Isotope Effect Evidence for the Zinc Hydroxide Mechanism of Carbonic Anhydrase Catalysis†

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ABSTRACT: The carbon kinetic isotope effect on the enzymatic dehydration of HCO_3^- ion is $k^{12}/k^{13} = 1.011$ and is independent, within experimental error, of the addition of sucrose, substitution of D_2O for H_2O, and substitution of enzyme-bound Zn^{2+} by Co^{2+}. These results are consistent with a ping-pong mechanism in which proton transfer between enzyme and solvent is separated from HCO_3^- dehydration. For the dehydration half-reaction, diffusional processes are severalfold faster than dehydration, and the rate-determining step is the dehydration itself. The intrinsic isotope effect is approximately 1.011, indicating that hydration of CO_2 occurs by reaction of zinc-bound OH^-, rather than zinc-bound H_2O.

The mechanism of the hydration of CO_2 and its reverse, dehydration of bicarbonate ion, catalyzed by carbonic anhydrase remains controversial in spite of an enormous number of studies devoted to this area (Lindskog, 1982; Silverman & Vincent, 1984; Coleman, 1984; Pocker & Deits, 1984). Important questions include the role of diffusion and the intermediacy of a $Zn–OH^-$ intermediate. The successful application of heavy-atom kinetic isotope effects in elucidation of mechanisms of organic reactions (Melander & Saunders, 1980) and enzymatic reactions (Schowen, 1986) has prompted us to try this method for both the spontaneous and carbonic anhydrase catalyzed interconversions of carbon dioxide and bicarbonate. Some results of these studies have already been published (Marlier & O'Leary, 1984; Paneth & O'Leary, 1985a,b).

Because of the high rates of these reactions, a special procedure had to be developed for the measurement of kinetic isotope effects. We have reported earlier the protocols for the spontaneous (Marlier & O'Leary, 1984) and the enzyme-catalyzed (Paneth & O'Leary, 1985a) reactions. Carbon isotope effects on the spontaneous dehydration of bicarbonate ion are measured by comparison of the isotopic composition of CO_2 formed at low conversion with that produced at 100% conversion under conditions where CO_2 is removed from the solution as soon as it is formed. The carbon kinetic isotope effects for the spontaneous dehydration of bicarbonate in H_2O and in D_2O below pH 9, where the predominant mechanism is the acid-catalyzed mechanism (eq 1), led us to conclude

$$H^+ + HCO_3^- \rightleftharpoons H_2O + CO_2 \qquad (1)$$

(Paneth & O'Leary, 1985b; unpublished results) that at least two steps are involved in this process, the first being proton transfer to form a zwitterionic and the second its decomposition to CO_2 and H_2O.

At higher pH, the predominant mechanism is decomposition of HCO_3^- to form CO_2 and OH^- (eq 2), and the isotope effect is near 1.01 (P. Paneth and M. H. O'Leary, unpublished results).

$$HCO_3^- \rightleftharpoons CO_2 + OH^- \qquad (2)$$

The enzymatic reaction is harder to study than the spontaneous reaction because of its higher rate, because of the simultaneous presence of the spontaneous reaction, and because of the inevitable occurrence of the reverse reaction (CO_2 hydration) at high enzyme concentrations. The isotope effect on the enzymatic reaction is obtained by curve fitting of the apparent isotope effect as a function of carbonic anhydrase concentration. The validity of this method was demonstrated by ^{18}O exchange experiments (Paneth & O'Leary, 1985b). The isotope effect for the enzymatic reaction is smaller than that for the acid-catalyzed spontaneous reaction and similar in magnitude to that for the high-pH process. No evidence has been obtained for the zwitterionic intermediate which is formed in the spontaneous reaction. In this paper, we report the influence of D_2O, metal ion, and sucrose on the carbon kinetic isotope effect for the enzymatic reaction and comparison of these results with isotope effects for the spontaneous reaction.

Experimental Procedures

Materials. Carbonic anhydrase from bovine erythrocytes and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid were obtained from Sigma Chemical Co. $NaHCO_3$ was obtained from Columbus Chemical Industries, Inc., and the same bottle was used throughout, for isotopic consistency. Sucrose (AR) was obtained from Mallinckrodt Chemical Works, St. Louis, MO. 1,4-Dioxane (spectrophotometric grade) was obtained from Aldrich Chemical Co. Water was purified with a Millipore Super-Q water purification system. D_2O was obtained from Stohler Chemical Co. $H_2{}^{18}O$ (97% ^{18}O) was obtained from Mound Laboratories, Miamisburg, OH.

Methods. Carbonic anhydrase was assayed by the colorimetric method of Roughton and Booth (Waygood, 1955).

Carbonic anhydrase containing cobalt was obtained by the two-step equilibrium dialysis method of Hunt et al. (1977). In the first step, zinc was removed by dialysis in phosphate buffer containing pyridine-2,6-dicarboxylic acid. In the second step, the chelating agent was removed by dialysis against tris(hydroxymethyl)aminomethane (Tris) buffer. The apoenzyme so obtained was tested for activity by measuring the carbon isotope effect in the presence of 10 units of enzyme. The resulting isotope effect was that of the spontaneous re-

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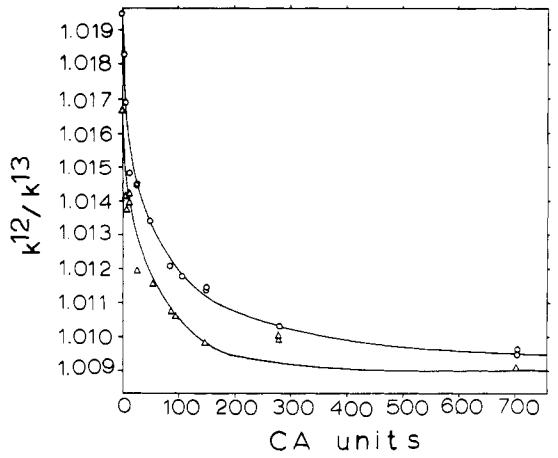

FIGURE 1: Observed isotope effect for the dehydration of HCO_3^- vs. carbonic anhydrase concentration at pH 8.2, 25 °C. Circles correspond to relative viscosity 3.5 and triangles to 2.2.

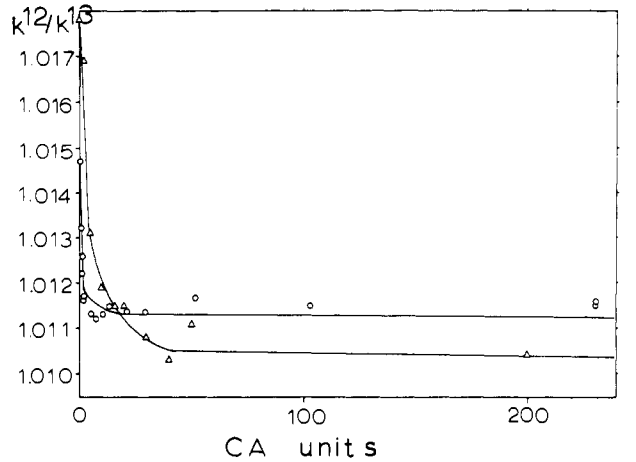

FIGURE 2: Observed isotope effect for the dehydration of HCO_3^- vs. carbonic anhydrase concentration at pH 8.2, 25 °C. Circles correspond to Co–carbonic anhydrase and triangles to D_2O.

Table I: Carbon Kinetic Isotope Effects on the Enzymatic Dehydration of Bicarbonate Ion at 25 °C

conditions	k^{12}/k^{13}
H_2O	1.0101 ± 0.0004[a]
D_2O	1.0107 ± 0.0007
Co–carbonic anhydrase	1.0115 ± 0.0004
sucrose solution of rel viscosity	
1.0	1.0101 ± 0.0004[a]
2.2	1.0104 ± 0.0006
3.5	1.0116 ± 0.0004

[a] Paneth & O'Leary (1985a).

action, which indicates no enzymatic activity. Afterward 1 equiv of $CoCl_2$ was added.

Isotope Effects. Experiments were carried out by the vacuum purge method of Marlier and O'Leary (1984), with the addition of a measured amount of carbonic anhydrase. All experiments were conducted at 25 °C in 0.5 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid buffer, pH 8.2. The ^{18}O control experiments were performed as described earlier (Paneth & O'Leary, 1985a). Samples from D_2O were equilibrated with normal water as described previously prior to isotopic analysis (Paneth & O'Leary, 1985b).

Isotope ratios were measured on a Finnigan Delta-E isotope ratio mass spectrometer. All ratios were corrected for oxygen-17 (Craig, 1957).

Results

Carbon isotope effects on the enzymatic dehydration of HCO_3^- in aqueous sucrose solutions of varying viscosity were obtained from a plot of the apparent isotope effect vs. carbonic anhydrase concentration (Paneth & O'Leary, 1985a). Results are shown in Figure 1, and isotope effects are summarized in Table I. Isotope effects at relative viscosity = 1.0 have been reported previously (Paneth & O'Leary, 1985a). The effectiveness of the CO_2 purge in viscous solutions was tested by measuring the rate of ^{18}O incorporation into CO_2 from $H_2{}^{18}O$ as a solvent and proved to be satisfactory.

The plots of the apparent isotope effect vs. carbonic anhydrase concentration for D_2O as the solvent and for the cobalt–enzyme are presented in Figure 2. Isotope effects are summarized in Table I.

Discussion

The stoichiometry of HCO_3^- dehydration requires proton transfer (eq 1). A number of lines of evidence indicate that in the carbonic anhydrase catalyzed reaction this proton transfer is temporally separated from HCO_3^- dehydration and that the overall reaction follows a "ping-pong" mechanism, with dehydration being one half-reaction and proton transfer being the other (Silverman & Vincent, 1984). Among the principal lines of evidence are the large solvent isotope effect on V_{max}, which apparently reflects the proton transfer half-reaction, and the lack of a solvent isotope effect on V_{max}/K_m, which apparently reflects the dehydration half-reaction (Silverman & Vincent, 1984).

The carbon isotope effect for the carbonic anhydrase catalyzed dehydration of HCO_3^- is the same in H_2O as in D_2O. If the reaction were not ping-pong, and proton transfer and dehydration occurred within the same sequence, then the carbon isotope effect would be expected to be different in the two isotopic solvents. The equality of isotope effects in the two solvents is consistent with the suggestion of a ping-pong mechanism in which there is no solvent isotope effect on the dehydration half-reaction. For such a mechanism, the carbon isotope effect is independent of the rate of the proton transfer half-reaction.

We can picture the dehydration half-reaction as

$$E + HCO_3^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}HCO_3^- \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{–}CO_2 \xrightarrow{k_5} E + CO_2 \quad (3)$$

where the first step is binding of bicarbonate ion to the enzyme, the second step is chemical interconversion between bicarbonate and carbon dioxide, and the third step is release of CO_2 from the enzyme. If we assume that only k_3 and k_4 show significant isotope effects, then the observed carbon isotope effect is given by

$$k^{12}/k^{13} = \frac{(k_3{}^{12}/k_3{}^{13})/(k_4{}^{12}/k_4{}^{13}) + (k_3{}^{12}/k_3{}^{13})(k_5/k_4) + k_3k_5/k_2k_4}{1 + k_5/k_4 + k_3k_5/k_2k_4} \quad (4)$$

Thus, the observed isotope effect depends on the isotope effects on k_3 and k_4 and on the partition factors k_3/k_2 and k_5/k_4. The former partition factor reflects the relative rates of dehydration and dissociation of enzyme-bound HCO_3^-. The latter factor reflects the relative rates of dissociation and rehydration of enzyme-bound CO_2. The ratio of isotope effects $(k_3{}^{12}/k_3{}^{13})/(k_4{}^{12}/k_4{}^{13})$ equals the overall equilibrium isotope effect, which is 1.0090 (Mook et al., 1974). The observed isotope effect will be smaller than the intrinsic carbon isotope effect unless the dehydration step is entirely rate determining.[1]

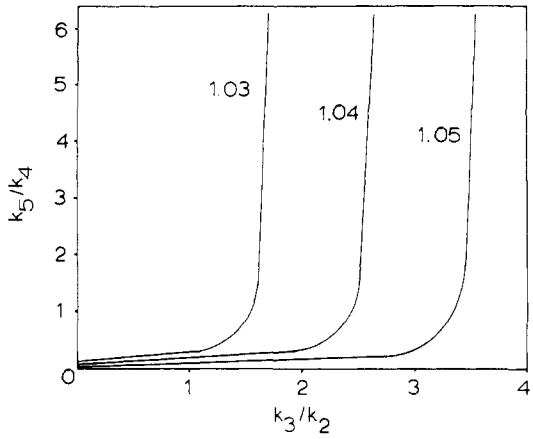

FIGURE 3: Possible values of k_3/k_2 and k_5/k_4 for the enzymatic dehydration of HCO_3^- assuming an observed isotope effect of k^{12}/k^{13} = 1.0110. The three curves are for three assumed values of the intrinsic isotope effect (1.03, 1.04, and 1.05).

Our further analysis of the carbon isotope effects must begin with an assumption about the intrinsic isotope effect on k_3. This value, in combination with the known equilibrium isotope effect (Mook et al., 1974), permits calculation of the isotope effect on k_4. Once these values are determined, it is possible to estimate pairs of values of k_3/k_2 and k_5/k_4 that are consistent with the observed isotope effects. Unique solutions for individual ratios are never obtained but only pairs of values of the two partition ratios.

As we have noted previously (Paneth & O'Leary, 1985b), the dehydration of HCO_3^- can be viewed as a type of decarboxylation, in the sense that a carboxylate ion $R-CO_2^-$ is converted into CO_2 and some other product. Intrinsic isotope effects observed in other decarboxylation thus serve as a starting point for estimating the intrinsic isotope effect in this reaction. We begin with two possibilities: First, the intrinsic isotope effect is large—in the range k^{12}/k^{13} = 1.03–1.06. Alternatively, the intrinsic isotope effect may be small—near k^{12}/k^{13} = 1.01 or slightly larger. These two possibilities will be explored in the next two sections.

Large Intrinsic Isotope Effect. We begin by assuming an intrinsic isotope effect in the range 1.03–1.06. This is the range seen for intrinsic isotope effects in both enzymatic and nonenzymatic decarboxylations (Melander & Saunders, 1980; O'Leary, 1977; O'Leary & Piazza, 1981; Hermes et al., 1982, 1984a,b; Rosenberg & O'Leary, 1985). It is also the range for the nonenzymatic acid-catalyzed dehydration of HCO_3^- (Paneth & O'Leary, 1985b; unpublished results) and thus might be expected if the mechanism of the enzymatic reaction resembles that of the acid-catalyzed reaction. With this assumed isotope effect, we can use eq 4 to estimate pairs of values of k_3/k_2 and k_5/k_4 which are consistent with the observed isotope effects. These are shown in Figure 3 for three assumed values of the intrinsic isotope effect. Although a unique fit to the experimental data is not possible, it is clear that if this assumption is correct, then dehydration is not the rate-limiting step in the overall sequence. Instead, diffusional steps (either substrate binding or product dissociation or some combination) are largely rate limiting. The dehydration step on the enzyme surface is nearly at equilibrium, and the observed isotope effect principally reflects the equilibrium isotope effect on dehydration.

Substitution of Co^{2+} for Zn^{2+} at the active site of carbonic anhydrase leads to a significant decrease in V_{max} (Silverman & Vincent, 1984) and no significant increase in the carbon isotope effect. The simplest explanation of the rate decrease in the presence of Co^{2+} is that the effect of metal substitution on diffusion rates is negligible and the dehydration step occurs more slowly than in the presence of Zn^{2+}. Thus, k_3/k_2 would decrease, k_5/k_4 would increase, and the carbon isotope effect should be significantly larger in the presence of Co^{2+} (cf. eq 4). Experimentally, the lack of a change in an isotope effect with metal substitution indicates that k_3/k_2 and k_5/k_4 do not change with metal. Thus, the isotope effect in the presence of Co^{2+} seems inconsistent with this mechanism.

Interpretation of the effect of D_2O on the carbon isotope effect is also instructive. In the nonenzymatic acid-catalyzed dehydration of HCO_3^-, there is a solvent isotope effect on the rate of the dehydration step (Paneth & O'Leary, 1985b) and on the carbon isotope effect. If the mechanism of the enzymatic reaction is basically similar, we would expect solvent isotope effects on the enzymatic reaction as well. Unless substrate binding and dissociation steps show the same solvent isotope effect as the chemical step (and this seems unlikely, as these are simply diffusional processes), the carbon isotope effect should be larger in D_2O than in H_2O. However, no solvent isotope effect is observed (Silverman & Vincent, 1984), and the carbon isotope effect for the enzymatic reaction is the same in H_2O as in D_2O. Thus, the lack of a D_2O effect on the rate and on the carbon isotope effect appears to be inconsistent with this mechanism.

Hasinoff (1984) has measured the effect of glycerol on the enzymatic rate of hydration of CO_2 and dehydration of HCO_3^- as a way of identifying kinetically significant diffusional processes. Similar arguments have been made for other enzymatic reactions (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984), although recent studies indicate that sucrose and glycerol can have other effects on enzymatic reactions in addition to their effects on viscosity (Bazelyansky et al., 1986). The rate of enzymatic dehydration of HCO_3^- at low substrate concentration decreases by about a factor of 2 as glycerol concentration increases. This has been interpreted in terms of rate-determining diffusion of HCO_3^- and CO_2 on and off the enzyme (Hasinoff, 1984). Such an effect of viscosity is consistent with the idea that the intrinsic isotope effect is large, diffusion is principally rate determining, and thus the observed isotope effect is small.

We used sucrose as a viscogen and measured carbon isotope effects over the same range of viscosities reported by Hasinoff (1984). If the above explanation is correct, then the rate of the dehydration step (k_3) should be independent of viscosity. Consequently, k_3/k_2 should increase and k_5/k_4 should decrease with increasing viscosity, and the observed isotope effect should decrease. Such a decrease is not seen. Thus, we conclude that the effect of viscogens on the kinetics and carbon isotope effects is not consistent with the existence of a large intrinsic isotope effect.

Thus, the lack of a variation in carbon isotope effect with metal substitution, with D_2O, and with viscosity is inconsistent with a mechanism in which the intrinsic isotope effect is large and the overall rate is limited principally by diffusional processes. Although any one of these approaches might be subject to criticism, the consistency of the results from three kinds of studies lends considerable strength to this conclusion. We show in the following paragraphs that all data are nicely consistent with the alternate mechanism, in which the dehydration step is entirely rate limiting.

[1] An intrinsic isotope effect is the isotope effect for a single isotope-sensitive step, in the absence of contributions from other steps.

Small Intrinsic Isotope Effect. Alternatively, we can assume that the intrinsic isotope effect is near 1.01. This is an unusual intrinsic isotope effect for a "decarboxylation" reaction. To our knowledge, only one case is known where the intrinsic isotope effect is less than 1.03: The dehydration of HCO_3^- to give CO_2 and OH^- (that is, the high-pH dehydration of HCO_3^-; eq 2) shows an isotope effect near 1.01 (P. Paneth and M. H. O'Leary, unpublished results). The small size of the isotope effect probably arises from the very late transition state for this reaction, which is unlike those observed in other CO_2-forming reactions. This reaction is, of course, analogous to one of the mechanisms which has been suggested for carbonic anhydrase involving zinc-bound OH^- as the reactive species. For such a mechanism, the intrinsic isotope effect might be near 1.01. In the following paragraphs, we explore this possibility in light of our carbon isotope effects.

In this case, the observed isotope effect is equal or nearly equal to the intrinsic isotope effect for the dehydration step. Diffusion of substrates and products on and off the enzyme must be fast (k_3/k_2 must be small and k_5/k_4 must be large).

The effect of Co^{2+} on the rate and the lack of an effect of Co^{2+} on the carbon isotope effect fit nicely with this explanation. Because a single step is rate determining, partitioning does not enter, and the isotope effect does not change, even though the rate of dehydration changes by 2-fold.

The rate of the spontaneous dehydration of HCO_3^- is unaffected by D_2O, unlike the rate of the acid-catalyzed reaction (Pocker & Bjorquist, 1977). This is consistent with the fact that the rate of enzyme-catalyzed dehydration is also unaffected by D_2O (Silverman & Vincent, 1984). Pocker and Bjorquist (1977) have presented a model for CO_2 hydration in which reaction of CO_2 occurs with a Co^{3+} hydroxo complex. For this reaction, the solvent isotope effect is unity. Similarly, the carbon isotope effect on the spontaneous dehydration is unaffected by D_2O, whereas the carbon isotope effect on the acid-catalyzed reaction increases in the presence of D_2O (Paneth & O'Leary, 1985b; unpublished results). The carbon isotope effect on the enzymatic reaction is unaffected by D_2O both because the intrinsic isotope effect is independent of solvent deuteriation and because the dehydration step is entirely rate determining, so that the solvent dependence of partitioning factors does not enter.

The effect of viscogens is somewhat more puzzling. If the mechanism currently under consideration is correct, then there is only a single kinetically significant step (dehydration), and diffusion is substantially faster than this step. Under these conditions, addition of viscogens would not be expected to change the isotope effect, even if they affect the rate. This is, of course, what is observed. On the other hand, diffusion is not limiting in this mechanism, and changes in viscosity should not affect the rate. This is, of course, contrary to what is observed (Hasinoff, 1984). It should be noted that an explanation strictly in terms of *viscosity* is also not possible if the intrinsic isotope effect is large (vide supra).

The resolution of this dilemma probably lies in effects of glycerol on phenomena other than viscosity. This has been noted in other recent studies (Bazelyansky et al., 1986). Glycerol is also known to affect the rates of other enzymatic CO_2-forming reactions (O'Leary & Piazza, 1981), presumably through a medium effect, and the same might be occurring here.

Thus, the carbon isotope effect data are more consistent with the possibility that the intrinsic carbon isotope effect is small and the chemical step is totally rate determining. The small isotope effect indicates that the reaction involves zinc-bound hydroxide as the reactive species, rather than any other possibility.

Conclusion. Lack of a D_2O effect on the carbon isotope effect on the enzymatic dehydration of HCO_3^- requires that the reaction occur via a ping-pong mechanism, with proton transfer to or from the enzyme occurring in a separate half-reaction from the dehydration/hydration step. Two possibilities have been considered for the dehydration step. The possibility that the reaction rate is principally determined by diffusion appears to be eliminated by the constancy of the observed carbon isotope effect in the face of metal substitution, deuteriation of the solvent, and addition of viscogen to the solvent. On the other hand, these same observations are nicely consistent with a mechanism in which the dehydration itself is entirely limiting, and diffusional processes are manyfold faster than this step. This in turn requires that the intrinsic isotope effect is near 1.01, indicating that the hydration of CO_2 occurs by reaction of enzyme-bound CO_2 with zinc-bound OH^-.

Registry No. HCO_3^-, 71-52-3; ^{13}C, 14762-74-4; carbonic anhydrase, 9001-03-0.

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