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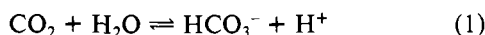
## Carbon Dioxide Hydration Activity of Carbonic Anhydrase in Mixtures of Water and Deuterium Oxide†

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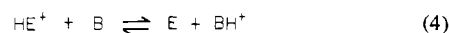
**ABSTRACT:** We have measured the hydration of CO<sub>2</sub> catalyzed by bovine red cell carbonic anhydrase in mixtures of H<sub>2</sub>O and D<sub>2</sub>O at pH(D) which is in the plateau region of the pH-activity profile. The steady-state parameters for hydration  $V_{\max}$  and  $K_m$  both show an exponential dependence on the atom fraction of deuterium in solvent water. These observed solvent isotope effects cannot be fit to a model involving one hydrogen but must arise from the contributions of two or more hydrogens which change their fractionation factor in the transition state

of the rate-limiting step of the catalysis. On the basis of previous evidence, this step is taken to be an intramolecular proton transfer which our results suggest involves water bridges. The dependence of the rate constant for the uncatalyzed reaction between CO<sub>2</sub> and water has a nonlinear dependence on the atom fraction of deuterium in the solvent. We conclude that the observed isotope effect must arise from more than one hydrogen in the transition state.

The zinc-containing metalloenzyme carbonic anhydrase catalyzes the reaction of eq 1. Current understanding of the

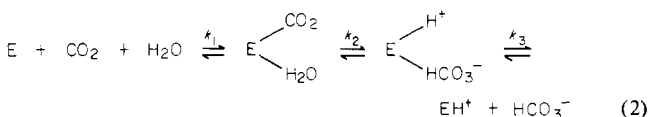


catalytic mechanism of the human carbonic anhydrase II (the high-activity isozyme) and the kinetically similar bovine carbonic anhydrase from red cells is based in part on steady-state kinetic studies carried out in H<sub>2</sub>O and D<sub>2</sub>O. Steiner et al. (1975) and Pocker & Bjorkquist (1977a) observed a kinetic solvent isotope effect of magnitude between 3 and 4 for both  $k_{\text{cat}}$  and  $K_m$  for hydration, whereas the isotope effect on  $k_{\text{cat}}/K_m$  was very close to unity. The corresponding values for dehydration were rather similar. Steiner et al. (1975) used these results to suggest a catalytic mechanism in which the hydration of CO<sub>2</sub> and a rate-limiting, intramolecular proton transfer occur in distinct stages. Elements of the proposed mechanism are given in eq 2-4. Equation 2 rep-



resents the catalytic interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> in which EH<sup>+</sup> designates the form of enzyme with a protonated, activity-controlling group of pK<sub>a</sub> near 7. Equation 3 describes the transfer of a proton between the activity-controlling group and a proton acceptor in the enzyme, designated by HE<sup>+</sup>, which acts as a proton shuttle group. Histidine-64 has been suggested as a possible proton shuttle group (Steiner et al., 1975); with a pK near 7 and ~6 Å from the zinc, it is suited to act as either a donor or acceptor of protons from the activity-controlling group. The transfer of a proton between the enzyme and buffer in solution (eq 4) has been found to limit the rate of catalysis of the hydration of CO<sub>2</sub> only at buffer concentrations less than ~10 mM (Silverman & Tu, 1975; Jonsson et al., 1976).

We report here an extension of the work of Steiner et al. (1975) and Pocker & Bjorkquist (1977a,b) by measuring the kinetic solvent isotope effect as a function of the atom fraction of deuterium in solvent water for the catalyzed and uncatalyzed hydration of CO<sub>2</sub>. These results provide information, through the Gross-Butler relationship, concerning the number and fractionation factor of hydrogenic sites which undergo bonding changes in the transition state of the rate-limiting event in catalysis (Schowen, 1978). Since proton transfer is believed



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to play a prominent and limiting role in the catalytic hydration of CO<sub>2</sub>, such information has significance with regard to the mechanism.

### Materials and Methods

**Enzyme and Chemicals.** Bovine carbonic anhydrase was isolated from erythrocytes by the affinity chromatography procedure described by Khalifah et al. (1977) and dialyzed exhaustively against distilled water. Enzyme concentrations were estimated from the molar extinction coefficient at 280 nm of  $5.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Coleman, 1967). All buffers and indicators were obtained from Aldrich Chemical Co.; imidazole was twice recrystallized from benzene; 1,2-dimethylimidazole was distilled under reduced pressure; *p*-nitrophenol and *m*-cresol purple were used as obtained.

Saturated solutions of CO<sub>2</sub> were prepared by bubbling CO<sub>2</sub> gas into deionized, distilled H<sub>2</sub>O or D<sub>2</sub>O in a vessel maintained at 25 °C in a water bath. Aliquots were withdrawn by allowing the solution to flow by gravity into a gas-tight syringe. Pocker & Bjorkquist (1977b) have reported the concentration of CO<sub>2</sub> when saturated in H<sub>2</sub>O ( $3.38 \times 10^{-2} \text{ M}$ ) and in D<sub>2</sub>O ( $3.81 \times 10^{-2} \text{ M}$ ) at 25 °C. Solutions of different CO<sub>2</sub> concentration were made by dilution of saturated CO<sub>2</sub> solutions with degassed H<sub>2</sub>O or D<sub>2</sub>O. This procedure was carried out by using two syringes with a gas-tight connection.

**Stopped-flow Methods.** Initial velocity measurements of the hydration of CO<sub>2</sub> were carried out on a Durrum-Gibson stopped-flow spectrophotometer. The initial velocity of hydration  $v$  was calculated by using the equation

$$v = \left[ \frac{d(\text{CO}_2)}{dt} \right]_{\text{initial}} = -Q_0 \left( \frac{dA}{dt} \right)_{\text{initial}}$$

with  $dA/dt$  determined by least-squares analysis of values of absorbance and time accumulated in an interfaced Hewlett-Packard 9835B calculator. The absorbance of *p*-nitrophenol was followed at 400 nm and *m*-cresol purple was followed at 578 nm. Care was taken to measure  $dA/dt$  only in the region near  $t = 0$ . Data were taken for the entire reaction but only data from  $t = 5 \text{ ms}$  up to 10% of completion were used to measure initial velocity. The buffer factor  $Q_0$  relating changes in absorption to changes in (H<sup>+</sup>) was estimated by the method of Khalifah (1971) for the buffer-indicator pairs of imidazole with *p*-nitrophenol and 1,2-dimethylimidazole with *m*-cresol purple.

All experiments were performed at  $25 \pm 0.2$  °C and in all experiments total ionic strength was maintained at 0.1 with Na<sub>2</sub>SO<sub>4</sub>. The values of pL reported here are uncorrected pH meter readings. We have relied on the fact that the correction of a pH meter reading in 100% D<sub>2</sub>O [pD = (meter reading) + 0.4; Glasoe & Long, 1960] is approximately offset by the change in ionization state of buffer in D<sub>2</sub>O ( $\text{p}K_{\text{D}} - \text{p}K_{\text{H}} = 0.5 \pm 0.1$  for almost all acids with pK values between 3 and 10; Bell, 1959). Consequently, we expect all reactive species to maintain approximately the same ratio of acidic to basic forms in these experiments at pL = 8.2. Moreover, all kinetic solvent isotope effects for the catalyzed hydration of CO<sub>2</sub> reported here were determined at pL = 8.2, a value for which both  $k_{\text{cat}}$  and  $K_{\text{m}}$  are in a plateau region showing almost no change for small variations ( $\pm 0.2$ ) in pL (Pocker & Bjorkquist, 1977a).

### Results

We use the notation of Gibbons & Edsall (1963) for the uncatalyzed hydration of CO<sub>2</sub> at pH below 7:

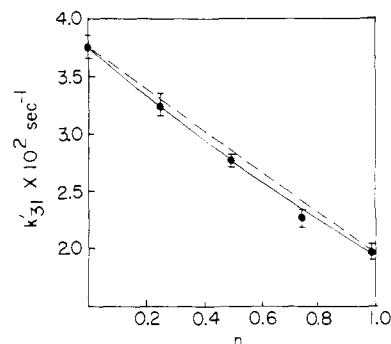
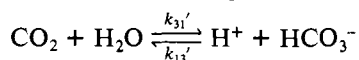


FIGURE 1: Variation of  $k'_{31}$ , the rate constant for the uncatalyzed hydration of CO<sub>2</sub>, with  $n$ , the atom fraction of deuterium in solvent water, at 25 °C. The rate constants were obtained in imidazole buffers (total buffer concentration 10 mM) with *p*-nitrophenol as the indicator. The pL was 6.95, and the total ionic strength of solution was maintained constant at 100 mM with Na<sub>2</sub>SO<sub>4</sub>. The solid curve corresponds to a two-proton model and obeys the equation  $k'_{31} = 0.0375(1 - n + 0.72n)^2$ . Data are given as the average and standard deviation.

Table I: Observed Rate Constants for the Uncatalyzed and Catalyzed Hydration of CO<sub>2</sub> at Various Values of  $n$ , the Atom Fraction of Deuterium in Solvent Water

$n$	$k'_{31}{}^a \times 10^2$ (s <sup>-1</sup> )	$V_{\text{max}}{}^b \times 10^2$ (M s <sup>-1</sup> )	$K_{\text{m}}{}^b$ (mM)
0.0	3.75 ± 0.10	6.00 ± 0.30	15.6 ± 0.6
0.248	3.26 ± 0.08	4.40 ± 0.20	11.9 ± 1.1
0.496	2.77 ± 0.08	3.40 ± 0.20	9.1 ± 0.7
0.744	2.66 ± 0.10	2.35 ± 0.12	6.9 ± 0.5
0.992	1.94 ± 0.08	1.85 ± 0.12	5.2 ± 0.4

<sup>a</sup> The rate constant for the uncatalyzed reaction between CO<sub>2</sub> and H<sub>2</sub>O at  $25 \pm 0.2$  °C measured in 10 mM imidazole buffer at pL 6.95. <sup>b</sup> Measured in 50 mM 1,2-dimethylimidazole at  $25 \pm 0.2$  °C and pL 8.2. Median values with standard deviations from three to five experiments are given.

Because of its small concentration relative to (HCO<sub>3</sub><sup>-</sup>), H<sub>2</sub>CO<sub>3</sub> is neglected. In this pH range, the rate constant  $k_{\text{OH}}$  describing the reaction of CO<sub>2</sub> and OH<sup>-</sup> can also be neglected. Values of  $k'_{31}$  measured at various atom fractions of D<sub>2</sub>O at pL of 6.95 and 25 °C are given in Figure 1 and Table I; the buffer imidazole (pK<sub>a</sub> = 7.1) was present at 10 mM and the indicator *p*-nitrophenol was  $5 \times 10^{-5} \text{ M}$ . The concentration of CO<sub>2</sub> used for the data of Figure 1 was 8.5 mM. The variation of  $k'_{31}$  with  $n$  is not linear but slightly bulging down, where  $n$  is the atom fraction of deuterium in the solvent water. The ratio  $(k'_{31})_0/(k'_{31})_1 = 1.93 \pm 0.13$  (average and standard deviation), in which the subscript 0 refers to  $n = 0.016$  and subscript 1 refers to  $n = 0.992$ .

Double-reciprocal plots for the hydration of CO<sub>2</sub> catalyzed by bovine carbonic anhydrase are presented in Figure 2 for a range of CO<sub>2</sub> concentrations and values of  $n$ . The variations in  $V_{\text{max}}$  and  $K_{\text{m}}$  as a function of  $n$  appear in Figures 3 and 4 and Table I. The values plotted are median values of the direct linear plot (Eisenthal & Cornish-Bowden, 1974) with standard deviations from three to five experiments. These experiments were performed at pL 8.2 with the buffer 1,2-dimethylimidazole (pK<sub>a</sub> = 8.2) at 50 mM and the indicator *m*-cresol purple at  $3.0 \times 10^{-5} \text{ M}$ . The data for both  $V_{\text{max}}$  and  $K_{\text{m}}$  as a function of  $n$  are nonlinear and bulging down. The ratios for H<sub>2</sub>O and D<sub>2</sub>O are  $(V_{\text{max}})_0/(V_{\text{max}})_1 = 3.24 \pm 0.37$  and  $(K_{\text{m}})_0/(K_{\text{m}})_1 = 3.0 \pm 0.35$ . These are comparable to the values of  $(k_{\text{cat}})_0/(k_{\text{cat}})_1 = 3.3$  and  $(K_{\text{m}})_0/(K_{\text{m}})_1 = 3.0$  determined for hydration of CO<sub>2</sub> catalyzed by bovine enzyme (Pocker & Bjorkquist, 1977a) with similar values for human II enzyme (Steiner et al., 1975). It has also been reported for both the bovine and human II enzyme that  $(k_{\text{cat}}/K_{\text{m}})_0/(k_{\text{cat}}/K_{\text{m}})_1$  is

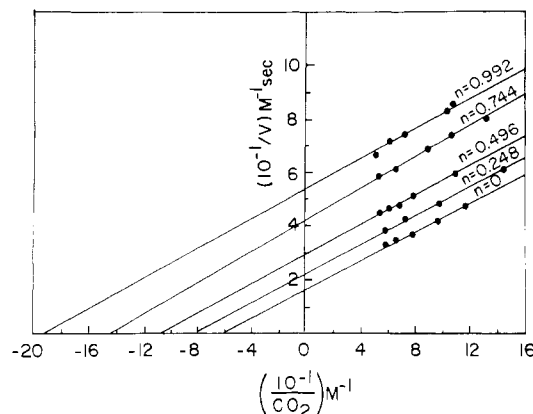


FIGURE 2: Lineweaver-Burk plots for the hydration of  $\text{CO}_2$  at various values of  $n$ , the atom fraction of deuterium in solvent water. The concentration of bovine red cell carbonic anhydrase was  $4 \times 10^{-8}$  M. The rates were measured at  $25 \pm 0.2^\circ\text{C}$  in a solution containing 50 mM 1,2-dimethylimidazole buffer with *m*-cresol purple as the indicator. The pL was 8.20, and the total ionic strength of the solution was maintained at 100 mM by using  $\text{Na}_2\text{SO}_4$ .

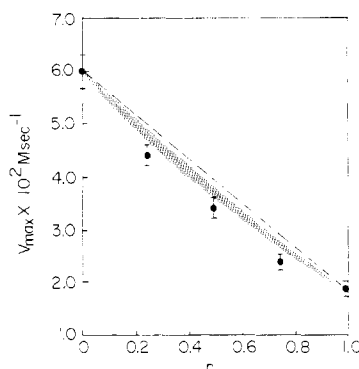


FIGURE 3: Maximum velocity of the catalyzed hydration of  $\text{CO}_2$ ,  $V_{\max}$ , plotted against  $n$ , the atom fraction of deuterium in solvent water. The conditions were the same as those described in Figure 2. The shaded region contains those values of  $V_{\max}$  predicted for a model in which two equivalent hydrogens change their fractionation factors during catalysis. The reactant-state fractionation factors for this model were assumed to be in the region  $0.5 \leq \phi^R \leq 1.2$ . The transition-state fractionation factors  $\phi^T$  are fixed by the values of  $\phi^R$  and the value of the observed isotope effect,  $(V_{\max})_0/(V_{\max})_1 = 3.24$ .

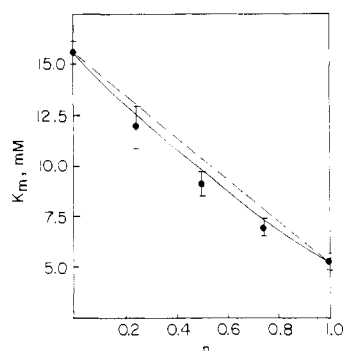


FIGURE 4: Michaelis constant for the hydration of  $\text{CO}_2$ ,  $K_m$ , plotted as a function of the atom fraction of deuterium in solvent water,  $n$ . The experimental conditions are described in Figure 2. The solid curve contains values of  $K_m$  predicted by a two-proton model which obeys the equation  $K_m = 15.6(1 - n + 0.577n)^2$ .

approximately equal to 1 (Pocker & Bjorkquist, 1977a; Steiner et al., 1975). From the data reported here,  $(V_{\max}/K_m)_0/(V_{\max}/K_m)_1 = 1.08 \pm 0.25$ , and experiments at intermediate values of  $n$  confirm that, within experimental error,  $k_{\text{cat}}/K_m$  is invariant with  $n$ . We note here that although our isotope effects at  $n = 0$  and  $n = 1$  are consistent with the literature

values, the value for  $k_{\text{cat}}$  in 100%  $\text{H}_2\text{O}$  that we obtain at pH 8.2,  $1.5 \times 10^6 \text{ s}^{-1}$  for hydration, is larger than the maximum value of  $k_{\text{cat}}$  of  $8.3 \times 10^5 \text{ s}^{-1}$  reported by Pocker & Bjorkquist (1977a).

## Discussion

The variation of a rate constant  $k_n$  with  $n$ , the atom fraction of deuterium in the solvent, is described by the Gross-Butler equation (Gold, 1969):

$$k_n = k_0 \frac{\prod_i (1 - n + n\phi_i^T)}{\prod_j (1 - n + n\phi_j^R)} \quad (5)$$

Here  $\phi_i^T$  is the isotopic fractionation factor for each of the  $\nu$  exchangeable hydrogenic sites of the transition state and  $\phi_j^R$  is the similar factor for each of the  $\nu$  sites in the reactant state. The isotopic fractionation factor  $\phi$  of any given site is defined as the ratio of the preference of the site for deuterium compared to protium relative to the preference of a solvent site for deuterium compared to protium (Schowen, 1978). In some cases,  $\phi_i^T$  may be obtained from experimental values of  $k_n$  vs.  $n$  by fitting the data with known or assumed  $\phi_j^R$  to give an inventory of transition-state protons and the isotope effect associated with each one. Proton inventories of this type have been carried out on both model and enzymic reactions (Schowen, 1977; Schowen, 1978, and references cited therein).

If all the  $\phi_j^R$  are equal to unity, then eq 6 results:

$$k_n = k_0 \prod_i (1 - n + n\phi_i^T) \quad (6)$$

Under these conditions, only transition-state hydrogenic sites contribute to the overall solvent isotope effect. Also,  $k_n$  will vary linearly with  $n$  if only one transition-state hydrogen contributes to the isotope effect. A nonlinear "bulging down" plot for  $k_n$  vs.  $n$  arises if more than one hydrogen contributes to the overall isotope effect and all  $\phi^T < 1$ . In this case we obtain information about the number of transition-state hydrogenic sites and their individual contribution to the overall isotope effect. Nonlinear, "bulging up" plots arise when (1) a large inverse isotope effect (i.e., one or more of the  $\phi_i^T > 1.0$ ) is only partially cancelled by a smaller normal isotope effect or (2) a change in the rate-determining step occurs as  $n$  is varied (Schowen, 1978). It is critical in such interpretations that the contribution of the reactant-state terms, the denominator of eq 5, be accurately accounted for. It is possible that a series of small contributions with  $\phi_j^R \neq 1.0$  may have the effect of cancelling important  $\phi_i^T$  terms in eq 5 (Kresge, 1973).

**Uncatalyzed Hydration of  $\text{CO}_2$ .** The variation of  $k_{31}'$  with  $n$  is shown in Figure 1. The plot is nonlinear and bulging down with  $(k_{31}')_0/(k_{31}')_1 = 1.93$ , a value also reported by Pocker & Bjorkquist (1977b). For this uncatalyzed reaction between  $\text{CO}_2$  and water, there is no reactant-state contribution to eq 5; that is, the fractionation factor of water in the reactant state is known to be 1.0 (Schowen, 1978). Consequently, eq 6, considering only the  $\phi_i^T$ , is valid for this situation. This leads to the conclusion that the bulging down curvature in Figure 1 arises from two or more protons which undergo a change in fractionation factor between the initial and the transition state.

The data for  $k_{31}'$  vs.  $n$  fit a model in which two equivalent hydrogens undergo bonding changes in the transition state. The solid curve in Figure 1 is calculated for such a model by using the equation

$$(k_{31}')_n = 0.0375(1 - n + 0.72n)^2 \quad (7)$$

in which each of the hydrogens has a fractionation factor of  $\phi^T = 0.72$  [ $\phi^T = [(k_{31}')_1/(k_{31}')_0]^{1/2} = (0.0194/0.0375)^{1/2} = 0.72$ ; see Schowen (1978) for details of this procedure]. As demonstrated in Figure 1, the values of  $(k_{31}')_n$  generated by eq 7 agree well with experimental data. Although inspection of Figure 1 allows us to differentiate between a one-proton mechanism and a two-proton mechanism, very high precision would be required to differentiate a two-proton model from other models when the overall isotope effect is 1.9. Consequently, these data alone cannot serve to select among the possible mechanisms for the uncatalyzed reaction between CO<sub>2</sub> and water, three of the most likely of which are described by Pocker & Bjorkquist (1977b). As discussed by these authors, the value of  $(k_{31}')_0/(k_{31}')_1$  is also insufficient to choose among the possible mechanisms.

**Catalyzed Hydration of CO<sub>2</sub>.** Has a conformational change accompanying the change of solvent from H<sub>2</sub>O to D<sub>2</sub>O affected the kinetics of catalysis? Several kinetic parameters which reflect the rate of eq 2 remain unaffected, within experimental errors, upon changing solvent from H<sub>2</sub>O to D<sub>2</sub>O:  $k_{\text{cat}}/K_m$  for initial velocity of hydration of CO<sub>2</sub> (Steiner et al., 1975; Pocker & Bjorkquist, 1977a);  $k_{\text{cat}}^{\text{exch}}$  measured from <sup>13</sup>C NMR line widths of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> at chemical equilibrium (Simonsson et al., 1978); the rate of interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> as measured by <sup>18</sup>O exchange at chemical equilibrium (Silverman et al., 1979). It is also known that the pK<sub>a</sub> of the apparent activity-controlling group in the human and bovine enzymes is ~0.5 unit larger in D<sub>2</sub>O than in H<sub>2</sub>O (Pocker & Bjorkquist, 1977a; Steiner et al., 1975), in agreement with nearly all weak acids with pK<sub>a</sub> values between 3 and 10 (Schowen, 1978). Moreover, solutions of the bovine enzyme stored in D<sub>2</sub>O for many days give the same rates of hydration immediately after dilution in a large amount of H<sub>2</sub>O as do solutions of bovine enzymes stored in H<sub>2</sub>O. These facts suggest that no major conformational changes contribute to the observed solvent deuterium isotope effects.

There is always a concern in interpreting kinetic solvent isotope effects for enzymatic processes that the fractionation factors for the reactant state  $\phi_j^R$  are not accurately known. All reactant-state fractionation factors which represent a hydrogenic site involved in a kinetically important step will be reflected in the plots of rate constants vs.  $n$ . Actually, the fractionation factor for many hydrogenic sites on proteins (imidazole side chains; carboxylic acids; amino groups; hydroxyl groups) show  $\phi^R$  nearly identical with unity (Schowen, 1978). However, in the case of carbonic anhydrase, the zinc-bound hydroxide which is believed to be involved in the catalysis for hydration (Pocker & Sarkanen, 1978) needs special consideration. The fractionation factor  $\phi^R$  for zinc-bound hydroxide will be 1.0 if there is no charge on the oxygen. This is the fractionation factor found for the exchangeable hydrogens of water, alcohols, and carboxylic acids (Schowen, 1978). If zinc-bound hydroxide bears a negative charge, then the value of  $\phi^R$  depends on the degree of solvation: for aqueous hydroxide ion the value  $\phi^R = 0.5$  can be explained by a threefold hydration of the ion, with  $\phi^R$  for the hydrogen of OH<sup>-</sup> itself being ~1.2 and  $\phi^R = 0.7$  for each of three hydrogens, one on each solvating water (Gold & Grist, 1972). Thus, in the extreme that the zinc-bound hydroxide of carbonic anhydrase bears a full negative charge with no waters of solvation,  $\phi^R \approx 1.2$ . We consider it unlikely that the true  $\phi^R$  is as great as 1.2 because of the partial solvation of the zinc-bound hydroxide and at least partial neutralization of the charge on OH<sup>-</sup> by zinc. Nevertheless, we allow for a range of values for  $\phi^R$  of zinc-bound hydroxide in our calculation:

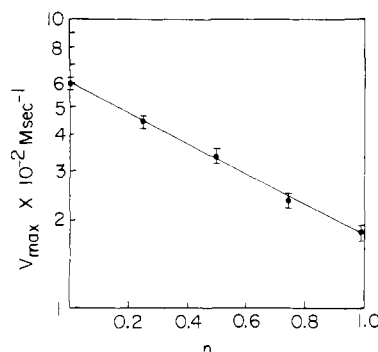


FIGURE 5: Data of Figure 3 plotted with a logarithmic ordinate.

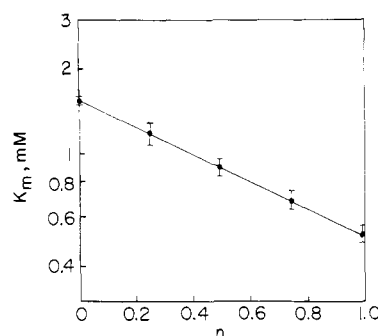


FIGURE 6: Data of Figure 4 plotted with a logarithmic ordinate.

$0.5 \leq \phi^R \leq 1.2$ . This range would also include  $\phi^R$  for zinc-bound water.

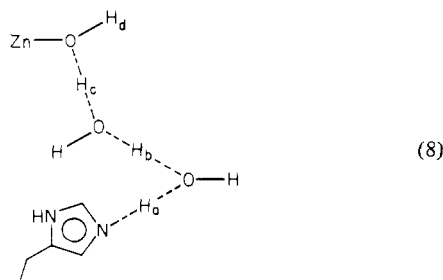
As shown in Figures 3 and 4, plots of  $(V_{\text{max}})_n$  and  $(K_m)_n$  are clearly nonlinear and bulge down. This indicates that the transition state of the rate-limiting step involves changes in the fractionation factor of two or more hydrogens but eliminates the possibility of a mechanism which involves bonding changes for one hydrogen. Figure 3 is an attempt to fit the data for  $V_{\text{max}}$  to a mechanism involving translation of two equivalent protons; the shaded area in Figure 3 contains those values of  $(V_{\text{max}})_n$  consistent with such a model,  $(V_{\text{max}})_n = (V_{\text{max}})_0 (1 - n + n\phi^T)^2 / (1 - n + n\phi^R)^2$ , assuming that the reactant-state fractionation factors are in the region  $0.5 \leq \phi^R \leq 1.2$ . The transition-state factors  $\phi^T$  are fixed by the values of  $\phi^R$  and the value of the observed isotope effect,  $(V_{\text{max}})_0 / (V_{\text{max}})_1 = 3.24$ . This mechanism is marginally consistent with the data and becomes more acceptable when the value of  $V_{\text{max}}$  at  $n = 0$ , taken as one end point in the calculation, is allowed to vary within the limits of the experimental uncertainty.

Going further, we note that for a reaction involving many protons, all contributing a small but approximately equal normal isotope effect, a plot of  $\ln k_n$  vs.  $n$  will be linear (Schowen, 1978). Included in such a multiproton model is the case in which a change in conformation or in charge distribution occurring during the enzymatic reaction alters the fractionation factors of solvating water molecules, thus generating a secondary isotope effect. Figures 5 and 6 show that  $V_{\text{max}}$  and  $K_m$  for catalyzed hydration of CO<sub>2</sub> fit this interpretation better than a one- or two-proton mechanism. This is the first case, to our knowledge, of an enzymic process for which a logarithmic relationship has been shown between the steady-state rate constants and  $n$ . This opens the possibility that the solvent deuterium isotope effect observed for  $k_{\text{cat}}$  and  $K_m$  is caused entirely or in part by solvation changes in the active site which occur in the rate-determining step of catalysis. There is no way with the present data to estimate the number of hydrogens which change their fractionation factor on going from reactant to transition state for these processes; extraordinary precision would be required to determine the

number of protons in translation in a multiproton mechanism.

We now interpret these results using the proposal of Steiner et al. (1975) that an isotope effect localized to the rate-determining step of eq 3 predicts identical isotope effects on  $k_{\text{cat}}$  and  $K_m$ , leaving  $k_{\text{cat}}/K_m$  unaffected, as actually observed for bovine and human carbonic anhydrase II (Steiner et al., 1975; Pocker & Bjorkquist, 1977a). Such a scheme is also consistent with product inhibition of the hydration of  $\text{CO}_2$ , with the pH dependence of  $k_{\text{cat}}$  and lack of pH dependence of  $K_m$ , and with the kinetics of interconversion of  $\text{CO}_2$  and  $\text{HCO}_3^-$  determined from  $^{13}\text{C}$  magnetic resonance line widths (Simonsson et al., 1979). The transfer of a proton between the enzyme and buffer in solution, the step in eq 4, has been found rate limiting only at lower buffer concentration, less than 10 mM (Silverman & Tu, 1975; Jonsson et al., 1976). Since all data reported here were obtained at large buffer concentration, 50 mM 1,2-dimethylimidazole, we do not consider the step of eq 4 to be rate determining for our studies.

Interpreting these results within the proposal of Steiner et al. (1975), we estimate that nearly all of the observed isotope effect results from the rate-limiting proton transfer of eq 3. Moreover, the excellent fit of  $\ln V_{\text{max}}$  and  $\ln K_m$  vs.  $n$  of Figures 5 and 6 suggests that the proton transfer of eq 3 involves water in the active site to a significant extent; a mechanism involving water bridges to relay protons is likely. A possible mechanism which involves water bridges in the transfer of a proton from an imidazole side chain of His-64 to zinc-bound hydroxide is shown in eq 8 and is consistent with the data; three hydrogens



( $\text{H}_a$ ,  $\text{H}_b$ , and  $\text{H}_c$ ) and possibly a fourth ( $\text{H}_d$ ) change bonding in the transition state. The one-proton mechanism involving the direct transfer of a proton from the imidazole to the catalytic group is ruled out based on the data. The magnitude of the observed isotope effect in  $k_{\text{cat}}$  and  $K_m$ , between 3 and 4, is consistent with proton transfer between two groups of approximately equal basicity as observed in model systems (Bergman et al., 1978; Cox & Jencks, 1978). We note that the zinc-bound water and histidine-64 of human carbonic anhydrase II both have values of  $\text{p}K_a$  near 7 (Campbell et al., 1975) and that a rate-limiting intramolecular proton transfer during catalysis between two groups with  $\text{p}K_a$  values near 7 is also indicated by  $^{18}\text{O}$  exchange (Silverman et al., 1979). The proton transfer between zinc-bound water and Glu-106 suggested by Kannan et al. (1977) is also a possibility but requires an unusually high  $\text{p}K_a$  for Glu-106.

It has been suggested by Steiner et al. (1975) that an intramolecular proton transfer might be rate limiting in  $\text{D}_2\text{O}$  whereas in  $\text{H}_2\text{O}$  the catalysis might not be limited by a single step. However, the data for  $V_{\text{max}}$  and  $K_m$  in Figures 2 and 3 eliminate the possibility of the following type of change in rate-determining step as  $n$  is varied: a sequential pathway in which the step or steps limiting in  $\text{H}_2\text{O}$  have a smaller isotope effect than the step limiting in  $\text{D}_2\text{O}$ . If such a change in rate-limiting step exists, we would observe a dependence of  $V_{\text{max}}$  and  $K_m$  on  $n$  that bulges up (Schowen, 1978).

The hydration of acetaldehyde and the hydrolysis of *p*-

nitrophenyl acetate when catalyzed by bovine carbonic anhydrase show an inverse isotope effect on  $k_{\text{cat}}$ , on  $K_m$ , and often on  $k_{\text{cat}}/K_m$  (Pocker & Meany, 1965; Pocker & Stone, 1968; Steiner et al., 1975). For the hydration of acetaldehyde ( $(k_{\text{cat}})_0/(k_{\text{cat}})_1 = 0.5$  (Pocker & Meany, 1965) while for the hydrolysis of *p*-nitrophenyl acetate it is 0.75 (Pocker & Stone, 1968). The observed isotope effect of 0.5 and 0.75 can possibly arise from the reactant-state contribution of zinc-bound hydroxide in the slower catalytic hydrolysis of esters or hydration of acetaldehyde. This would occur when  $\phi^R < \phi^T$  in the rate-limiting step. That such an inverse isotope effect is observed suggests that a step analogous to eq 2 is rate limiting for ester and aldehyde substrates, whereas eq 3 is partially or fully rate limiting for the more rapid hydration of  $\text{CO}_2$ . An inverse isotope effect on the step described by  $k_2$  in eq 2 means that a fractionation factor for zinc-bound hydroxide at the active site less than unity must be considered in the denominator of the Gross-Butler equation. However, as explained in previous comments, a reactant state contribution of  $\phi^R < 1.0$  does not improve the fit of the data to a one-proton mechanism, and it remains a main conclusion of this work that two or more hydrogens undergo bonding changes in the transition state of the rate-limiting step in the hydration of  $\text{CO}_2$  catalyzed by bovine carbonic anhydrase.

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## Formation of Hybrid Luciferases from Subunits of Different Species of *Photobacterium*<sup>†</sup>

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**ABSTRACT:** Enzyme divergence within three species of the genus *Photobacterium* (*P. fischeri*, *P. leiognathi*, and *P. phosphoreum*) was studied by comparing the catalytic characteristics and quaternary interactions of bacterial luciferases isolated from each species. Each luciferase was composed of two subunits of different molecular weights as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subunits were isolated in quantity by DEAE-Sephadex gel filtration in 7 M urea. Isolated subunits had no luciferase activity after renaturation in buffer, but active enzyme could be recovered by renaturation of the heavy and light subunits together. Renaturation of hybrid pairs (containing one subunit

from each of two different species) yielded active luciferases, but only in cases where a heavy subunit of one species was combined with a light subunit of another. These hybrids exhibited in vitro catalytic characteristics most like those of the parent luciferase from which the heavy subunit was derived. The light subunit of *P. leiognathi* luciferase conferred an increased thermal stability to all enzymes containing it. The heavy subunit of each of the three *Photobacterium* species was sensitive to trypsinization. Thus, on the basis of structural and functional analogies with the luciferase from *Beneckea harveyi*, the heavy and light subunits of *Photobacterium* species have been designated  $\alpha$  and  $\beta$ , respectively.

**B**acterial luciferase is an  $\alpha$ - $\beta$  heterodimer catalyzing the mixed-function oxidation by molecular oxygen of reduced flavin mononucleotide and a long-chain aliphatic aldehyde, yielding oxidized flavin, the corresponding acid, water, and a blue-green luminescence (Hastings & Nealson, 1977). The two dissimilar subunits of luciferase can be easily isolated after urea denaturation, and, while the individually renatured subunits have no activity, renaturing them together yields a high recovery of active enzyme (Friedland & Hastings, 1967a).

One of the fundamental questions in luciferase biochemistry has concerned the functions of the two subunits. Studies involving chemical or mutational modifications of one or the other of the subunits isolated from the luminous species *Beneckea harveyi* have indicated that the active center is located on the heavier, or  $\alpha$ , subunit, while the lighter, or  $\beta$ , subunit may be structural (Cline & Hastings, 1972; Meighen et al., 1971). Such investigations have not been reported with any of the other species of marine luminous bacteria.

The taxonomic relationships between different strains of luminous bacteria have recently been clarified (Hastings & Nealson, 1977), and five species in two genera, *Beneckea* and *Photobacterium*, have been designated (Reichelt & Baumann, 1973; Reichelt et al., 1976): *B. harveyi*, *B. splendida*, *P. fischeri*, *P. leiognathi*, and *P. phosphoreum*. It was thus of interest to investigate the possibility of producing active hybrid luciferases by combining in vitro a purified subunit from the luciferase of one species with that of another. Using active

hybrid luciferases prepared in this manner, it was possible to examine whether the species-specific catalytic and physical properties of each native luciferase (Hastings et al., 1969) were determined primarily by one or both of its subunits.

### Materials and Methods

**Materials.** Trypsin TPCK<sup>1</sup> was purchased from Worthington Biochemical Corp.; nonanal, decanal, and dodecanal were obtained from Aldrich Chemical Co.; tridecanal was provided by T. Nakamura, Department of Biology, Osaka University, Japan. Urea (Ultra-Pure) was a product of Schwarz/Mann.

Bacterial luciferase was isolated as previously described (Gunsalus-Miguel et al., 1972) from *B. harveyi*, M17 (Ulitzur & Hastings, 1978); *P. fischeri*, ATCC 7744; *P. leiognathi*, PL 721; and *P. phosphoreum*, NZ-11-D (Ruby & Morin, 1978).

**Luciferase Assay.** Luciferase activity was determined at 25 °C in an assay buffer (20 mM potassium phosphate, pH 7.0, and 0.2% BSA) with the addition of 0.2% dodecanal as substrate aldehyde unless otherwise noted. The reaction was initiated by the injection of 1 mL of a solution of 50  $\mu$ M catalytically reduced flavin mononucleotide, and the light emission was monitored with a photomultiplier-photometer and a strip chart recorder (Hastings et al., 1978). The turnover rate of the reaction was determined from the first-order rate constant ( $k$ ) for the decay of luminescence intensity observed during the reaction. The relative quantum yield ( $cq$ ) of the reaction was calculated from the expression  $cq = I_0 k^{-1}$ , where  $I_0$  is the peak luminescence of the reaction. Both the rate

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<sup>1</sup> Abbreviations used: BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.