Molecularity of Water in Enzymic Catalysis. Application to Carbonic Anhydrase II

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Abstract: In recent papers from this laboratory (Pocker, Y.; Janjić, N. *Biochemistry* **1987**, *26*, 2597; *Biochemistry* **1988**, *27*, 4114), we have examined the carbonic anhydrase II catalyzed CO_2 hydration and HCO_3^- dehydration reactions and their dependence on solution viscosity increase using several cosolutes (glycerol, sucrose, and ficoll). Previous work in this growing field has emphasized the diffusion-controlled feature of fast enzymic processes, whereas our study focuses on the cooperative nature of isomerization processes affecting functionally important motions. In this article we wish to elaborate on the notion that great care must be taken in interpreting such data because the "viscosity" variation method may give rise to a "cosolute" effect by perturbing the original system and that significant reduction in water concentration produced by the addition of viscogenic cosolutes remains a viable and exciting explanation for the observed rate decrease. Analysis of the dependence of the second-order rate constant, k_{cat}/K_m, on water concentration in the carbonic anhydrase II catalyzed CO_2 hydration and HCO_3^- dehydration is of fundamental importance in diagnosing the reaction mechanism and is consistent with the conception that two water molecules participate during turnover.

The upper limit to any bimolecular process is restricted by the encounter frequency between the reacting species as determined by their diffusion rates.[1,2] The diffusion rates are, in turn, proportional to solution viscosity, and the dependence of a chemical reaction on viscosity has traditionally been used as an estimate of the extent to which a bimolecular reaction is diffusion-controlled. Recently, the use of nondenaturing, inert viscogenic probes has become increasingly popular as a novel mechanistic tool for analyzing fast enzymic processes.[3-14] Additionally, these studies have gained a broader importance since the extent to which an enzymic reaction has reached the diffusion limit may provide an index of evolutionary perfection of the catalyst.[15] With a turnover number close to 1×10^6 s^{-1} for CO_2 hydration at pH 9 and 25 °C, the high activity form of carbonic anhydrase (isozyme II) is one of the fastest enzymes known.[11,16-18] In recent papers from this laboratory, we examined the rates of the forward (CO_2 hydration) and reverse (HCO_3^- dehydration) reactions catalyzed by carbonic anhydrase II, eq 1, on solution viscosity increase using

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

several cosolutes (glycerol, sucrose, and ficoll).[10-12] It has been shown through detailed circular dichroic studies that the tertiary structure of the enzyme remains unchanged in these mixed solvents. Additionally, the equilibrium constant for the reversible hydration of CO_2 (Haldane relationship), as calculated from the Michaelis–Menten parameters, did not change with added cosolutes. These rate reductions were interpreted in terms of viscosity induced decreases of the cooperative isomerization rates affecting functionally important motions.[11,12] We have indicated, however, that great care must be taken in interpreting the data because the "viscosity" variation method may give rise to a "cosolute" effect by perturbing the original system.[12] In this article we wish to elaborate on this notion and point out that the significant reduction in water concentration produced by the addition of viscogenic cosolutes remains a viable and exciting explanation for the observed rate decrease.

Experimental Section

Enzyme Solutions. Dialyzed, lyophilized bovine erythrocyte carbonic anhydrase (carbonic anhydrase II) was purchased from Sigma (2700 W-A units/mg). Stock solutions were prepared by dissolving the purified protein in distilled, deionized water. The stock solutions were found to retain full activity for several weeks when stored at 5 °C. Concentration of the active enzyme was determined by correcting the spectrophotometric concentration (ϵ_{280} = 54 000) by a factor obtained from titration of enzymic activity with acetazolamide.

Table I. Water Concentration in Solutions with Glycerol, Sucrose, and Ficoll[a]

	$\rho^{25}{}_4$[b]	$[H_2O]$,[c] M	$[H_2O]_{rel}$[d]	η(cP)[e]
[glycerol], % w/w				
7.46	1.0153	52.15	0.9388	1.05
14.6	1.0320	48.92	0.8806	1.28
23.9	1.0550	44.57	0.8022	1.76
34.9	1.0835	39.15	0.7048	2.66
45.3	1.1110	33.73	0.6072	4.05
[sucrose], % w/w				
6.69	1.0235	53.01	0.9542	1.06
16.1	1.0620	49.46	0.8903	1.45
24.8	1.1010	45.96	0.8273	2.10
30.3	1.1270	43.60	0.7849	2.78
35.6	1.1525	41.20	0.7416	3.79
[ficoll], % w/w				
1.6	1.0028	54.77	0.9859	1.20
3.2	1.0081	54.17	0.9750	1.60
5.2	1.0151	53.42	0.9615	2.21
6.4	1.0194	52.96	0.9534	2.67
8.0	1.0252	52.36	0.9424	3.47

[a] At 25.0 °C. [b] Densities of glycerol–water and sucrose–water solutions are taken from ref 19; densities of ficoll–water solutions are taken from ref 20. [c] Molar concentrations were calculated from the weight percent and solution density values. [d] $[H_2O]_{rel} = [H_2O]/[H_2O]_0$, with subscript zero denoting values in aqueous solutions without cosolutes. [e] Solution viscosities were determined as described in ref 11 and 12.

Substrate Solutions. Saturated solutions of CO_2 were prepared by bubbling CO_2 gas (Airco, grade 4, 99.99% pure) through a fine glass frit for over 20 min into degassed, distilled, deionized water thermostated at 30.0 ± 0.05 °C (solutions that are saturated at 30 °C are undersaturated

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at 25 °C and are therefore considerably more stable; this technique significantly improves the reproducibility of the kinetic measurements). Solutions were then rapidly transferred into the stopped-flow syringe avoiding air contact and thermostated at 25.0 ± 0.02 °C. Concentrations of saturated CO_2 solutions in water and mixed aqueous solvents were determined by titration with standardized $Ba(OH)_2$ solution as described previously.[11]

Reagent grade sodium bicarbonate was purchased from Baker and used without purification. In order to adjust the ionic strength to 0.1, an appropriate amount of sodium sulfate (Baker; reagent grade) was added. Because bicarbonate solutions rapidly absorb CO_2 from the atmosphere, they were kept in stoppered, air-tight syringes and were used within 12 h.

Buffer Components. The following zwitterionic buffers were purchased from Sigma and used as obtained: 2-(*N*-morpholino)ethanesulfonic acid (MES), pK_a = 6.1 and 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS), pK_a = 8.4. Indicators bromocresol purple (pK_a = 6.2, ϵ_{589} = 71 400) and *m*-cresol purple (pK_a = 8.2, ϵ_{578} = 32 000) were obtained from Sigma and used without purification. Buffer concentration in all kinetic assays was 20 mM, with the ionic strength adjusted to 0.1 with Na_2SO_4.

Solution Properties. Densities of aqueous solutions of glycerol, sucrose, and ficoll were obtained from literature sources.[19,20] Molar concentrations of water were calculated from the weight percent and solution density values. Viscosities of these mixed aqueous solvents were determined as previously described.[11,12] The pertinent data are summarized in Table I.

Kinetic Analyses. Initial rates of proton release in CO_2 hydration (or proton uptake in HCO_3^- dehydration) were measured by the changing indicator method described in detail elsewhere.[11,21-23] All buffer factors were determined directly by injecting microliter amounts of standardized HCl into 3 mL of a buffer–indicator system and measuring the resulting changes in the absorbance. The component of the rate due to enzymic catalysis was obtained by subtracting the buffer (uncatalyzed) velocity from the total observed velocity. Values of the buffer rates typically accounted for <10% of the overall rate. Values of k_{cat} and K_m for carbonic anhydrase II catalyzed CO_2 hydration and HCO_3^- dehydration were determined as previously described.[11]

Instrumentation. An extensively modified Durrum-Gibson Model 1300 stopped-flow spectrophotometer was used for all CO_2 hydration and HCO_3^- dehydration runs. Both the apparatus and the experimental technique have been described previously.[11,23] A Cary Model 210 UV–visible double-beam spectrophotometer interfaced with an Apple II/e microcomputer was used for all other spectral analyses. A Radiometer Model PHM 84 research pH meter equipped with a Cole-Palmer Ag/AgCl glass electrode was used to obtain the buffer pH readings.

Results and Discussion

Organization of H_2O molecules in the active site of enzymes in general[24-27] and in carbonic anhydrases in particular[28-32] undoubtedly plays a critical role in the catalytic cycle. In addition to being a formal reactant for the CO_2 hydration reaction (and product for the HCO_3^- dehydration reaction), water also serves to bridge in carbonic anhydrase II the gap between the catalytically important proton transferring groups.[33] The availability *and* appropriate alignment of the catalytically important water molecules are certainly important for maximal turnover rates.

The available evidence from a number of X-ray crystallographic,[34-36] kinetic,[10-12,18,37,38] chemical modification,[39-41] and site-directed modification studies[42] strongly suggests that histidine-64 residue plays an important role as a mediator of protons between the zinc-coordinated water or hydroxide and external buffer. Located near the conical opening of the active site ~6 Å away from the zinc ion,[34-36] this residue serves as the only contact between inner water molecules and bulk water. A recently refined X-ray crystallographic structure of carbonic anhydrase II emphasizes that the distances between histidine-64 and its surrounding water molecules are too short for van der Waals contacts but too long for hydrogen bonds.[36] Consequently, the observed position of this residue represents an average of two conformations that differ by a 180° turn of the imidazole ring.

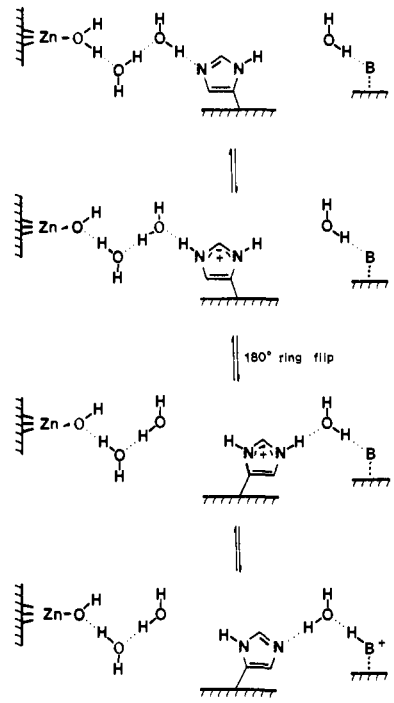

Figure 1. Proposed mechanisms of protonation/deprotonation in the active site of carbonic anhydrase II. If transfer of a proton between the zinc–aquo (or zinc–hydroxo) complex and the imidazole (or imidazolium) side chain of histidine-64 is (at least partially) rate limiting, as is frequently inferred, then the bridging water molecules must be of critical importance for turnover. The external buffer species, B (or BH^+), known to be essential for maximal catalytic rates, is depicted, for convenience, as being bound in vicinity of the active site throughout the proton-transferring cycle. It has recently been shown that when histidine-64 is replaced with alanine, the enzymic activity was only reduced by ca. 2-fold.[42] Therefore, this group need not be essential for high CO_2 hydratase/HCO_3^- dehydratase activity as an imperative proton-transferring group. It is likely, however, that due to the size difference between histidine and alanine side chains, the entry of small buffer molecules into the active site is more facile in the mutant enzyme (in comparison to the native enzyme). Indeed, the mutant Ala-64 enzyme is ideally suited to recognize and bind imidazole buffers. Consequently, the relative importance of the *inter*molecular proton transfer is expected to be increased.

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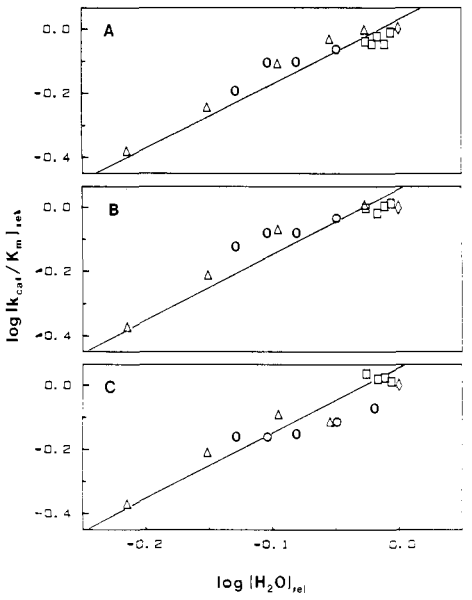

Figure 2. Plots of log $(k_{cat}/K_m)_{rel}$ vs log $[H_2O]_{rel}$ (where $(k_{cat}/K_m)_{rel} = (k_{cat}/K_m)/(k_{cat}/K_m)_0$ and $[H_2O]_{rel} = [H_2O]/[H_2O]_0$, with subscript zero denoting values in aqueous solutions without cosolutes) for carbonic anhydrase II catalyzed HCO_3^- dehydration at pH 5.90 [20 mM 2-(*N*-morpholino)ethanesulfonic acid buffer] (A), CO_2 hydration at pH 5.90 [20 mM 2-(*N*-morpholino)ethanesulfonic acid buffer] (B), and CO_2 hydration at pH 8.80 [20 mM 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid buffer] (C) performed in purely aqueous (◊), glycerol–water (△), sucrose–water (○), and ficoll–water (□) solutions. A slope of 2.0 gives the best integer fit of the data points presented in panels A, B, and C. Michaelis–Menten parameters were obtained from the corresponding Lineweaver–Burk plots. Ionic strength of all solutions was adjusted to 0.1 with Na_2SO_4, and the kinetic runs were performed at 25.0 °C.

Although this rotation appears to be sterically unhindered,[36] it must be recognized that if the ring flip is accompanied by extensive reorganization of hydrogen bonding in the active site, the energy barrier to "rotation" about a single bond may be significantly higher than normally expected. This mechanism is illustrated in Figure 1.

The solvent deuterium isotope effect on k_{cat} of 3–4 observed at saturating buffer concentrations[23,43] indicates that events involving some kind of proton transfer are at least partially rate limiting during turnover. It has been proposed that under such conditions, the intramolecular proton transfer between the active site zine–aquo (or zinc–hydroxo) complex and the imidazole (or imidazolium) side chain of histidine-64 operates through several water bridges and may become partially rate limiting.[18,23,37,38] Further evidence for the importance of histidine-64 comes from numerous chemical modification studies: all known alkylations that target this residue significantly reduce the effectiveness of carbonic anhydrase II as a CO_2 hydratase/HCO_3^- dehydratase.[39-41] While there is little question that this group is involved in the catalytic cycle, the exact extent of its importance for the maximal turnover rates is not clear. It was recently reported that when this residue was replaced with alanine by site-directed mutagenesis techniques, the activity was only reduced by ~2-fold.[42] It must be realized, however, that due to the size difference between histidine and alanine side chains, the "gate" that leads to the zinc-coordinated water or hydroxide ion is expected to be bigger for the mutant enzyme. This change would facilitate the entry of external buffer molecules, especially imidazole, into the active site and therefore increase the relative importance of *inter*molecular proton transfer. It is relevant to point out that a detailed examination of buffer dependence and efficiency in CO_2 hydration and HCO_3^- dehydration reactions catalyzed by wild type carbonic anhydrase II revealed the participation of two distinct families of buffers.[10a,b] The mutant enzymes would be expected to be even more sensitive to the specific properties of buffer species with regard to their ability to fit into the active site in an orientation suitable for accepting (or donating) a proton from (or to) the zinc-coordinated water (or hydroxide ion).

In Figure 2, the logarithm of the second-order rate constant, k_{cat}/K_m, for carbonic anhydrase II catalyzed CO_2 hydration and HCO_3^- dehydration is plotted against the logarithm of water concentration. Slopes of these plots, calculated from the least-squares fit of the data points, are close to 2. This observation is of value in diagnosing the reaction mechanism and is consistent with the idea that two water molecules participate during turnover. It is important to realize, however, that this analysis assumes that the concentration of water in the active site of the enzyme parallels that in the bulk solvent, and this need not always be the case.[26,44-46] Furthermore, binding of water molecules in the interior of the active site is analogous to substrate binding and is therefore expected to display saturation behavior. Consequently, the kinetic order with respect to water is only meaningful if the binding sites for water are not completely occupied throughout this study. The experimental difficulties associated with working in exceedingly viscous solvents, however, preclude us from extending this analysis to the more interesting lower water concentration region. Nevertheless, at least one of the common viscogenic cosolutes (i.e., glycerol) is small enough to penetrate into the active site and directly perturb the organized water structure, thus diminishing the effective water concentration. The fact that several primary and secondary alcohols have been shown to be inhibitory toward carbonic anhydrase II catalyzed aldehyde hydration (with K_i values ranging from 0.08 to 1.2 M) is consistent with this idea.[47]

The experimentally observed reduction in rate of carbonic anhydrase II catalysis toward its natural substrates can therefore be the result of either an increase in solvent viscosity *or* a decrease in water concentration. Additional studies with nondenaturing reagents that can significantly decrease the water concentration without inducing an increase in viscosity, or vice versa, should further clarify this important question.

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