. Public Health Service Grant 1R01 GM25877, and a grant from the University of California at Riverside Committee on Research. The UCR Bruker WH90D-18 multinuclear FTNMR spectrometer was provided by Biomedical Sciences Grant 5S05RR 07010-09 from the National Institutes of Health and National Science Foundation Grant MPS75-06138.

- Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. & Strandberg, B. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), 3rd Ed., Vol. 5, pp. 587–665.
- Wyeth, P. & Prince, R. H. (1977) *Inorg. Perspect. Biol. Med.* **1**, 37–71.
- Lindskog, S. & Coleman, J. E. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2505–2508.
- Coleman, J. E. (1967) *J. Biol. Chem.* **242**, 5212–5219.
- Pocker, Y. & Bjorkquist, D. W. (1977) *Biochemistry* **16**, 5698–5707.
- Bailey, D. B., Ellis, P. D. & Cardin, A. D. (1978) *J. Amer. Chem. Soc.* **100**, 236–237.
- Drakenberg, T., Lindman, B., Cave, B. & Parello, J. (1978) *FEBS Lett.* **92**, 241.
- Armitage, I. H., Pajer, R. T., Schoot-Uiterkamp, A. J. M., Chlebowski, J. F. & Coleman, J. E. (1976) *J. Amer. Chem. Soc.* **98**, 5710–5711.
- Armitage, I. H., Schoot-Uiterkamp, A. J. M., Chlebowski, J. F. & Coleman, J. E. (1978) *J. Magn. Reson.* **29**, 375–392.
- Sudmeier, J. L. & Bell, S. J. (1977) *J. Amer. Chem. Soc.* **99**, 4499–5000.
- Khalifah, R. G., Strader, D. J., Bryant, S. H. & Gibson, S. M. (1977) *Biochemistry* **16**, 2241–2247.
- Osborne, W. R. A. & Tashian, R. E. (1975) *Anal. Biochem.* **64**, 297–303.
- Henderson, L. E., Henriksson, D. & Nyman, P. O. (1976) *J. Biol. Chem.* **251**, 5457–5463.
- Andersson, B., Nyman, P. O. & Strid, L. (1972) *Biochem. Biophys. Res. Commun.* **48**, 670–677.
- Hunt, J. B., Rhee, M. J. & Storm, C. B. (1977) *Anal. Biochem.* **79**, 614–617.
- Coleman, J. E. (1971) in *Progress in Bioorganic Chemistry*, eds. Kaiser, E. T. & Kezdy, F. J. (Interscience, New York), Vol. 1, pp. 159–344.
- Khalifah, R. G. & Edsall, J. T. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 172–176.
- Bertini, I., Canti, G., Luchinat, C. & Scozzafava, A. (1977) *Biochem. Biophys. Res. Commun.* **78**, 158–160.
- Jacob, G. S., Brown, III, R. D. & Koenig, S. H. (1978) *Biochem. Biophys. Res. Commun.* **82**, 203–209.
- Bauer, R., Limkilde, P. & Johansen, J. T. (1976) *Biochemistry* **15**, 344–348.

21. Tibell, L. A. E. & Lindskog, S. (1978) *FEBS Proc. Meet.*, 2262 (abstr.).
22. Vallee, B. L. & Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. USA* 59, 498-505.
23. Grell, E. & Bray, R. C. (1971) *Biochim. Biophys. Acta* 236, 503.
24. Kannan, K. K., Petef, M., Frideborg, K., Cid-Dresdner, H. & Lövgren, S. (1977) *FEBS Lett.* 73, 115-119.
25. Taylor, J. S. & Coleman, J. E. (1971) *J. Biol. Chem.* 246, 7058-7067.
26. Bauer, R., Limkilde, P. & Johansen, J. T. (1977) *Carlsberg Res. Commun.* 42, 325-339.
27. Nordstrand, B., Vaara, I. & Kannan, K. K. (1975) in *The Isozymes*, ed. Markert, C. L. (Academic, New York), Vol. 1, pp. 575-599.