the trifunctional reagent TCEA provides a new tool for studying protein-protein contacts in various proteins which possess multisubunit and multidomain structures. Its utility best can be appreciated in this paper by the success in the cross-linking of three domains of myosin S-1.

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Registry No. ATPase, 9000-83-3; TCEA, 555-77-1; BCEA, 51-75-2.

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Origin of Viscosity Effects in Carbonic Anhydrase Catalysis. Kinetic Studies with Bulky Buffers at Limiting Concentrations†

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ABSTRACT: In our earlier paper we showed that the rates of CO_2 hydration and HCO_3^- dehydration catalyzed by the high-activity form of mammalian erythrocyte carbonic anhydrase (CA II) were dependent on solution viscosity increase and that the effect was linked to some kind of proton-transfer-related event [Pocker, Y., & Janjič, N. (1987) *Biochemistry 26*, 2597-2606]. In order to further elucidate the source of the observed viscosity effect, the dependence of k_{cat} and K_m for CA II catalyzed HCO_3^- dehydration at pH 5.90 on sucrose-induced viscosity increase was investigated at several concentrations of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, including the very low buffer concentration region (<10 mM) where the proton transfer between the shuttle group on the enzyme and buffer becomes rate limiting. In all examined cases, k_{cat} steadily decreased with added sucrose while K_m remained independent of the viscosity increase. The extent to which this reaction was dependent on viscosity was found to be constant, within experimental error, over the entire range of MES buffer concentrations studied (1-20 mM). Furthermore, the viscosity effect was qualitatively and quantitatively the same when an exceptionally large buffer (i.e., bovine serum albumin) was used instead of the more commonly used biological buffer (i.e., MES). We conclude from these observations that it is unlikely that reduction in translational diffusion rates of the substrate or the buffer species contributes significantly to the observed viscosity effect and present evidence that supports our assertion that the rate-limiting proton transfer between the zinc-water or zinc-hydroxide and buffer is affected through viscosity- (or cosolute-) induced changes in intramolecular isomerization rates involving functionally important motions.

The only known physiological role of carbonic anhydrase is in catalyzing the interconversion of CO_2 and HCO_3^- (eq 1).

$$EH^+ + HCO_3^- \rightleftharpoons E + CO_2 + H_2O \quad (1)$$

With a turnover number close to 1×10^6 s^{-1} at 25 °C, the high-activity form of mammalian erythrocyte carbonic anhydrase (CA II)[1] is one of the fastest enzymes known (Edsall, 1967; Khalifah, 1971; Pocker & Sarkanen, 1978; Lindskog,

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[1] Abbreviations: CA II, carbonic anhydrase, high-activity isozyme; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; BCP, bromocresol purple.

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1986). According to Albery and Knowles (1976), the extent to which an enzymatic process has reached the diffusion limit is a measure of its evolutionary perfection. Viscogenic probes have recently been used as an important tool in mechanistic studies of a number of fast enzymatic processes (Nakatani & Dunford, 1979; Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984; Bazelyansky et al., 1986). In our earlier paper in this series (Pocker & Janjić, 1987) we examined the dependence of CA II catalysis on solution viscosity increase induced by addition of glycerol, sucrose, and ficoll (a copolymer of sucrose and epichlorohydrin). Ficoll, as anticipated, was found to be unsuitable as a viscogenic probe because it failed to appreciably decrease the mobilities of smaller species despite its capacity to greatly increase the macroscopic viscosity of the medium. In view of the inhibitory tendency of glycerol and that of its trace impurities, we prefer to regard the weaker effect induced by sucrose as the most reliable index of the "true" viscosity effect. Analysis of individual Michaelis–Menten parameters, k_{cat} and K_m, corresponding to the classical one-substrate, one-product mechanism, revealed that for both CO_2 hydration and HCO_3^- dehydration the viscosity effect was confined to the turnover term and that this term decreased as the viscosity increased. Since the Michaelis constant was found to be independent of viscosity in both the forward and reverse directions of the enzymatic catalysis and because K_m is predominantly determined by the rate constants describing association and dissociation of the enzyme with its substrate (Rowlett, 1984; Lindskog, 1986), we concluded that the observed viscosity-induced rate decrease was in all probability not due to reduced diffusion rates of the substrate species.

In order to complete a full round of catalysis, the enzyme has to abstract a proton following the dehydration reaction in eq 1. It has by now been widely recognized (Khalifah, 1973; Prince & Wooley, 1973; Jonsson et al., 1976; Lindskog & Coleman, 1973; Pocker et al., 1981, 1985, 1986; Rowlett & Silverman, 1982; Lindskog, 1986) that the external buffer species, normally present at moderate concentration in a kinetic assay, plays a principal role in this process (eq 2). We have

$$E + BH^+ \rightleftharpoons EH^+ + B \qquad (2)$$

considered in earlier papers from our laboratory the possibility that an internally located group B_iH^+ (or B_i) with a pK_a (or pK_b) near 7 may be involved in the dynamics of proton transfer between the zinc-coordinated H_2O or OH^- and the buffer system of the medium, eq 3 (Pocker & Meany, 1965; Pocker

$$ZnOH|B_i|BH^+ \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} ZnOH|B_iH^+|B \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} ZnOH_2|B_i|B \qquad (3)$$

& Bjorkquist, 1977; Pocker et al., 1986; Pocker & Janjić, 1987). The proton relay depicted for HCO_3^- dehydration is envisioned as occurring in two distinct stages: an *inter*molecular stage a and an *intra*molecular stage b.[2] Diminished diffusion rates of the buffer molecules would affect stage a and the overall rate, provided that this step is rate limiting. Stage b, on the other hand, could be affected through inter alia viscosity-dependent conformational changes involving intramolecular isomerization rates and other functionally important motions (Frauenfelder & Wolynes, 1985; Perutz et al., 1987). In this paper we endeavor to provide additional insight into the source of the observed viscosity effect by extending the study of CA II catalysis to low buffer concentrations, a region where proton-transfer processes become rate limiting. We also make use of an exceptionally bulky physiological buffer whose diffusion rates are several orders of magnitude slower than those of the smaller, more commonly employed, buffers. Serious use of such exceptionally bulky buffers is being extended in an attempt to scrutinize the catalytic mechanism of other enzymes.

[2] BH^+ may be bound to E so that intermolecular stage may imply BH^+ acting as second substrate along with HCO_3^-.

Experimental Procedures

Enzyme Solutions. Dialyzed, lyophilized bovine carbonic anhydrase (CA II) was obtained from Sigma (2500 Wilbur–Anderson units/mg of protein). The enzyme was dissolved in distilled, deionized water to make 10^{-5} M stock solutions and the spectrophotometric concentration determined from the absorbance at 280 nm (ϵ_{280} = 54 000 M^{-1} cm^{-1}). Titration of enzymatic activity with acetazolamide yielded a factor by which the spectrophotometric concentration needed to be divided in order to obtain the concentration of active enzyme. A single batch of CA II was used throughout this work. In all of the enzyme-catalyzed kinetic runs, the CA II concentration was 2.3×10^{-7} M upon 1/1 dilution in the stopped-flow cell. All buffer–CA II solutions were made fresh and used within 48 h of preparation.

Bicarbonate Solutions. Reagent-grade sodium bicarbonate was purchased from Baker and used as substrate in all kinetic experiments. Bicarbonate solutions were prepared by dissolving a measured amount of this compound in distilled, deionized, CO_2-free water. The ionic strength of all solutions was adjusted to 0.1 by addition of an appropriate amount of anhydrous sodium sulfate (Baker, reagent grade). Kept in stoppered, airtight syringes, bicarbonate solutions were good for 24 h but were normally used within 3 h of preparation.

Buffer–Indicator System. Initial rates of proton uptake in the dehydration of HCO_3^- were measured by the changing indicator method described in detail elsewhere (DeVoe & Kistiakowsky, 1961; Gibbons & Edsall, 1964; Khalifah, 1971; Pocker & Bjorkquist, 1977). 2-(*N*-Morpholino)ethanesulfonic acid (MES), pK_a = 6.10, purchased from Sigma, was found to be a monohydrate (M_r 213.2) and was used without purification. Extensively dialyzed (against distilled water), deionized, fraction V bovine serum albumin (BSA) was obtained from Sigma and used without further treatment. BSA solutions of 10 mg/mL (1.5×10^{-4} M using M_r 65 000) were used in the kinetic runs. The ionic strength of all buffer solutions was adjusted to 0.1 with sodium sulfate. Bromocresol purple indicator (BCP) was added to the buffer solutions in concentrations of ca. 1×10^{-5} M in order to give initial absorbances of 0.2 in the stopped-flow cell after 1/1 dilution. The acidity constant for BCP at the ionic strength of 0.1 and 25 °C was determined in MES and BSA buffers by a standard spectrophotometric procedure to have the values of 6.20 and 6.30, respectively. The λ_{max} value corresponding to the basic form of the indicator was shifted from 589 nm (ϵ_{589} = 71 400) to 603 nm (ϵ_{603} = 76 600) in the pH 5.8–7.0 region when BSA was in greater than sixfold excess over BCP. Indeed, interaction of indicator dyes with albumin has been extensively studied (Steinhardt & Reynolds, 1969). All buffer factors [=d[H^+]/d(absorbance)] were determined experimentally by measuring the absorbance changes of the buffer–indicator system at 589 and 603 nm for MES–BCP and BSA–BCP solutions, respectively, upon addition of microliter aliquots of 1.00 N NaOH.

Viscosity. Viscosities of aqueous solutions of sucrose (Baker, reagent grade) at 25.0 ± 0.01 °C determined on a Cannon-Ubbelohde viscometer were in good agreement with values in the literature (Swindells et al., 1958). Somewhat higher values were measured when sucrose solutions contained 1.5×10^{-4} M BSA (1% w/v).

Table I: Viscosity Dependence of k_{cat} and K_m for CA II Catalyzed HCO_3^- Dehydration at Several Concentrations of MES Buffer[a]

	20 mM[b]		10 mM[b]		5 mM[b]		3 mM[b]		1 mM[b]	
solution viscosity[c] (cP)	k_{cat}[d]	K_m[e]	k_{cat}[d]	K_m[e]	k_{cat}[d]	K_m[e]	k_{cat}[d]	K_m[e]	k_{cat}[d]	K_m[e]
0.89 (0)[f]	3.90	20.5	3.52	17.7	2.40	12.5	1.68	8.3	1.20	5.4
1.45 (16.1)	3.61	22.0	2.91	18.1	2.00	11.7	1.65	9.3	0.87	4.3
2.10 (24.8)	3.23	21.5	2.51	17.5	1.76	11.2	1.50	9.5	0.73	4.0
2.78 (30.3)	3.24	21.7	2.39	18.2	1.66	12.3	1.17	7.8	0.64	4.3
3.79 (35.6)	2.36	19.4	2.06	17.6	1.45	11.8	1.06	7.9	0.57	4.6

[a] Kinetic runs were performed at pH 5.90, ionic strength 0.1 (adjusted with Na_2SO_4), and 25.0 °C. [b] Concentrations of MES buffer. [c] Viscosity of aqueous sucrose solutions at 25.0 °C. [d] Reported values are $k_{cat} \times 10^{-5}$ (s^{-1}). [e] In mM. [f] Sucrose concentrations (% w/w).

Instrumentation. All HCO_3^- dehydration runs were performed on an extensively modified Durrum–Gibson Model 1300 stopped-flow spectrophotometer. Both the stopped-flow apparatus and the experimental technique have been described in detail in earlier papers from our laboratory (Pocker & Bjorkquist, 1977; Pocker & Fong, 1980). A Cary Model 210 UV–visible double-beam spectrophotometer interfaced with an Apple II/e microcomputer was used for determination of spectrophotometric concentrations of CA II stock solutions and evaluation of buffer factors. Since it has been shown that the glass electrode method yields correct values of hydrogen ion activities in aqueous organic solvents (Marshall & Grunwald, 1953; Bacarella et al., 1958), a Radiometer Model PHM 84 research pH meter equipped with a Cole–Palmer Ag/AgCl glass electrode was used to obtain buffer pH readings.

Evaluation of Catalytic Parameters. Experimentally determined initial slopes of absorbance vs time plots were multiplied by the buffer factor [=d[H^+]/d(absorbance)] in order to obtain velocities. The component of the rate due to enzymatic catalysis was calculated by subtracting the buffer (uncatalyzed) velocity from the total velocity. Values of k_{cat} and K_m for CA II catalyzed HCO_3^- dehydration were determined from the initial rate measurements according to the formalism of Lineweaver and Burk. Ordinate and abscissa intercepts of the $1/V$ vs $1/[S]$ plots were determined by least-squares analysis of the data points. All kinetic runs were performed at 25.0 ± 0.02 °C.

Results

In our earlier papers (Pocker et al., 1986; Pocker & Janjić, 1987), we showed that a remarkable similarity existed between the viscosity effects associated with CA II catalyzed CO_2 hydration and HCO_3^- dehydration reactions. Furthermore, the equilibrium constant for the reversible hydration of CO_2 was not perturbed by the enzyme in the presence of viscogenic cosolutes. The fact that k_{cat} was the only parameter affected by the viscosity increase indicated that some kind of proton-transfer-related event was primarily responsible for the observed rate decrease. In this paper, the viscosity dependence of k_{cat} and K_m for CA II catalyzed HCO_3^- dehydration[3] was investigated at several concentrations of MES buffer (Table I, Figure 1), including the very low buffer concentration region where the proton transfer between the shuttle group and buffer becomes rate limiting (Silverman & Tu, 1975; Jonsson et al., 1976; Rowlett & Silverman, 1982; Pocker et al., 1986; Y. Pocker and C. H. Miao, unpublished observations). It may be noticed that, in all examined cases, k_{cat} steadily decreased with added sucrose while K_m remained independent of the viscosity increase.

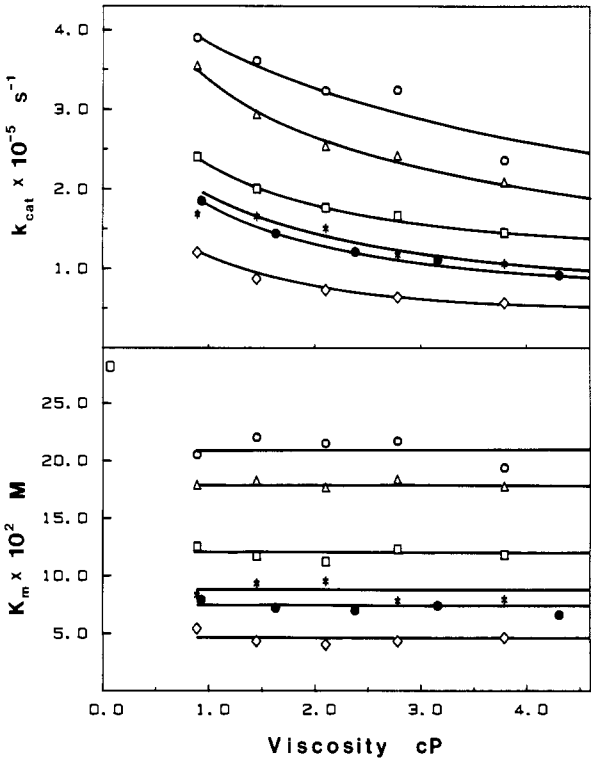

FIGURE 1: Dependence of k_{cat} and K_m for CA II catalyzed HCO_3^- dehydration on sucrose-induced solution viscosity increase at pH 5.90, ionic strength of 0.1, and 25.0 °C in 20 (○), 10 (△), 5 (□), 3 (*), and 1 (◇) mM MES and "2.6 mM" (●) BSA buffers. The value in quotation marks represents the effective buffering capacity of BSA at this pH and corresponds to 1.5×10^{-4} M BSA (see text for details). Note that the viscosities of solutions containing BSA are higher, as expected.

The theoretical basis for quantitating the extent to which CA II catalysis is subject to changes in diffusion rates and/or viscosity has been discussed in detail recently (Pocker et al., 1986; Pocker & Janjić, 1987); consequently, only a brief overview consistent with clarity will be given here. Combining the Stokes–Einstein equation for the diffusion of spherically symmetric particles in a liquid of given viscosity (η) with the Smoluchowski expression (1917) for the second-order diffusion-controlled rate constant, as modified by Collins and Kimball (1949), one obtains eq 4, where k_{obsd} is the observed

$$1/k_{obsd} = 1/k_0 + 1/k_d = 1/k_0 + 3000 r_E r_X \eta / 2RT(r_E + r_X)R_e \quad (4)$$

second-order rate constant for the reaction ($=k_{cat}/K_m$), k_0 is the intrinsic rate constant (i.e., the limiting value of k_{obsd} at zero viscosity), r_E and r_X are the hydrodynamic radii of the enzyme and the substrate or the buffer species, respectively,

[3] At pH values where CA II is most efficient as a dehydratase (i.e., below pH 6), nonenzymatic rates of HCO_3^- dehydration are essentially independent of sucrose concentration. On the other hand, at values above pH 8 where the enzyme is a most efficient hydratase, nonenzymatic rates of CO_2 hydration are strongly catalyzed by sucrose and other viscogenic cosolutes (Pocker & Janjić, 1987). Furthermore, the rate of CA II catalysis is known to be reduced at low concentrations of buffer. For these reasons we believe that the study of viscosity dependence of CA II catalyzed HCO_3^- dehydration at low pH (5.90) yields the most reliable results.

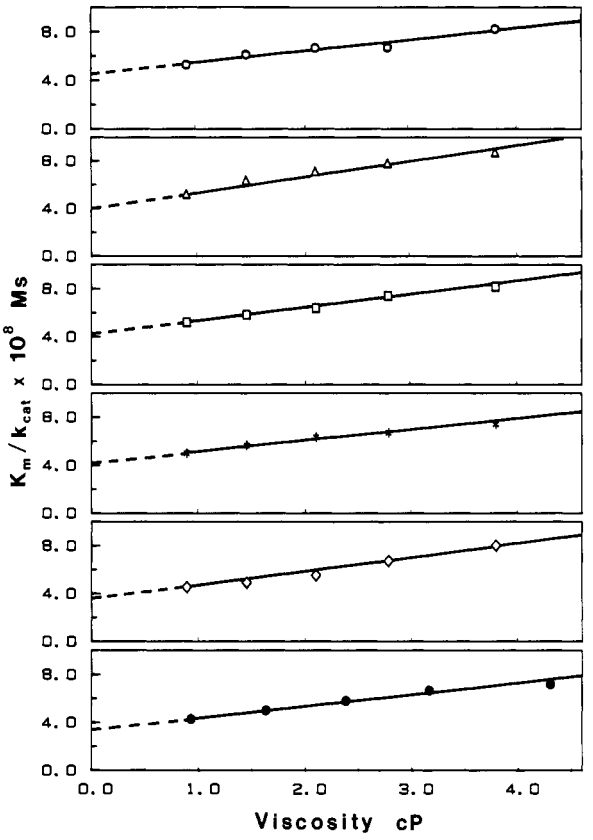

FIGURE 2: Dependence of K_m/k_{cat} on solution viscosity for HCO_3^- dehydration. Conditions and symbols are the same as in Figure 1.

R is the gas constant, T is the absolute temperature, and R_e is the encounter radius. Clearly, the form of eq 4 dictates that a plot of K_m/k_{cat} against solution viscosity should be linear, which is indeed observed for CA II catalyzed CO_2 hydration and HCO_3^- dehydration (Pocker & Janjič, 1987). Here we show that good linearity of these plots is maintained at all studied concentrations of external buffer (Figure 2). Slopes of the above-mentioned plots may be used to obtain an estimate of the apparent degree of diffusion-controlled character for this reaction at a given viscosity, δ, by comparison with the slope of the line representing the diffusion limit, eq 5. Values

$$\delta = 1 - k_{obsd}/k_0 \qquad (5)$$

of δ for HCO_3^- dehydration at viscosity equal to 0.890 cP (i.e., viscosity of water at 25.0 °C) obtained from the weighted linear least-squares fit of the plots in Figure 2 are clearly independent of the MES buffer concentration.[4]

Variations in the size of the reacting molecules do not affect the calculated diffusion-controlled rate unless the ratio of their hydrodynamic radii is significantly altered (Caldin, 1964). Additionally, if the encounter radius, R_e, is identified with the sum of the hydrodynamic radii of the reactants,[5] it can be shown that the value of the diffusion-controlled rate constant, k_d, will be smallest when $r_E = r_X$. If k_d is closer in magnitude to the observed second-order rate constant, k_{obsd}, and if the diffusion of buffer species (partially) limits the rate, then the effect of increased viscosity on CA II catalysis should be more pronounced. For these reasons, we chose to employ a buffer with molecular dimensions similar to those of CA II, namely, bovine serum albumin (BSA).

Table II: Viscosity Dependence of k_{cat} and K_m for CA II Catalyzed HCO_3^- Dehydration in Solutions Buffered with BSA[a]

	"2.6 mM" BSA[b]	
solution viscosity[c] (cP)	$k_{cat} \times 10^{-5}$ (s^{-1})	K_m (mM)
0.93 (0)[d]	1.85	7.9
1.63 (16.1)	1.44	7.2
2.38 (24.8)	1.21	7.0
3.16 (30.3)	1.11	7.4
4.31 (35.6)	0.92	6.6

[a] Conditions are the same as in Table I. [b] This value is the effective buffer concentration and corresponds to 1.5×10^{-4} M BSA (see text for details). [c] Viscosities of aqueous sucrose solutions with 1.5×10^{-4} M BSA. Note that the values are somewhat higher than the values in Table I. [d] Sucrose concentration (% w/w).

The use of albumin as the only mediator of protons in CA II catalysis requires that its effective buffering capacity at this pH be quantitatively related to the buffering capacity of standard biological buffers (e.g., MES). Because our kinetic measurements make use of the changing indicator technique, buffer factors [Q = d[H^+]/d(absorbance)] are evaluated experimentally by measuring the changes in absorbance of the buffer–indicator system following injections of small aliquots of standardized acid or base. This quantity can also be calculated by eq 6, where $[B]_t$ and $[I]_t$ refer to the total stoi-

$$Q = \frac{[B]_t}{[I]_t}\frac{K_a^B}{K_a^I}\frac{1}{\epsilon d}\left(\frac{K_a^I + [H^+]}{K_a^B + [H^+]}\right)^2 \qquad (6)$$

chiometric concentrations of buffer and indicator, respectively, K_a^B and K_a^I are the acid dissociation constants of buffer and indicator, respectively, ϵ is the extinction coefficient of indicator, and d is the optical path length. For substances commonly employed as buffers, all quantities on the right-hand side of eq 6 can be determined with good accuracy by standard methods, and indeed, the calculated values of Q are typically in excellent agreement with the experimental values. When BSA is used as buffer, however, neither $[B]_t$ nor K_a^B are well-defined quantities. Nevertheless, if we can estimate the $[B]_t$ of BSA for this pH region, a value of K_a^B can be obtained provided that Q is known from experiment. Fortunately, hydrogen ion equilibria of serum albumins from human (HSA) and bovine sources have been studied intensively by the physical chemists (Edsall & Wyman, 1958). It has been reported that the titration behavior of HSA and BSA in the pH region between 5.5 and 8 can be adequately described by considering ionizations of all histidine residues (Tanford, 1950; Tanford et al., 1955). Assuming that the effective buffer strength of 1.5×10^{-4} M BSA solution is 17 times greater (BSA has 17 histidine residues) at pH 5.90 and using $Q = 5.60 \times 10^{-3}$, $[I]_t = 6.60 \times 10^{-6}$ M, $pK_a^I = 6.30$, $\epsilon_{603} = 7.66 \times 10^4$ M^{-1} cm^{-1}, and $d = 1$ cm, we obtain a value for pK_a^B of 6.1, indeed a reasonable average value for ionization of imidazolium side chains of histidines. Consequently, we will refer to 1.5×10^{-4} M BSA solutions as the "2.6 mM" BSA buffer.

Listed in Table II are the values of k_{cat} and K_m for HCO_3^- dehydration determined in such a system at several solution viscosities. The similarity of both catalytic parameters with the values determined in MES buffer of similar effective concentration (Table I, Figure 1) is indeed remarkable.

[4] Because our data fit an expression for a second-order diffusion-controlled rate constant, we have used the values of δ as a convenient index for comparing the extent of dependence of CA II catalysis on sucrose concentration. The possibility that the "viscosity" effect is actually a "cosolute" effect induced by sucrose on the equilibrium structure of the enzyme cannot be ruled out.

[5] Transfer of a proton from the core of the active site (i.e., $Zn-OH_2$) to a region near the surface of the protein is believed to be mediated by an internally located group (i.e., histidine-64) so that the external buffer species need not penetrate deeply into the enzyme interior. Consequently, in this case, it is reasonable to equate the encounter radius with the sum of hydrodynamic radii of the reactants.

Table III: Buffer Dependence of Apparent Degrees of Diffusion Control, δ, for CA II Catalyzed HCO_3^- Dehydration in Sucrose Solutions at 25 °C[a]

	δ ($\pm 2\sigma$)
[MES] (mM)	
1	0.22 ± 0.03
3	0.16 ± 0.03
5	0.18 ± 0.01
10	0.23 ± 0.05
20	0.16 ± 0.04
[BSA] (mM)	
"2.6"[b]	0.21 ± 0.04[c]

[a] Calculated from weighted least-squares analysis of K_m/k_{cat} vs viscosity plots according to eq 5. [b] This value is the effective buffer concentration of 1.5×10^{-4} M BSA (see text for details). [c] The somewhat higher viscosities corresponding to sucrose solutions with 1.5×10^{-4} M BSA (Table II) were used in the calculation.

Furthermore, the use of BSA as the only buffer does not affect the extent of dependence of K_m/k_{cat} on viscosity (Figure 2, Table III). Thus, these BSA solution assays may also be regarded as being in the limiting buffer concentration region.

Discussion

The participation of buffer in the rapid protonation/deprotonation of the active site of CA II is now supported by several lines of evidence; initial rates of CA II catalyzed CO_2 hydration and HCO_3^- dehydration (Jonsson et al., 1976; Rowlett & Silverman, 1982; Pocker et al., 1986; Y. Pocker and C. H. Miao, unpublished observations) as well as the catalytic exchange of ^{18}O between CO_2 and H_2O (Silverman & Tu, 1975; Silverman et al., 1978; Tu et al., 1981) are appropriately reduced at low buffer concentrations, and the rate of proton transfer between enzyme and external buffer is a function of the pK difference between donor and acceptor species (Rowlett & Silverman, 1982; Pocker et al., 1986), as expected from theoretical considerations (Eigen, 1964). The catalytic cycle of CA II pertaining to the reversible hydration of CO_2 can thus be subdivided into the turnover step that includes product release (eq 1) followed by the proton-transfer step that regenerates the original protonation state of the active site (eq 2). It has been pointed out previously that this reaction pathway is essentially the same as the classical two-step transfer (ping-pong) mechanism (Jonsson et al., 1976; Rowlett & Silverman, 1982; Rowlett, 1984; Y. Pocker and C. H. Miao, unpublished observations) with buffers acting as a second substrate. A steady-state expression suitable for analysis of initial rates of HCO_3^- dehydration can be written in the form of eq 7, where $K_m^{BH^+}$ and $K_m^{HCO_3^-}$ are the Michaelis constants

$$\frac{[E]_t}{V} = \frac{1}{k_{cat}} \frac{K_m^{BH^+}}{[BH^+]} + \frac{1}{k_{cat}} \left(1 + \frac{K_m^{HCO_3^-}}{[HCO_3^-]} \right) \quad (7)$$

for the protonated buffer and bicarbonate, respectively. It is apparent that at saturating buffer concentrations (i.e., when $[BH^+] \gg K_m^{BH^+}$), eq 7 is reduced to the standard one-substrate, one-product Michaelis–Menten expression. Furthermore, the form of eq 7 predicts that the apparent values of k_{cat} and K_m, determined from the ordinate and abscissa intercepts of the $1/V$ vs $1/[HCO_3^-]$ plots, respectively, be reduced at low buffer concentrations, as is observed (Table I, Figure 1). The second-order rate constant, k_{cat}/K_m, which is proportional to the slope of the above plots, should not be dependent on the buffer concentration, and this is also in agreement with experiment (Figure 2). The apparent Michaelis constant for the buffer, $K_m^{BH^+}$, has been recently estimated for a large number of buffers with values in the millimolar range (Jonsson et al., 1976; Rowlett & Silverman, 1982; Y. Pocker and C. H. Miao, unpublished observations). However, caution should be exercised when interpreting the physical meaning of this quantity since, as pointed out by Pocker and Miao, the value corresponding to the "true" dissociation constant of the enzyme–buffer complex is actually 1–2 orders of magnitude larger, so that only a rather loose binding need be invoked.[6]

With the maximal value of 5×10^8 M^{-1} s^{-1}, the second-order rate constant describing transfer of a proton between enzyme and buffer is very close to the diffusion-controlled limit (Pocker et al., 1986; Y. Pocker and C. H. Miao, unpublished observations). If this process was truly a simple bimolecular hydrogen ion exchange between donor and acceptor moieties that becomes rate determining at limiting buffer concentrations (<10 mM), then the reaction should become increasingly dependent on viscosity as the buffer concentration is decreased. This effect should be even more pronounced with an exceptionally large buffer like BSA. However, the extent of dependence of CA II catalysis on viscosity is here clearly shown to be independent of the buffer concentration over a 1–20 mM range. Additionally, the viscosity effect was found to be the same, within experimental error, in MES and BSA buffers. These facts, taken together with the observation that the Michaelis constant for substrate binding is also viscosity independent, render it unlikely that translational diffusion events are the source of the observed viscosity effect as the rate-limiting step in CA II catalysis.

There are now numerous kinetic (Steiner et al., 1975; Pocker & Bjorkquist, 1977; Rowlett & Silverman, 1982; Lindskog, 1986; Pocker et al., 1986; Y. Pocker and C. H. Miao, unpublished observations), X-ray crystallographic (Liljas et al., 1972; Kannan et al., 1975; Eriksson et al., 1986), and chemical modification studies (Göthe & Nyman, 1972; Khalifah & Edsall, 1972; Y. Pocker and C. H. Miao, unpublished observations; Pocker & Janjić, 1988) that strongly suggest the important role of histidine-64 as the mediator of protons between the zinc-coordinated water or hydroxide ion and external buffer. The solvent deuterium isotope effect on k_{cat} of 3–4 observed at saturating buffer concentrations (Steiner et al., 1975; Pocker & Bjorkquist, 1977) indicates that events involving some kind of proton transfer must be at least partially rate limiting during turnover. It has been proposed that it is the intramolecular proton transfer between the active site zinc–aquo or zinc–hydroxo complex and the imidazole group of histidine-64 through several water bridges that determines the rate under such conditions (Steiner et al., 1975; Rowlett, 1984; Lindskog, 1986). When the availability of buffering

[6] In the classical two-step transfer (ping-pong) mechanism

$$EH^+ + HCO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH^+ \cdot HCO_3^- \xrightarrow{k_2} E + CO_2 + H_2O$$

$$E + BH^+ \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E \cdot BH^+ \xrightarrow{k_4} EH^+ + B$$

the steady-state value of the apparent Michaelis constant for the protonated form of the buffer, K_m, is equal to $[k_2/(k_4 + k_2)][(k_{-3} + k_4)/k_3]$. Since there is evidence that k_4 is 1–2 orders of magnitude larger than k_2 (Y. Pocker and C. H. Miao, unpublished observations), the true dissociation constant of the enzyme–buffer complex, $(k_{-3} + k_4)/k_3$, is larger than K_m by approximately the same amount. Most buffers are very weak inhibitors of CA II catalyzed CO_2 hydration and HCO_3^- dehydration, with K_i values >0.1 M (C. H. Miao, unpublished observations). Additionally, buffers affect the visible spectrum of Co(II)-substituted CA II only at concentrations higher than 0.5 M [it must be kept in mind, however, that the binding which perturbs the Co(II) spectrum may be different from other productive binding].

species becomes reduced (i.e., at low buffer concentrations), another step, namely, the transfer of a proton from the enzyme to the external buffer, becomes rate limiting. The fact that the observed viscosity effect is essentially the same at both saturating and limiting buffer concentrations indicates that similar molecular mechanisms of proton transfer are most likely operative in both steps. We have suggested in our earlier paper (Pocker & Janjić, 1987) the possibility that stage b of the proton relay depicted in eq 3 could be affected through viscosity- (or cosolute-) induced changes in intramolecular isomerization rates involving functionally important motions (Frauenfelder & Wolynes, 1985; Lumry & Gregory, 1986). Provided that the external buffer is bound to the enzyme prior to proton exchange, stage a of eq 3 could also be affected by the same phenomenon.

Additional evidence supporting the notion that fluctuations in enzymatic structure are important during turnover and/or proton exchange between the active site and the bulk solvent comes from a recently refined X-ray crystallographic study on the structure of CA II (Eriksson et al., 1986). These authors have shown that histidine-64, located near the opening of the active site about 6 Å away from the zinc atom, serves as the only contact between the nine inner water molecules and bulk water. Furthermore, since distances between histidine-64 and its surrounding waters are too short for van der Waals contacts but too long for hydrogen bonds, the observed position of this residue represents an average of two conformations that differ by a 180° turn of the imidazole ring. If isomerization of the two alleged conformational states involves fluctuation of a large portion of the protein in a diffusion-like motion (Karplus & McCammon, 1983), increase in solvent friction induced by viscogenic cosolute may retard the rate of this process.

It has been known for many years that polyhydric alcohols and sugars stabilize biological molecules in solution (Ball et al., 1943; Tanford et al., 1962; Bradbury & Jacoby, 1972; Arakawa & Timasheff, 1982). Recent studies on protein–solvent interactions have indicated that the stabilization results from preferential exclusion of such cosolutes from the immediate domain of proteins (Lee & Timasheff, 1981; Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982). This thermodynamically unfavorable interaction thus favors the more compact conformations with minimal surface area exposed to the solvent that generally correspond to the native state of many proteins. Destabilization of the unfolded state is clearly evidenced by a gradual increase in the activation energy of denaturation for α-chymotrypsin, chymotrypsinogen, and ribonuclease with incremental increase in sucrose concentration up to 1 M (Lee & Timasheff, 1981).

The catalytic role of CA II and other enzymes may involve an interplay of several functionally related conformational states (Koshland, 1959, 1963, 1970), and the relative stability of these states may very well be offset by added sucrose (and other similar cosolutes), with the more open conformations being preferentially destabilized relative to the more compact structures. If such conformational isomerizations occur along the reaction coordinate of CA II catalyzed reversible hydration of CO_2, the effect of sucrose on this reaction may be due to an increase in the free energy of activation for these structural isomerizations.

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Nuclear Magnetic Resonance Study on Rabbit Skeletal Troponin C: Calcium-Induced Conformational Change†

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ABSTRACT: Rabbit skeletal muscle troponin C (TnC) was investigated by means of ^{1}H NMR in the presence of dithiothreitol that prevents dimerization of the protein. Two-dimensional (2D) ^{1}H NMR spectra were observed in order to assign resonances to specific amino acids. One-dimensional ^{1}H NMR spectra were observed as a function of Ca^{2+} concentration. The Ca^{2+}-induced spectral change is categorized into two types: type 1 corresponds to the conformational change of the C-terminal-half domain (Ca^{2+} high-affinity sites) and type 2 to that of the N-terminal-half domain (Ca^{2+} low-affinity sites). From the 2D NMR spectra and Ca^{2+} titration data, it was suggested that (1) amide protons of Gly-108, Ile-110, Gly-144, and Ile-146 are hydrogen-bonded when the C-terminal-half domain binds 2 mol of Ca^{2+} and (2) hydrogen bonds of Gly-108, Ile-110, Gly-144, and Ile-146 are destroyed or weakened when the C-terminal-half domain releases 2 mol of Ca^{2+}. Nuclear Overhauser enhancement difference spectra as well as the Ca^{2+} titration data suggested that a hydrophobic cluster is formed in the C-terminal-half domain when the C-terminal-half domain binds 2 mol of Ca^{2+}. A hydrophobic cluster exists in the N-terminal-half domain without regard to Ca^{2+} binding to the N-terminal-half domain. The spectra of Tyr-10 showed both types of spectral change during the Ca^{2+} titration. The results suggested that Tyr-10 of apo-TnC interacts with the C-terminal-half domain.

Troponin C (TnC)[1] is a component of the troponin complex protein in thin filaments of myofibril, together with the other components TnI and TnT (Ebashi, 1974). The binding of Ca^{2+} to TnC induces a conformational change. This conformational change is transmitted to the other two components and results in a release of the inhibition of the actin–myosin interaction, which triggers the contractile activity (Gergely & Leavis, 1980).

Rabbit skeletal muscle TnC is a single polypeptide of 159 residues (Collins et al., 1975) and contains four calcium binding sites, two low-affinity Ca^{2+}-specific sites ($K_{Ca^{2+}} = 2 \times 10^5$ M^{-1}) and two high-affinity Ca^{2+} sites which can bind also Mg^{2+} ($K_{Ca^{2+}} = 2 \times 10^7$ M^{-1}, $K_{Mg^{2+}} = 5 \times 10^4$ M^{-1}) (Potter & Gergely, 1975). On the basis of the crystal structure of parvalbumin, Kretsinger and Barry predicted that the four Ca^{2+}-binding sites of TnC are in the "helix–loop–helix" conformation (Kretsinger & Barry, 1975). Sin and co-workers

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[1] Abbreviations: NMR, nuclear magnetic resonance; TnC, calcium binding component of troponin; TnI, inhibitory component of troponin; TnT, tropomyosin binding component of troponin; Ca_2TnC, TnC with 2 mol of calcium at high-affinity sites; Ca_4TnC, TnC with 4 mol of calcium at high- and low-affinity sites; CaM, calmodulin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; β-Me, β-mercaptoethanol; 2D, two-dimensional; COSY, correlated spectroscopy; NOE, nuclear Overhauser enhancement effect; NOESY, nuclear Overhauser enhancement effect spectroscopy; RCT, relayed coherence transfer in COSY; COCONOSY, combined COSY/NOESY experiment; QD, quadrature detection; TSP-d_4, (trimethylsilyl)propionic-d_4 acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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