quickly and uneventfully effected in 35% yield to deliver **1**, a highly volatile waxy solid, sublimed at ~100 °C, mp 180 °C (rapid heating in sealed capillary). The HRMS of 1,4-bishomohexaprismane (**1**) exhibited molecular ion peak at 184.12558 (calcd for $C_{14}H_{16}$ 184.12528) and the ^1H NMR spectrum (100 MHz) had three resonances at δ 2.38 (br s with st), 2.0 (br s), 1.2 (dd, J = 1.3 Hz) in a ratio of 2:1:1, respectively. The ^{13}C NMR spectrum exhibited three lines at δ 43.0, 36.3, and 33.5 in accordance with its symmetry. With the acquisition of parent **1**, we are adapting the flexible strategy delineated here to obtain its functionalized derivatives that will be amenable to ring contraction to hexaprismane.

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Supplementary Material Available: ^1H NMR spectrum (100 MHz) of 1,4-bishomohexaprismane (1 page). Ordering information is given on any current masthead page.

^{15}N NMR Studies of the Complex of Carbonic Anhydrase with the Novel Carbonyl Hydration Substrate Pyruvamide. Evidence for the Coordination of the Deprotonated Amide Group to the Active Site Zinc

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Carbonic anhydrase, an extremely efficient catalyst of the reversible hydration of carbon dioxide,[1-5] also catalyzes the reversible hydration of carbonyl groups of aldehydes and some ketones[3] and the hydrolysis of aromatic and α-keto esters.[3,4] It is almost universally assumed that a zinc-bound OH is a nucleophile or general base in the mechanism of catalysis of all these substrates.[2-7] Due to weak binding and limited solubility, it has hitherto not been possible to study tightly formed 1:1 enzyme complexes with hydration substrates, so that the role of the metal as a Lewis acid catalyst has not been adequately explored. We now report the identification of pyruvamide as a novel carbonyl hydration substrate of carbonic anhydrase and ^{15}N NMR studies of its tight enzyme complex in which the coordination of a *deprotonated* primary amide to the active site zinc of a metalloenzyme is uniquely demonstrated.

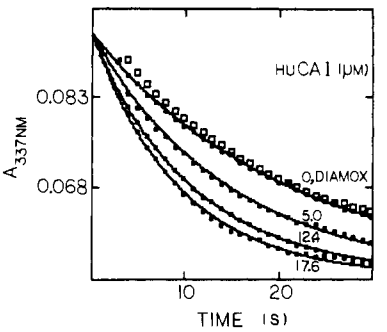

Figure 1. Catalysis of hydration of the keto group of pyruvamide by human carbonic anhydrase I at pH 6.0 in 0.05 M MES buffer. The reaction was followed spectrophotometrically at the keto absorption band (337 nm) and was carried out at ambient (24 °C) temperature. Substrate, as a 10-μL aliquot of a stock solution in *p*-dioxane, was added to 1.0 mL of buffer containing the micromolar concentrations of enzyme shown in the figure. The open squares represent an experiment in which the buffer contained 12.4 μM enzyme and 0.1 mM diamox inhibitor.

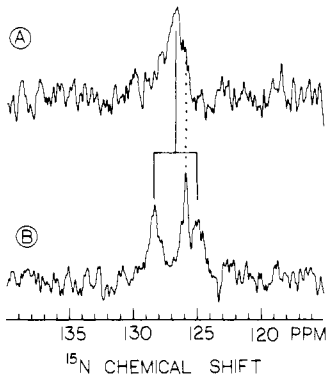

Figure 2. ^{15}N NMR (20.28 MHz) spectra of human carbonic anhydrase I (6.26 mM) containing 1 equiv of 99%-enriched [^{15}N]pyruvamide in 5.0 mM bis-Tris buffer at pH 7.0 in 18% D_2O. Acquisition conditions were as follows: (A) 0.5 W proton broadband decoupling; 130 K scans; decoupler gated off for 3 s following each pulse; (B) no proton decoupling, no delay, 715 K scans. In both cases temperature was 30-32 °C, and acquisition time was 0.48 s, with 8 K data and 16 K transforms, and 3-Hz line broadening.

Figure 1 shows that human carbonic anhydrase I (formerly HCAB) specifically catalyzes the hydration of pyruvamide to the *gem*-diol. The rate increase is linear with enzyme concentration, and catalysis is prevented by the prior addition of the specific inhibitor diamox (acetazolamide). Similar results were obtained with the bovine and human isozymes II. Pocker and co-workers have previously reported that the enzyme catalyzes the hydration of the related pyruvic acid and its alkyl esters,[8] but the affinity of these substrates for the enzyme is very weak. In contrast, we have independently determined[15] that the dissociation constant for binding of pyruvamide approaches 10^{-4} M, making its 1:1 enzyme complexes more favorable for study than any known substrate.

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(1) (a) Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561-2573. (b) Khalifah, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1986-1989.
(2) Silverman, D. N.; Vincent, S. H. *CRC Crit. Rev. Biochem.* **1983**, *14*, 207-255.
(3) Pocker, Y.; Sarkanen, S. *Adv. Enzymol.* **1978**, *47*, 149-274.
(4) (a) Lindskog, S.; Henderson, L. E.; Kannan, K. K.; Liljas, A.; Nyman, P. O.; Strandberg, B. *Enzymes, 3rd Ed.* **1971**, *5*, 587-665. (b) Lindskog, S. *Adv. Inorg. Biochem.* **1982**, 4, 115-170. (c) Lindskog, S. In *Zinc Enzymes, Metal Ions in Biology*; Spiro, T. G., Ed.; Wiley-Interscience: New York, 1983; pp 78-121.
(5) Lindskog, S.; Coleman, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2505-2508.
(6) Buckingham, D. A. In *Biological Aspects of Inorganic Chemistry*; Addison, A. W., Cullen, W. R., Dolphin, D., James, B. R., Ed.; Wiley-Interscience: New York, 1977; pp 141-196.
(7) Harrowfield, J. M.; Norris, V.; Sargeson, A. M. *J. Am. Chem. Soc.* **1976**, *98*, 7282-7289.
(8) (a) Pocker, Y.; Meany, J. E. *J. Am. Chem. Soc.* **1965**, *87*, 1809-1811. (b) Pocker, Y.; Meany, J. E. *Biochemistry* **1967**, *6*, 239-246. (c) Pocker, Y.; Dickerson, D. G. *Biochemistry* **1968**, *7*, 1995-2004. (d) Pocker, Y.; Meany, J. E. *J. Phys. Chem.* **1970**, *74*, 1486-1492. (e) Pocker, Y.; Meany, J. E.; Davis, B. C. *Biochemistry* **1974**, *13*, 1411-1416. (f) Pocker, Y.; Meany, J. E.; Davis, B. C.; Arrigoni, J.; Stein, J. E. *J. Am. Chem. Soc.* **1978**, *100*, 2883-2885. (g) Pocker, Y.; Meany, J. E.; Jones, R. C. *J. Am. Chem. Soc.* **1982**, *104*, 4885-4889.

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Recent *indirect* evidence suggests that *deprotonated* amides and anionic inhibitors bind in a similar manner by competing with solvent hydroxyls for coordination to the active site metal of carbonic anhydrase.[9] In view of the implications of such binding to the mechanism of pyruvamide hydration catalysis and in view of the favorable potential for using pyruvamide to elucidate the mode of binding of amides, we have utilized ^{15}N NMR to characterize the enzyme–pyruvamide *equilibrium* complex. The proton-decoupled ^{15}N NMR spectrum of 99% ^{15}N-enriched pyruvamide[10] shows two resonances at 101.4 and 103.4 ppm[11] (integral ratio of 5:3) that we tentatively assign to the amide resonances of the keto and *gem*-diol forms, respectively.[12] Figure 2A shows the proton-decoupled ^{15}N NMR spectrum of labeled pyruvamide in the presence of 1 equiv of enzyme. Only a single resonance can be seen at 126.7 ppm whose positive NOE (determined in a separate experiment) confirms its assignment to the enzyme-bound pyruvamide.[14] ^{13}C NMR studies on C-2 labeled pyruvamide[15] unambiguously demonstrate that the bound pyruvamide *within the complex* is overwhelmingly in the keto form.

By using the approach pioneered by Kanamori and Roberts,[16a] we examined the *proton-coupled* ^{15}N NMR spectrum of the complex to see whether it is a triplet ($-NH_2$) or doublet ($-NH^-$). Careful examination of the proton-coupled spectrum (Figure 2B) reveals a doublet ($^1J_{N-H}$ = 68 Hz) centered at the decoupled position (126.7 ppm) and an additional resonance about 0.7–0.8 ppm upfield from the doublet center. The amide group is expected to be about 18% deuteriated, since 18% D_2O was used for locking purposes. Independent experiments in which the extent of deuteriation was varied confirmed that the additional component is the resonance of a deuteriated ^{15}N. Very similar ^{15}N isotope shifts *per deuterium* have been reported for ammonia.[17] Although the deuterium-shifted resonance is an unresolved shoulder in Figure 2A, it is clearly resolved at the higher field of 7.05 T. The doublet structure in the proton-coupled spectrum of labeled pyruvamide unambiguously shows that *pyruvamide is bound as the deprotonated amide anion.*[16] Studies on metal ion complexes indicate that this is almost certainly due to substitution of an amide proton by the zinc.[18] Such complexes are normally stabilized with respect to alkaline metal ion hydrolysis by formation of chelate structures.[18] The active site can provide stabilizing interactions, such as hydrogen bonding to the NH and CO of the amide,[19] and the keto group could orient toward the metal to form a five-membered chelate.[20] The unexpectedly small N–H coupling constant we see suggests that the presumably coordinated nitrogen is largely pyramidal in character.[21]

It should be emphasized that *any relation of the dominant pyruvamide binding we see at equilibrium to the catalytically productive binding mode remains to be established.* Should they prove to be the same, our results would have important implications.[22] Since anionic ligands compete with solvent hydroxyls for binding at the active site metal of carbonic anhydrase,[5] our results raise the important question of whether hydration catalysis can be achieved in this enzyme in absence of the zinc–hydroxide mechanism. The striking dissimilarities reported[3,8] in the pH profiles of different carbonyl hydration substrates, along with our present observations, suggest that this enzyme may be capable of a hitherto unrecognized mechanistic diversity in its hydration catalysis.

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(9) Rogers, J. I.; Mukherjee, J.; Khalifah, R. G. *Biochemistry* **1987**, *26*, 5672–5679.

(10) Pyruvamide was synthesized by treating acetylbromide with $KC^{15}N$ followed by carrying out a limited hydrolysis of the resulting pyruvonitrile.[15]

(11) Chemical shifts are reported as being downfield from ammonia, by using aqueous $Na^{15}NO_3$ (assumed to be at 376.0 ppm[21]) as an external reference.

(12) The hydration equilibrium constant for pyruvamide has been reported to be 0.75–0.80.[13]

(13) (a) Fischer, G.; Sieber, M.; Schellenberger, A. *Bioorg. Chem.* **1982**, *11*, 478–484. (b) Fischer, G.; Kullertz, G.; Schellenberger, A. *Tetrahedron* **1976**, *32*, 1503–1505.

(14) At 4.7 T, dipolar calculations suggest that an NOE near 0.7 should be obtained for an immobilized pyruvamide on carbonic anhydrase.

(15) Mukherjee, et al., manuscript in preparation.

(16) (a) Kanamori, K.; Roberts, J. D. *Biochemistry* **1983**, *22*, 2658–2664. (b) Blackburn, G. M.; Mann, B. E.; Taylor, B. F.; Worral, A. F. *Eur. J. Biochem.* **1985**, *153*, 553–558.

(17) Litchman, W. M.; Alei, M.; Florin, A. E. *J. Chem. Phys.* **1969**, *50*, 1897.

(18) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385–426.

(19) (a) Vedani, A.; Meyer, E. F. *J. Pharm. Sci.* **1984**, *73*, 352–358. (b) Vedani, A.; Dunitz, J. D. *J. Am. Chem. Soc.* **1985**, *107*, 7653–7658.

(20) Bertini, I.; Luchinat, C.; Scozzafava, A. *Bioinorg. Chem.* **1978**, *9*, 93–100.

(21) Levy, G. C.; Lichter, R. L. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; Wiley-Interscience: New York, 1979.

(22) Our results also contribute to understanding the mode of binding of the CO_2 competitive inhibitor imidazole.[23] In view of the similarity in pK_a and coordination potential of deprotonated amides and deprotonated "pyrrole" nitrogens of imidazole,[18] our present findings make the proposed inhibition of carbonic anhydrase by the imidazole anion at high pH[23] much more tenable.

(23) (a) Bertini, I.; Luchinat, C. *Acc. Chem. Res.* **1983**, *16*, 272–279. (b) Khalifah, R. G.; Rogers, J. I.; Mukherjee, J. *Biochemistry*, in press.

$^{11}C/^{14}C$ Kinetic Isotope Effects

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Kinetic isotope effect (KIE) measurements employing the isotopes of hydrogen as well as many heavy elements have frequently been utilized in the elucidation of organic and enzymatic reaction mechanisms.[1] In this paper we report a method for the determination of $^{11}C/^{14}C$ KIE.[2] The radionuclide ^{11}C is a positron emitter with a half-life of 20.34 min. There are several reasons why the combined use of ^{11}C and ^{14}C may be useful in isotope effect studies. (1) A large mass range of carbon isotopes is used, resulting in a large rate ratio. (2) Both isotopes are radioactive and can be analyzed with high precision. (3) Direct rate measurements can be performed reacting the isotopic species in the same reaction pot, thus eliminating interexperimental errors. There is, of course, also a fundamental interest in this new carbon KIE.

The main obvious drawbacks in using ^{11}C are the restrictions imposed by its short half-life and the need for accelerator facilities. However, the increased use of positron emission tomography (PET) in biomedical research as well as in clinical applications[3] have accelerated the development of rapid labeling synthesis. Today, a large range, including quite complex, ^{11}C labelled molecules, is available.[4] The use of ^{11}C in the study of physio-

(1) See, e.g.: Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; John Wiley and Sons: New York, 1980.

(2) (a) See Matsson (Matsson, O. *Abstracts of Uppsala Dissertations from the Faculty of Science*; 1984; 723) for a preliminary report of the method. (b) This work was presented in part at the IVth Conference on the Application of Medical Cyclotrons, Turku, Finland, 1986, the VIIIth IUPAC Conference in Physical Organic Chemistry, Tokyo, Japan, 1986, and the Gordon Conference on Isotopes in Chemistry and Physics, Oxnard, CA, 1986.

(3) (a) *The Metabolism of the Human Brain Studied with Positron Emission Tomography*; Greitz, T., Ingvar, D. H., Widén, L., Eds.; Raven Press: New York, 1985. (b) *Positron Emission Tomography and Autoradiography: Principles and Applications for the Brain and Heart*; Phelps, M., Mazziotta, I., Schelbert, H., Eds.; Raven Press: New York, 1986. (c) *Biomedical Imaging*; Hayaishi, O., Torizuka, K., Eds.; Academic Press: New York, 1986.

0002-7863/87/1509-7233$01.50/0