A Study of the Binding of Two Sulfonamides to Carbonic Anhydrase

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Abstract: Rate constants and inhibition constants have been determined for the binding of two sulfonamide inhibitors, 4-hydroxy-3-nitrobenzenesulfonamide and pentafluorobenzenesulfonamide, to carbonic anhydrase. Two alternative proposals for the pathway of sulfonamide–carbonic anhydrase complex formation have been examined. In the case of the pentafluorobenzenesulfonamide–carbonic anhydrase complex, the rate data measured are not compatible with a scheme where it is postulated that the un-ionized sulfonamides react with the form of the enzyme in which the water molecule bound to the active site Zn(II) is ionized. The association rate constants calculated for pentafluorobenzenesulfonamide, according to this model, exceed the theoretical diffusion limit by about two orders of magnitude. However, the rate constants measured for both 4-hydroxy-3-nitrobenzenesulfonamide and pentafluorobenzenesulfonamide are consistent with a scheme where the ionized sulfonamides react with the form of the enzyme in which the water ligand bound to the active site Zn(II) ion is un-ionized. A reasonable suggestion for carbonic anhydrase–sulfonamide complex generation is that there is rapid preequilibrium formation of an outer-sphere complex between the sulfonamide anion and the Zn(II) ion at the active site, which then loses water as the rate-determining step of inner sphere inhibitor–enzyme complex formation.

The study of inhibitor binding to metalloenzymes provides a valuable means of examining reactions of the metal ion while it is bound to the enzyme. Carbonic anhydrase is particularly suitable for this kind of investigation since it is a well-characterized metalloenzyme for which numerous primary sulfonamides are tightly bound specific inhibitors.[1,2] Strong evidence exists that the sulfonamide group binds directly to the metal at the active site replacing the water ligand present in the free enzyme.[3-7] A previous communication from this laboratory presented the preliminary results of a study of the binding of 4-hydroxy-3-nitrobenzenesulfonamide to carbonic anhydrase.[8] Here we include a full account of that study and also present the results of a similar investigation conducted with a new inhibitor containing a very acidic sulfonamide group, pentafluorobenzenesulfonamide.

Experimental Section

Materials. Enzyme. Commercial preparations of bovine erythrocyte carbonic anhydrase (BCA), which are mixtures of coenzymes A and B, were obtained from Mann Research Laboratories, Nutritional Biochemicals Corp., and Worthington Biochemical Corp.; the enzyme was treated as described below. The commercial sample was dissolved in a minimum volume of 0.05 *M* Tris buffer pH 8.7 and dialyzed against several changes of the same solvent. The solution was then passed over a 10 cm × 1.5 cm DEAE-50 Sephadex column, eluting with 0.1 *M* Tris, pH 8.7. The fractions containing significant amounts of enzyme were pooled and dialyzed against deionized water or an appropriate buffer. After dialysis, the solutions were either lyophilized and then stored, desiccated, in a freezer or were stored as solutions in a refrigerator at 4°. On rare occasion, an enzyme solution appeared turbid and was clarified by ultracentrifugation. All operations were performed in the cold room. After treatment, a BCA solution having $A_{280} = 2.5$ had A_{410} of only 0.006, indicating that essentially all contaminating hemoglobin had been removed. The stock BCA solutions retained unchanged activity, as assayed by 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone hydrolysis,[9] for over a year. The concentration of BCA was determined spectroscopically using a value of $\epsilon_{280} = 5.61 \times 10^4$.[10]

Human erythrocyte carbonic anhydrase isoenzyme B (HCAB) in $(NH_4)_2SO_4$ was kindly provided by Dr. F. Dorner of Professor J. T. Edsall's laboratory, Harvard University. The sample was extensively dialyzed against an appropriate buffer or deionized water, and it was clarified by ultracentrifugation. A value of $\epsilon_{280} = 4.9 \times 10^4$ [10] was used to determine the HCAB concentrations in solution.

Inhibitors. 4-Hydroxy-3-nitrobenzenesulfonamide was synthesized as described by Kaiser and Lo.[10] Stock solutions were prepared by dissolving the sulfonamide in buffer (usually Tris–Na_2SO_4 buffer, $\mu = 0.25$, pH 7.6) containing sufficient acetone (approximately 8% v/v in a 1×10^{-2} *M* inhibitor solution) to keep the sulfonamide in solution.

Pentafluorobenzenesulfonamide was kindly supplied by Imperial Smelting Ltd., and was used without further purification. Stock solutions were prepared by dissolving the sulfonamide in a mixture of deionized water with approximately 250 μl of 1 *M* NaOH per 10 ml of solution.

Other Chemicals. Deionized water was obtained by passing distilled water through a mixed-bed ion-exchange column supplied by Continental Demineralization Service.

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) was obtained from Mann Research Laboratories; the ultrapure grade was used.

2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone was kindly provided by Dr. K.-W. Lo of this laboratory. Stock solutions of the sultone in acetone (5×10^{-3} *M*) were kept tightly stoppered and refrigerated.

Stock solutions of CO_2 were prepared by bubbling pure CO_2, "Bone Dry" grade supplied by J. T. Baker Chemical Co., for 45 min into fresh deionized water. At room temperature, this gives approximately a 3.5×10^{-2} *M* solution.

All other chemicals used were reagent grade.

Dialysis tubing was boiled for 10 min in bicarbonate solution, rinsed exhaustively with tap water and deionized water, and then soaked in about 20 changes of deionized water. It was stored and refrigerated in deionized water.

Instrumentation. The Durrum–Gibson stopped-flow spectrophotometer and its use has been described in great detail earlier.[11]

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(1) T. H. Maren, *Physiol. Rev.*, **47**, 595 (1967).
(2) P. W. Taylor, R. W. King, and A. S. V. Burgen, *Biochemistry*, **9**, 2638 (1970).
(3) K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander, and G. Wiren, *J. Mol. Biol.*, **25**, 505 (1967).
(4) J. E. Coleman, *NASA Spec. Publ.*, **No. SP-188**, 141 (1968).
(5) M. E. Riepe and J. H. Wang, *J. Biol. Chem.*, **243**, 2779 (1968).
(6) K. K. Kannan, A. Liljas, I. Waara, P.-C. Bergsten, S. Lövgren, B. Strandberg, U. Bengtsson, U. Carlbom, K. Fridborg, L. Jarup, and M. Petef, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 221 (1972).
(7) S. Lindskog, L. E. Henderson, K. K. Kannan, A. Liljas, P. O. Nyman, and B. Strandberg in "The Enzymes," Vol. V, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, p 587.
(8) J. Olander and E. T. Kaiser, *J. Amer. Chem. Soc.*, **92**, 5758 (1970).
(9) K.-W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966).
(10) E. T. Kaiser and K.-W. Lo, *J. Amer. Chem. Soc.*, **91**, 4912 (1969).
(11) G. Tomalin, M. Trifunac, and E. T. Kaiser, *ibid.*, **91**, 4912 (1969).

An Aminco–Morrow stopped-flow spectrophotometer was also used during this investigation; the mode of operation is similar for both instruments though the design differs somewhat. A 10-mm path-length cuvette was used in the Aminco spectrophotometer and a 20-mm cuvette in the Durrum instrument. Treatment of the data depended on the kind of kinetic measurements being made. If the total change in transmittance exceeded 5%, the oscilloscope trace readings were converted to optical density units before further analysis.

A Cary 15 spectrophotometer and the Beckman DU spectrophotometer equipped with a Gilford 220 optical density converter and a recorder were also used. All of the spectrometers had thermostated cell compartments, generally at 25.0 ± 0.1°. Lauda water baths were employed.

pH measurements were made either on the Radiometer Model PHM 4C or the Beckman Research pH meters which were standardized against Fisher Certified buffers or standard buffers described by Meites.[12] Three types of electrodes were used: Thomas combination microelectrode pH 0 to 12, 4858 L-15; Thomas combination microelectrode pH 0 to 14, 4858 L-60; and Beckman E-1 micro glass electrode with a fiber junction reference microelectrode.

Methods. Assay. The activity of carbonic anhydrase stock solutions was periodically checked by assaying with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone substrate. Sultone concentrations in excess of the enzyme were used, and the pseudo-first-order kinetics of hydrolysis were observed by recording the change in the absorbance at 410 nm. Most assays were performed on the Cary 15 spectrophotometer using 1-cm cuvettes containing approximately 7×10^{-8} *M* enzyme in 3 ml of buffer; the reaction was initiated by rapidly adding and mixing 25 μl of the stock sultone solution (5×10^{-3} *M*) to the cuvette. Later, stopped-flow instruments were used in preference to the Cary spectrometer since higher concentrations of enzyme could be used for the assay, thus circumventing the "wall-effect," the adsorbance of enzyme onto the walls of a cuvette, which begins to be significant at concentrations below 7×10^{-8} *M*. Solutions of about 2×10^{-6} *M* enzyme in buffer were mixed in the stopped-flow instrument with about 1×10^{-4} *M* of sultone in deionized water. The sultone does not hydrolyze noticeably in deionized water over a period of 5 hr. The assays were done at 25.0° in Tris–Na_2SO_4 buffer, either $\mu = 0.25$ or $\mu = 0.125$, at pH 7.6. The BCA activity was considered unchanged if $k_{enz} = (k_{meas} - k_{spont})/[E]_0 = (3.8 \pm 0.3) \times 10^5\ M^{-1}\ sec^{-1}$ was obtained. k_{meas} is the measured pseudo-first-order rate constant for the enzyme catalyzed hydrolysis, k_{spont} is the first-order rate constant for spontaneous hydrolysis of sultone at the same pH and in the same buffer, and $[E]_0$ is the initial enzyme concentration.

Equilibrium Dialysis. Plexiglass equilibrium dialysis cells (1.2 ml) were assembled using cut dialysis tubing as the membrane, dried using a stream of nitrogen, and filled with exactly 1.0 ml of the desired solution using glass syringes equipped with plastic "needles." The experiments were typically run as described below. A 2×10^{-5} *M* BCA solution in 0.05 *M* Tris-0.065 *M* Na_2SO_4, pH 7.6, buffer was placed in side A of a cell and varying concentrations of the 4-hydroxy-3-nitrobenzenesulfonamide dissolved in the same buffer were placed in side B of the same cell. At each sulfonamide concentration, a control experiment was run in a second cell by dialyzing buffer in side C *vs.* the sulfonamide solution in side D. Dialysis was allowed to proceed for 7–10 days in the refrigerator with frequent manual shaking of the cells. After equilibrium was reached, the sulfonamide concentrations in sides B, C, and D were determined by measuring the absorbances at 400 nm, where $\epsilon_{400} = 4.14 \times 10^3$, using 1.0-ml volume, 1-cm path-length cells. If the absorbances of sides C and D were equal, the dialysis was considered to have reached equilibrium. The concentration of bound inhibitor = (concentration of sulfonamide in side D) − (concentration of sulfonamide in side B) and r = (moles of inhibitor bound)/(total moles of enzyme) can then be calculated. $[I]_{unbound}$ = concentration of sulfonamide in side B. The data were treated as described by Klotz, *et al.*[13] A plot of r *vs.* $1/[I]_{unbound}$ gives the number of inhibitor molecules that can be bound per molecule of enzyme. If the binding is 1:1, K_I, the dissociation constant, can be determined from eq 1.

$$1/r = 1 + K_I/[I]_{unbound} \qquad (1)$$

(12) L. Meites, "Handbook of Analytical Chemistry," McGraw-Hill, New York, N. Y., 1963, p 11-4.

(13) I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Amer. Chem. Soc.*, **68**, 1486 (1946).

We varied the ratio moles of inhibitor/moles of enzyme from 1.0 to 41.

Inhibition Constants. The inhibition of the BCA catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone by 4-hydroxy-3-nitrobenzenesulfonamide was measured by following the appearance of the *p*-nitrophenolate species produced from the sultone at 410 nm in the Durrum–Gibson stopped-flow spectrophotometer. Both the inhibitor (6×10^{-7} to 4×10^{-5} *M*) and the sultone (2.5×10^{-5} *M*) concentrations were in excess of the enzyme concentration (1×10^{-7} or 1×10^{-6} *M*). Therefore, since $K_m > [S]$[10] for the sultone and since $[I] = [I]_0$ under our chosen conditions, we could analyze the data according to eq 2.

$$\frac{[E]_0}{(k_{meas} - k_{spont})} = \left(\frac{[E]_0}{k_{meas,0} - k_{spont}}\right)\left(1 + \frac{[I]_0}{K_I}\right) \qquad (2)$$

In eq 2, k_{meas} and k_{spont} are defined as before, $k_{meas,0}$ is the rate constant measured in the absence of inhibitor, and $[I]_0$ is the initial concentration of inhibitor. The stopped-flow technique had to be employed because it allowed the use of relatively high enzyme concentrations, thus minimizing the "wall-effect." Solutions of BCA containing the appropriate concentration of inhibitor in Tris–Na_2SO_4 buffer, $\mu = 0.25$, pH 7.6, were mixed in the stopped-flow instrument with freshly prepared solutions of sultone in deionized water. The total concentration of acetone in the final, mixed solutions was about 1.1%. Full-scale oscilloscope traces were obtained.

The pentafluorobenzenesulfonamide–BCA inhibition constant was also measured in the presence of excess 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone substrate, but the inhibitor was not in significant excess over the enzyme. Since $K_m > [S]$,[10] noncompetitive inhibition was assumed and eq 3[14] was used to analyze the

$$[I]_0/(1 - \alpha) = [E]_0 + K_I/\alpha \qquad (3)$$

data where $\alpha = (k_{meas} - k_{spont})/(k_{meas,0} - k_{spont})$. This treatment makes it unnecessary to use excess inhibitor, but the total enzyme concentration must be held constant. The Aminco stopped-flow instrument was used for these experiments. The solutions were prepared and mixed in the same way as above and the final concentration of acetone was 1%.

Direct Measurement of the Inhibitor Association Rate Constant. The presence of the reporter group in 4-hydroxy-3-nitrobenzenesulfonamide allowed us to observe the kinetics of its binding to carbonic anhydrase directly;[10] rapid pseudo-first-order kinetics were observed in the presence of excess inhibitor. The first-order rate constants, k_{obsd}, measured are related to the rate constants of eq 4[14] by eq 5.[8]

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (4)$$

$$k_{obsd} = k_1[I]_0 + k_{-1} \qquad (5)$$

Thus, k_1 can be determined by varying the initial inhibitor concentrations. The enzyme (1.4×10^{-5} to 2.0×10^{-5} *M*) in Tris–Na_2SO_4 buffer, $\mu = 0.25$, pH 7.6, was mixed with sulfonamide (1.5×10^{-5} to 1.2×10^{-4} *M*) in the same buffer in the Durrum-Gibson stopped-flow apparatus and the reaction followed at 420 nm (slit = 1 mm). The 20-mm path length of the spectrophotometer's cuvette was crucial to these experiments since changes in transmittance of only 2% were being observed with BCA and only 1% with HCAB. Several oscilloscope traces were photographed and analyzed at each inhibitor concentration and the results averaged. Since this sulfonamide absorbs strongly at 420 nm, a greater than tenfold excess over enzyme of it could not be used without decreasing the sensitivity of the Durrum spectrophotometer too much. In all cases, however, no deviation from first-order kinetics was observed.

Indirect Measurement of the Inhibitor Association Rate Constant with CO_2 as the Substrate. This method involves the competition of CO_2 and inhibitor, both in excess, for BCA, and it has been described in detail by Kernohan[15] who derived eq 6 for analyzing the data.

$$\ln \Delta S_\infty = \ln \Delta S_0 - (k_{cat}[E]_0)/(k_1[I]) \qquad (6)$$

Here ΔS_∞ = amount of substrate left when the reaction of the

(14) S. Lindskog and A. Thorslund, *Eur. J. Biochem.*, **3**, 453 (1968).

(15) J. C. Kernohan, *Biochim. Biophys. Acta*, **118**, 405 (1966).

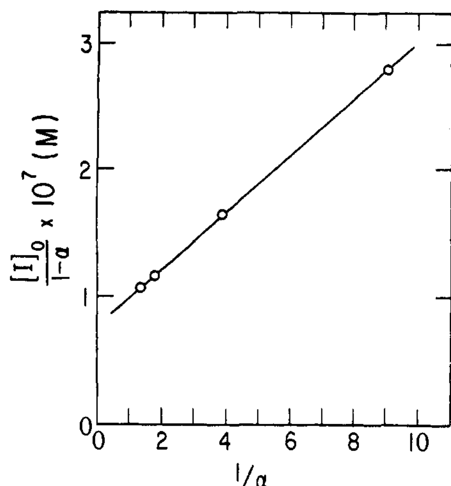


Figure 1. Determination of K_I for the inhibition by pentafluorobenzenesulfonamide of the action of BCA on 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone: $[S]_0 = 2.5 \times 10^{-8}$ M, $[E]_0 = 9.25 \times 10^{-8}$ M, and $[I]_0 = 1 \times 10^{-8}$–2.5×10^{-7} M; 0.025 Tris buffer containing 0.5 M Na_2SO_4 at 25.0° and pH 7.6 was used.

enzyme with the inhibitor is completed, subtracting the concentration of CO_2 left at equilibrium after the spontaneous hydration is completed; ΔS_0 = initial CO_2 concentration, subtracting this same concentration of CO_2 at equilibrium. ΔS_∞ and ΔS_0 were obtained by measuring vertical displacement of the oscilloscope trace.[15] We were careful to make our measurements in the flat portion of the curve which exists before the spontaneous hydration takes over. We followed Kernohan's method closely, mixing $[E]_0 \sim 1.9 \times 10^{-7}$ M, $[I]_0 = 4.2 \times 10^{-8}$ to 1.0×10^{-3} M, and 0.025 g/ml of bromophenol red indicator, all in 0.025 M Tris buffer containing 0.05 M Na_2SO_4 at pH 7.6, with $[CO_2] \sim 2.4 \times 10^{-3}$ M in the Aminco–Morrow stopped-flow spectrophotometer and following the reaction at 560 nm.

Indirect Measurement of the Inhibitor Association Rate Constant with Sultone as the Substrate. Equal concentrations of enzyme and inhibitor were used because the binding of pentafluorobenzenesulfonamide to BCA at 25.0° and pH 7.6 would otherwise be too rapid to measure on the Aminco–Morrow stopped-flow instrument. The kinetics observed are, therefore, second order and were analyzed according to eq 7,[2] where $F = [EI]_t/[EI]_\infty$; $[E]_\infty$ is the final equilibrium enzyme concentration at $t = \infty$ which can be calculated if K_I is known, as can $[EI]_\infty$, the equilibrium concentration of the complex.

$$\ln\left[\frac{[E]_0^2 - F[E]_\infty^2}{[E]_0^2(1 - F)}\right] = k_f\left[\frac{[E]_0^2 - [E]_\infty^2}{[E]_0}\right]t \qquad (7)$$

$[EI]_t$, the enzyme–inhibitor complex concentration at time t, can be determined by introducing the sultone substrate to compete with the inhibitor for BCA;[14] the concentration of sultone must be chosen such that the inhibitor binding reaction is slower than the enzyme-catalyzed sultone hydrolysis. Then, recalling that $K_m >$ [S] and taking $[S]_0 > [E]_0$, eq 8 can be derived, where α is defined as

$$[EI]_t = [E]_0(1 - \alpha_t) \qquad (8)$$

in eq 3 at a time t. The value of t is taken as the midpoint of the time interval used to calculate the slope, which is proportional to k_{meas}, from plots of $\ln (T_t - T_\infty)$ *vs.* t (where T is the transmittance).

We mixed BCA (4.58×10^{-8} M) in Tris-Na_2SO_4 buffer, μ = 0.25, pH 7.6, with pentafluorobenzenesulfonamide (4.53×10^{-6} M) and 2-hydroxy-5-nitro-2-toluenesulfonic acid sultone (5×10^{-5} M) in deionized water in the stopped-flow instrument and observed the reaction at 420 nm. Oscilloscope traces were taken on several different time scales from 10 to 200 msec. The kinetics in the absence of inhibitor under the same experimental conditions were also measured.

Results

4-Hydroxy-3-nitrobenzenesulfonamide. The kinetics of the binding of 4-hydroxy-3-nitrobenzenesulfonamide

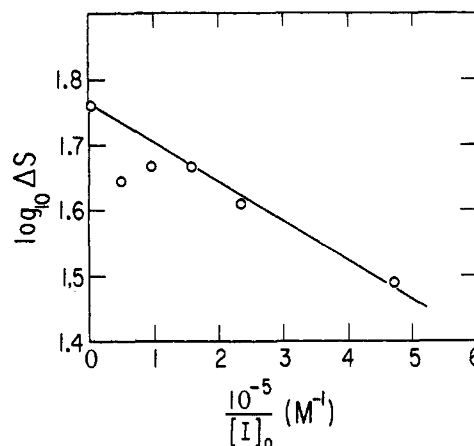


Figure 2. Data from the determination of the rate constants for the binding of pentafluorobenzenesulfonamide to BCA by competition experiments with CO_2 substrate: $[E]_0 \sim 9 \times 10^{-8}$ M, $[CO_2] \sim 1.2 \times 10^{-3}$ M; 0.025 M Tris buffer containing 0.05 M Na_2SO_4 at 25.0° and pH 7 was used.

to carbonic anhydrase at pH 7.6 can be followed directly at 420 nm[16] (see Experimental Section). As reported earlier,[8] the inhibitor association rate constants were found to be 3.7×10^4 M^{-1} sec^{-1} with bovine carbonic anhydrase (isozymes A + B) and 7.5×10^4 M^{-1} sec^{-1} with human carbonic anhydrase B, both in Tris-Na_2SO_4 buffer, μ = 0.25, pH 7.6, at 25.0°. The inhibitor association rate constant for the bovine enzyme has also been measured indirectly using the competition of CO_2 with sulfonamide, and a value of 5.4×10^4 M^{-1} sec^{-1} was obtained in Tris-Na_2SO_4 buffer, μ = 0.15, pH 7, and 25.0°.

The inhibition constant, K_I, measured for the bovine species as described in the Experimental Section is 3.2×10^{-6} M, and this value agrees with the dissociation constant of 4×10^{-6} M obtained from equilibrium dialysis experiments,[8] which also demonstrated that each enzyme molecule binds only one 4-hydroxy-3-nitrobenzenesulfonamide molecule.[8]

The ionizations of 4-hydroxy-3-nitrobenzenesulfonamide were measured spectrophotometrically in 0.1 M buffers and values of $pK_a = 4.89 \pm 0.04$ for the phenolic group and 10.9 ± 0.2 for the sulfonamide group were found.[8]

Pentafluorobenzenesulfonamide. The inhibition constant for the inhibition by pentafluorobenzenesulfonamide of the bovine carbonic anhydrase catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone is 2.3×10^{-8} M at pH 7.6 in 0.025 M Tris–0.05 M Na_2SO_4 buffer at 25.0° (Figure 1). Under the same buffer and temperature conditions the association rate constant for the inhibitor–enzyme binding reaction, as measured by the indirect method involving CO_2 competition with the sulfonamide for BCA ($[I]_0 < [E]_0$), yields a value of $k_1 = 4.6 \times 10^6$ M^{-1} sec^{-1} (Figure 2). The alternate technique involving competition of inhibitor with the sultone substrate ($[E]_0 = [I]_0$) gives $k_1 = 7.4 \times 10^6$ M^{-1} sec^{-1} in 0.025 M Tris buffer containing 0.037 M Na_2SO_4 at pH 7.6 (Figure 3).

A pK_a of 3.1 was determined for the ionization of the sulfonamide group by potentiometric titration of 5.16×10^{-3} M pentafluorobenzenesulfonamide in 0.042 M Na_2SO_4 ($\mu \sim 0.13$).

(16) K.-W. Lo, Ph.D. Thesis, The University of Chicago, Chicago, Ill., 1968.

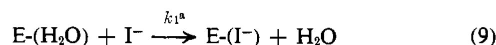
Discussion

From X-ray investigations with human carbonic anhydrase C, it is known that sulfonamides bind directly to the active site zinc ion.[3,4,7] Considerable evidence exists that the sulfonamides are bound to the metal ion in their anionic form. It has been argued by Chen and Kernohan,[17] for example, that only a small part of the large blue shift in the fluorescence emission spectrum of 5-dimethylaminonaphthalene-1-sulfonamide resulting from binding to bovine carbonic anhydrase can be attributed to the very hydrophobic environment of the binding site in the enzyme. They correlated the rest of the observed shift with the ionization of the sulfonamide group occurring on binding. The ultraviolet difference spectra of the *p*-nitrobenzenesulfonamide–human B and –human C carbonic anhydrase complexes compared with those of the free enzyme and unbound inhibitor were interpreted as indicating the presence of ionized sulfonamide species in these complexes.[18]

The red shift we have observed in the visible spectrum of 4-hydroxy-3-nitrobenzenesulfonamide when it binds to bovine carbonic anhydrase and to human carbonic anhydrase B is strikingly similar to that seen when the anion is generated from the sulfonamide.

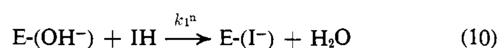
In addition to these measurements which support the hypothesis that sulfonamides bind to carbonic anhydrase in their anionic form, numerous similarities between the binding of sulfonamides and of small anionic inhibitors to the enzyme have been noted. Competition between sulfonamides and small anions for the enzymatic binding site has been demonstrated kinetically,[14] by spectrophotometric comparison of the changes in the Co(II) carbonic anhydrase spectrum resulting from inhibitor binding,[4] by direct binding studies,[19,20] by infrared studies,[5] and by an nmr investigation.[21]

Despite the strong evidence from most investigations that the sulfonamides are present in the anionic form in their complexes with carbonic anhydrase, there is disagreement concerning the stepwise pathway by which these complexes are formed. Two proposals have been favored by different groups of investigators. According to one proposal,[8,15,22] binding occurs as shown in eq 9



$$\text{E-(H}_2\text{O)} + \text{I}^- \xrightarrow{k_1^{\text{a}}} \text{E-(I}^-) + \text{H}_2\text{O} \qquad (9)$$

where E-(H_2O) represents the form of the enzyme in which the water bound to the active site zinc ion is un-ionized and I^- represents the sulfonamide anion. The alternative hypothesis is shown in eq 10 where



$$\text{E-(OH}^-) + \text{IH} \xrightarrow{k_1^{\text{n}}} \text{E-(I}^-) + \text{H}_2\text{O} \qquad (10)$$

E-(OH^-) represents the form of the enzyme in which hydroxide ion is bound to the active site zinc ion and IH represents the neutral sulfonamide.[23]

In Table I a summary is provided of most of the cases of sulfonamide inhibition of carbonic anhydrase for which k_1 and the pK_a values for ionization of the sulfonamide group have been measured. The "intrinsic" association rate constants calculated according to eq 9 and 10, using a pK_a value of 6.8 for the ionization of the zinc ion bound water are represented by k_1^a and k_1^n, respectively. Examination of the values of k_1^a reveals that with the exception of one inhibitor, *p*-(salicyl-5-azo)benzenesulfonamide (SABS),[2,24] for which k_1^a is 1.3×10^{10} M^{-1} sec^{-1}, the highest rate constant calculated is 2.5×10^9 M^{-1} sec^{-1}. Furthermore, neglecting the SABS result for the moment, there is only a slightly more than 100-fold variation in the values of k_1^a for a group of sulfonamides with pK_a values ranging from 3.1 for pentafluorobenzenesulfonamide to 10.7 for sulfanilamide, a spread of more than 7.5 pK units.

When the values of k_1^n are examined, a considerably different picture emerges. Except for the k_1^n values calculated in the present study for pentafluorobenzenesulfonamide (*e.g.*, 2.7×10^{11} M^{-1} sec^{-1}), the highest number obtained is 5.5×10^7 M^{-1} sec^{-1} for ethoxzolamide. Also, with inclusion of the data for pentafluorobenzenesulfonamide, the range of k_1^n values is nearly 10^7, a far greater range than that calculated for the k_1^a values, including the SABS data.

Neglecting electrostatic interactions and assuming that the active site is located on the surface of the enzyme, a theoretical diffusion-limited rate constant of approximately 2×10^9 M^{-1} sec^{-1} was calculated for the reaction of fumarate with fumarase.[25] Because SABS is a larger molecule than fumarate and would be expected to diffuse more slowly into an active site and because the active site of carbonic anhydrase is at the bottom of a cleft and not on the surface of the enzyme molecule, Taylor, *et al.*,[24] have argued that the diffusion-limited rate constant for the formation of a complex such as the one between SABS and carbonic anhydrase should be smaller than that for the association of fumarate with fumarase. Therefore, since the k_1^a value calculated for SABS is significantly above 2×10^9

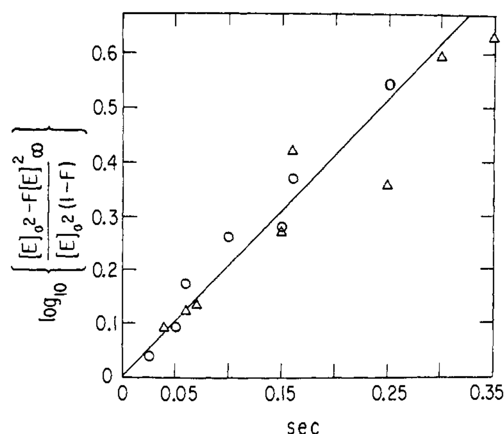


Figure 3. Data from the determination of the rate constants for the binding of pentafluorobenzene to BCA by competition experiments with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone. The two sets of data points represent the same experiment with two sets of freshly prepared solutions: $[S]_0 = 2.5 \times 10^{-5}$ M, $[E]_0 = 2.29 \times 10^{-6}$ M, $[I]_0 = 2.26 \times 10^{-6}$ M, all in Tris-Na_2SO_4 buffer, $\mu = 0.125$, pH 7.6, at 25.0°.

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Table I. Kinetic and Equilibrium Constants for Various Sulfonamides and Carbonic Anhydrase

Inhibitor	k_1, M^{-1} sec^{-1}	k_1^a, M^{-1} sec^{-1}	k_1^n, M^{-1} sec^{-1}	K_I, M	pH of reaction soln	pK_a for sulfonamide group	k_{-1}, sec^{-1}
4-Hydroxy-3-nitrobenzenesulfonamide	3.7×10^{4} [a]	5.4×10^{8}	4.3×10^{4}	3.2×10^{-6} [c]	7.6	10.9	0.14
	7.5×10^{4} [b]	1.1×10^{9}	8.7×10^{4}	4×10^{-6} [e]			
	5.4×10^{4} [d]	7.7×10^{8}	6.2×10^{4}				
	5.48×10^{4} [f]	2.1×10^{9}	1.6×10^{5}	4.17×10^{-6} [f]	6.5		0.216[f]
				3.95×10^{-6} [f]			
Pentafluorobenzenesulfonamide	4.6×10^{6} [d]	3.3×10^{7}	1.7×10^{11}	2.3×10^{-8} [c]	7.6	3.1	
	7.4×10^{6} [c]	5.4×10^{7}	2.7×10^{11}				
Sulfanilamide	7×10^{4} [g]	5.9×10^{8}	7.6×10^{4}	1.2×10^{-6} [g]	7.9	10.7[g]	
	3.9×10^{4} [h]	2.5×10^{8}	3.2×10^{4}	3×10^{-6} [h]	8.0		
Benzenesulfonamide	7×10^{4} [i]	3.9×10^{8}	1.6×10^{5}	3.2×10^{-6} [i]	6.7	10.2[i]	
	1.6×10^{5} [i]	1.4×10^{9}	1.8×10^{5}		7.7		
	1.06×10^{5} [f]	2.5×10^{9}	3.2×10^{5}	1.54×10^{-6} [f]	6.5		0.164[f]
p-Nitrobenzenesulfonamide	7.37×10^{5} [f,g]	7.0×10^{8}	2.2×10^{8}	6.25×10^{-8} [f]	6.5	9.30[j]	0.048[f]
				6.54×10^{-8} [f]			
Acetazolamide	4.83×10^{6} [f]	4.4×10^{7}	1.7×10^{7}	5.88×10^{-8} [f]	6.5	7.2[g]	0.068[f]
	5×10^{8} [i]	3.7×10^{7}	1.5×10^{7}	2.2×10^{-8} [i]	6.7		
	4×10^{6} [g]	6.5×10^{7}	2.6×10^{7}	1.2×10^{-8} [g]			
p-(Salicyl-5-azo)benzenesulfonamide (SABS)	1.13×10^{7} [f,g]	1.3×10^{10}	3.4×10^{7}	2.94×10^{-8} [f]	6.5	9.4[j]	0.033[f]
5-Dimethylaminonaphthalene-1-sulfonamide (dansylamide)	2.4×10^{5} [f]	1.1×10^{9}	7.2×10^{5}	1.72×10^{-6} [f]	6.5	10.0[j]	0.390[f]
				1.63×10^{-6} [f]			
Ethoxzolamide	2.9×10^{7} [i]	1.1×10^{9}	5.5×10^{7}	1.6×10^{-9} [i]	8.0	8.1[i]	0.05[i]
Chlorothiazide	4.5×10^{4} [i]	2.0×10^{7}	5.0×10^{4}	5.4×10^{-7} [i]	8.0	9.4[i]	0.02[i]
Cl 11,366	5.9×10^{8} [i]	3.0×10^{8}	9.4×10^{6}	9×10^{-9} [i]	8.0	8.3[i]	0.05[i]

[a] Direct measurement with BCA. [b] Direct measurement with HCAB. [c] Measured with BCA by competition with sultone substrate. [d] Measured with BCA by competition with CO_2 substrate. [e] Measured with BCA by equilibrium dialysis. [f] Reference 2. [g] Reference 14. [h] Reference 22. [i] Reference 15. [j] Reference 24.

M^{-1} sec^{-1}, they have concluded that the reaction scheme of eq 9 can be ruled out for enzyme–sulfonamide complex formation.

On the other hand, a substantial increase in the diffusion-limited rate constant can be produced by electrostatic attraction when ions of opposite charge react. Extrapolation to zero ionic strength gave a minimum value of 3×10^{10} M^{-1} sec^{-1} for the rate constant for association of fumarate with fumarase.[25] When a correction for the electrostatic attraction between the carboxylate groups of fumarate and oppositely charged groups (2 to 3) on the enzyme was made,[25] a theoretical diffusion-limited rate constant of 0.8–1.5 $\times 10^{10}$ M^{-1} sec^{-1} was calculated; for the 6 active sites on fumarase, this gave an overall calculated diffusion-limited rate constant of 4.8–9 $\times 10^{10}$ M^{-1} sec^{-1}. These values are approximately the same order of magnitude as the k_1^a value obtained by Taylor, *et al.*,[24] for the SABS–carbonic anhydrase complex. Considering the presence of the negatively charged carboxylate group in SABS and the positively charged zinc ion in the active site, it may not be surprising that formation of the carbonic anhydrase–SABS complex is accelerated relative to the formation of complexes between carbonic anhydrase and sulfonamides lacking such a group.[26] While *p*-carboxybenzenesulfonamide does not show a comparable rate acceleration for carbonic anhydrase complex formation,[2] this may only indicate that the positioning of the carboxylate group in the inhibitor molecule has a crucial effect on the electrostatic interaction between the inhibitor and enzyme molecules. An argument against the importance of the influence of electrostatic interactions on the association rate constants for sulfonamide–carbonic anhydrase complexes has been made[24] on the basis of the insensitivity of these rate parameters to changes in the ionic strength of the reaction medium. However, the ionic strength data reported by Taylor, *et al.*,[24] were obtained for *p*-nitrobenzenesulfonamide, a compound for which the k_1^a value is not unusually high as it is in the case of the carboxylate group substituted sulfonamide SABS.

In view of these considerations, it does not seem to us that the SABS data of Taylor, *et al.*,[24] rule out the reaction scheme of eq 9 for sulfonamide–carbonic anhydrase complex formation. Also, from Table I it can be seen that the association rate constant values, k_1^n, calculated for pentafluorobenzenesulfonamide, an inhibitor which does not contain any ionizable group other than the sulfonamide function, are about two orders of magnitude greater than the diffusion-limited rate constant of 2×10^9 M^{-1} sec^{-1} discussed above. Since there is no reason to expect unusually large electrostatic effects for the association of pentafluorobenzenesulfonamide with carbonic anhydrase, we conclude that the reaction scheme of eq 10 cannot account for our results. However, the values of k_1^a for sulfonamides which are as different structurally as pentafluorobenzenesulfonamide and 4-hydroxy-3-nitrobenzenesulfonamide are within the diffusion limit, and it appears to us that the reaction scheme of eq 9 is in satisfactory accord with our data.

The possibility that some sulfonamides might react according to the scheme of eq 9 and others by that of eq 10 cannot be ruled out at present.[7] However, for the

(26) It should be mentioned here that carboxylate anion species are known to form complexes with carbonic anhydrase very rapidly; see P. W. Taylor, J. Feeney, and A. S. V. Burgen, *Biochemistry*, 10, 3866 (1971).

reasons discussed above and because the evidence is strong that other organic inhibitors of carbonic anhydrase such as carboxylic acids[26] and aromatic thiols[27] react with the enzyme in their anionic form, we favor the hypothesis that sulfonamide–carbonic anhydrase formation occurs as illustrated by eq 9.

As suggested earlier,[8] the mechanism of carbonic anhydrase–sulfonamide complex formation appears to be similar to that for the binding of anionic ligands to Zn(II) in inorganic complexes.[28] According to this hypothesis, rapid pre-equilibrium formation of an outer-sphere complex between the sulfonamide anion and the active site zinc ion is followed by the loss of zinc-bound water in the rate-determining step and finally the entry of the sulfonamide anion into the inner sphere of the metal ion. The variation in the association rate constants for sulfonamide–carbonic anhydrase complex generation with structural changes in the inhibitor might seem at first to be somewhat greater than would be expected if this mechanism holds. However, the ligand geometry of the metal ion in the enzyme is different than it usually is in inorganic complexes,[4] and this may affect the observed rate behavior. Also, the outer-sphere association constants may be more variable than those for inorganic anion–Zn(II) complex formation because of substantial variations in the hydrophobic interactions of the enzymatic binding site and the aromatic or heteroaromatic rings present in the sulfonamide inhibitors.[22]

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Antibodies Specific for Conformationally Distinct Coenzyme–Substrate Transition State Analogs. A Fluorescence, Nuclear Magnetic Resonance, Circular Dichroism, and Antibody Study of *N*-(5-Phosphopyridoxyl)-3′-amino-L-tyrosine

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Abstract: Two different haptens have been constructed to resemble the Schiff base intermediate of pyridoxal phosphate dependent enzymes which utilize the substrate tyrosine. The synthesis of *N*-(5-phosphopyridoxyl)-3′-amino-L-tyrosine (II) and its cyclized derivative III was accomplished, and certain aspects of their conformation have been elucidated by spectroscopic means. Compound II is a flexible molecule which, in aqueous solution, behaves as though it is in an extended form. The detection of coupling interactions between its aromatic rings indicates that the molecule spends some time in a folded or stacked arrangement. Analog III is covalently locked into a compact configuration, with its tyrosyl and pyridoxyl rings in perpendicular apposition. These conformationally distinct haptens each evoked an antibody response specific for both the phosphopyridoxyl and tyrosine regions. Antibody directed against the unhindered form II reacted tenfold more effectively with it than with its cyclic counterpart III. Antibody specific for the cyclic molecule bound it 10 times more tightly than the extended compound II. The antibody obtained in response to these specifically designed haptens may provide valuable insight by simulating the effects of enzyme active sites.

The reversible formation of specific antibody–hapten complexes has long provoked analogy to the enzyme–substrate intermediate required for enzyme catalysis. Indeed, certain similarities are seen to exist when both of these ligand–protein interactions are examined at the level of their fundamental rate constants of complex formation and dissociation.[1,2] Most haptens used to elicit antibody response, however, bear little resemblance to enzyme substrates as they might appear while undergoing catalysis. Comparisons between the resulting antihapten antibodies and enzymes are thereby severely restricted. The nature of an antibody combining site is determined by the chemical and steric features of the hapten employed to initiate a given response.[3-5] We decided to specifically design a hapten for the purpose of eliciting antibody sites with binding properties characteristic of enzyme active sites. An attempt to select and synthesize such a molecule was therefore undertaken.

Schiff bases I formed by the condensation of amino

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