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Kinetics of Complex Formation between Human Carbonic Anhydrase B and Heterocyclic Sulfonamides

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SUMMARY

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The kinetics of both the association and dissociation reaction of two heterocyclic sulfonamide inhibitors (S), methazolamide and ethoxzolamide, with human carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) isoenzyme B (CA) has been studied as a function of pH, using fluorescence stopped-flow instrumentation. The dissociation rate constants are similar for the two inhibitors and are unaffected by pH changes in the range 6.0–10.0. The association rate constants exhibit bell-shaped curves as a function of pH, consistent with the rate being controlled by two ionizing groups. The evidence suggests that one is an ionizing group on the enzyme with a pK near 7.5, which controls the low-pH limb of the curve, while the other function, controlling the high-pH limb, is the ionization of the sulfonamide group of the inhibitor. The data are consistent with the enzyme-drug reaction being either CA + SH or $CAH^+ + S^-$.

INTRODUCTION

Human red cell carbonic anhydrases are inhibited by aromatic sulfonamides. The aromatic ring system may be benzenoid or heterocyclic, although heterocyclic inhibitors tend to have lower dissociation constants than most ring-substituted benzenesulfonamides (1). Taylor *et al.* (2) studied the kinetics of association and dissociation of sulfonamides with human carbonic anhydrases B and C at a single pH value, and also studied the effect of pH on these reactions for some benzenesulfonamides (3). Their results showed that while the rate of dissociation of the enzyme-inhibitor complex was independent of pH, the association rate exhibited a bell-shaped curve characterized by two pK values. Experiments using several sulfonamides with pK_a values covering a range of 0.7 pH unit showed that the high-pH limb was controlled by the dissociation of a hydrogen ion from the sulfonamide group. Other experiments, using the two commonly occurring isoenzymes of human carbonic anhydrase, showed that the low-pH limb was controlled by an ionizable group on the protein. The coincidence, in both isoenzymes, of the pK of this group with the pK of the group controlling enzymatic activity suggests that the same ionizing function may control both activity and inhibitor binding.

We have extended this work to include two potent heterocyclic sulfonamide inhibitors of

carbonic anhydrase, ethoxzolamide and methazolamide (structures shown in Table 1), whose single pK values for ionization of the sulfonamide are considerably lower than those of most benzenesulfonamides.

METHODS

Human red cell carbonic anhydrase, isoenzyme B, was prepared by the ion-exchange method of Armstrong *et al.* (4) from outdated human blood obtained from the blood bank. The enzyme was stored at 4° as a suspension in 60% (w/v) ammonium sulfate solution.

Concentrated (approximately 100 μM) stock solutions of the enzyme were prepared by dissolving an aliquot of the suspension in 20 mM Tris-sulfate buffer solution, pH 7.5, followed by gel filtration through Sephadex G-25 equilibrated with the same buffer. Enzyme concentration was determined spectrophotometrically, using a Zeiss PMQ II spectrophotometer, taking $\epsilon_{280} = 46.8\ mM^{-1}\ cm^{-1}$ (4, 5).

Solutions of enzyme used for stopped-flow experiments were prepared by diluting the stock solution approximately 100-fold with 20 mM buffer solutions of the appropriate pH.

Enzyme activity was monitored using *p*-nitrophenyl acetate as substrate (4).

Ethoxzolamide (lot 673L) was obtained from the Upjohn Company, and methazolamide, from Lederle Laboratories. Stock sulfonamide solutions were made up in water and diluted with buffer solution before use.

pK_a values of the sulfonamides were determined by alkaline titration and ultraviolet spectroscopy. For the alkalimetric titrations the sulfonamide was dissolved in 0.1 M KCl solution and titrated down to pH 3.5 in a vessel flushed with nitrogen. Then 0.1 N KOH solution was used to titrate the sulfonamide. Correction was made for a 0.1 M KCl solution blank, and the 50% neutralization pH was taken as the pK value.

Stopped-flow experiments were carried out using the fluorescence quenching technique described in detail by Taylor *et al.* (2). The excitation wavelength was set at 290 nm, and emission was observed at 345 nm. A 2-cm quartz cuvette containing saturated nickel sulfate solution was inserted between the lamp and the excitation monochromator to filter out the incident 345 nm radiation.

Reaction traces were collected by passing the amplified photomultiplier signal into the first channel of a two-channel, 200-point signal averager. When the photomultiplier output had reached a constant level the equilibrium signal was collected in the second channel, and the complete trace was displayed on an oscilloscope. The contents of both channels were then output on punched tape in a consecutive signal-baseline series. In this way it is possible to obtain 100 ordinate values from a single experiment. Since the stored signal is digitized as a six-bit word, the ordinate values are accurate to a mean of ±3%.

Association rates were measured by a second-order, first-order approach, using equimolar solutions of enzyme and inhibitor. Dissociation rates were measured by first-order displacement with a second sulfonamide of different quenching efficiency (details of both are given in ref. 2). All experiments were performed at 25° ± 0.2°. pH values on the graphs are those of the mixed solutions collected from the stopping syringe.

The results were processed using programs written in the FOCAL language for a PDP-8L computer (Digital Equipment Corporation). Visual display of the calculated points was included so that any deviation from linearity could be checked, and such results rejected. Points on the graphs are the means of a minimum of four or a maximum of 10 determinations.

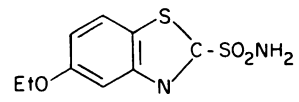
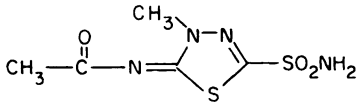
Buffer systems used were: pH 6.0–7.2, 20 mM cacodylic acid–NaOH; pH 7.2–8.8, 20 mM Tris-sulfate; pH 9.0–10.0, 2-amino-2-methyl-1,3-propanediol–sulfate.

RESULTS

The pK_a values of the sulfonamides are given in Table 1. The increased acidity of the sulfonamide group in these inhibitors over that of the benzenesulfonamides (pK_a values in the range 9–11, depending on ring substitution) is presumably due to the greater electronegativity of the nitrogen and sulfur atoms in the ring system stabilizing the anionic species. As can also be seen from

TABLE 1

pK values and fluorescence quenching efficiencies of ethoxzolamide and methazolamide

Sulfonamide	pK_s	Quenching of carbonic anhydrase B
		%
 (ethoxzolamide)	8.0 ± 0.1[a]	62
	8.05 ± 0.05[b]	
 (methazolamide)	7.2 ± 0.1[a]	
	7.3 ± 0.05[b]	57

Maximum quenching of the natural tryptophan fluorescence was measured at pH 7.5 by titration of the enzyme with sulfonamide solution.

[a] Measured by alkalimetric titration.

[b] Measured by ultraviolet spectroscopy.

Table 1, both the sulfonamides partially quenched the native enzyme fluorescence. Kernohan (6) has shown that ethoxzolamide quenches 58 % of the tryptophan fluorescence of bovine carbonic anhydrase, but found no quenching effect with methazolamide. We have noted that in the case of methazolamide, as with certain other inhibitors we have studied, although the free solution absorption spectrum has a very low intensity above 330 nm, the quenching efficiency in the complex is quite high.

Taylor *et al.* (2) have shown that the rate-limiting step in the displacement of a bound sulfonamide by another is the dissociation of the bound sulfonamide from the complex. As long as the displacing inhibitor has approximately similar values of association and dissociation rate constants to the bound inhibitor, a 100-fold molar excess over the concentration of the complex gives first-order kinetics, and the observed rate constant is an accurate measure of the dissociation rate. Figure 1 presents the results obtained from such first-order displacement reactions.

These results clearly confirm previous statements (3) that the dissociation rate of carbonic anhydrase–sulfonamide complexes is independent of hydrogen ion concentration over the pH range 6–10. Furthermore,

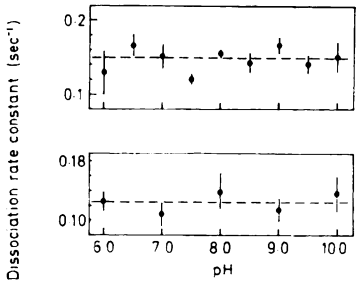


FIG. 1. *Effect of pH on dissociation rate constant of carbonic anhydrase B–sulfonamide complexes*

The first-order dissociation rate constants (sec^{-1}) of methazolamide (upper) and ethoxzolamide (lower) from carbonic anhydrase B were measured by displacement of the inhibitor from the complex by a 100-fold molar excess of *p*-carboxybenzenesulfonamide.

they show that this effect is independent of the nature of the ring system of the sulfonamide. The mean values of the dissociation rate constants, 0.15 sec^{-1} for methazolamide and 0.125 sec^{-1} for ethoxzolamide (dashed lines), are in the same range as those obtained by Taylor *et al.* (2) for various heterocyclic and substituted benzenesulfonamides at pH 6.5.

Figure 2 shows the results obtained for the measured association rates of methazol-

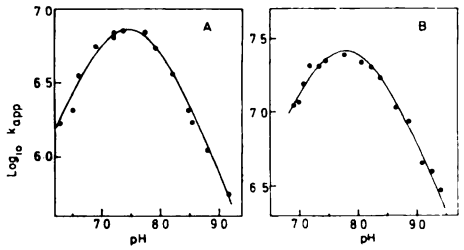


FIG. 2. *Effect of pH on association rate constant of carbonic anhydrase B and sulfonamides*

The apparent second-order association rate constants (k_{app}, M^{-1} sec^{-1}) of methazolamide (A) and ethoxzolamide (B) were measured using a second-order, first-order approach, by mixing equimolar solutions of enzyme and sulfonamide in the stopped-flow fluorometer. The solid lines were calculated as described in the text.

amide and ethoxzolamide with carbonic anhydrase B as a function of pH. The curves are bell-shaped, indicating that two ionizing groups control the formation of the complex. Taylor *et al.* (3) proposed that one of these ionizing groups is the sulfonamide group of the inhibitor, while the other is the enzymatic group controlling the activity.

Assuming that the equilibria in the mixed solution are $CAH^+ \rightleftharpoons CA + H^+$ and $SH \rightleftharpoons S^- + H^+$, the reaction might take place by combination between either or both forms of the enzyme (CA) and either or both forms of the sulfonamide. However, only the reactions

$$CA + SH \rightleftharpoons \text{complex} \quad (1)$$

and

$$CAH^+ + S^- \rightleftharpoons \text{complex} \quad (2)$$

will produce the observed bell-shaped curve of reaction rate constant as a function of pH.

The curves may be described mathematically by relating the measured association rate constant, k_{app}, to the intrinsic association rate constant, k_i, and the concentration of the reacting species at any given hydrogen ion concentration, by the following equations.

For reaction scheme 1

$$k_{app} = k_{i1} \frac{1}{(1 + K_s/[H^+])(1 + [H^+]/K_e)}$$

and for reaction scheme 2

$$k_{app} = k_{i2} \frac{K_s/K_e}{(1 + K_s/[H^+])(1 + [H^+]/K_e)}$$

K_s and K_e are the acidic dissociation constants of the sulfonamide and enzymatic groups, respectively.

Table 2 contains the results calculated from both schemes using the experimental values for k_{app} and K_s, and trial values for k_i and K_e, to obtain the best fit to the points. The curves obtained using best-fit values are drawn as solid lines in Fig. 2.

DISCUSSION

As pointed out by Taylor *et al.* (3), the maximum value of the association rate constant which can be attained by a small inhibitor combining with a receiving site on a macromolecule in a diffusion-controlled reaction, even if charge interaction is present, is in the region of 6×10^9 M^{-1} sec^{-1} at 25°. On the basis of results obtained with red cell human carbonic anhydrase C and the inhibitor salicylazobenzenesulfonamide, they concluded that the initial combination of the reactants took place via scheme 1, since the required *intrinsic* association rate constant (that is, the true bimolecular rate constant of the reacting species) for scheme 2 to operate was greater than 10^{10} M^{-1} sec^{-1}.

The values of the intrinsic association rate constant obtained for both the sulfonamides used in the present work, using either reaction scheme, fall fairly well below the maximum value for a diffusion-controlled reaction. It is impossible, therefore, to distinguish between the two reaction schemes on the basis of these results. It should be pointed

TABLE 2

Best-fit parameters for curves of k_{app} vs. pH

Using Eqs. 1 and 2 and the experimental values of K_s, the values of K_e and k_i were varied until the best fit was obtained to the experimental points.

Sulfonamide	pK_e	k_i of scheme 1	k_i of scheme 2
		M^{-1} sec^{-1}	M^{-1} sec^{-1}
Methazolamide	7.6	4.2×10^7	2.1×10^7
Ethoxzolamide	7.5	5.8×10^7	2.05×10^8

out, however, that the value of 2.05×10^8 M^{-1} sec^{-1} obtained using scheme 2 for ethoxzolamide combining with carbonic anhydrase B would still be an extremely fast rate for a reaction involving a biological macromolecule and a small, asymmetrical ligand (7).

Other recently published work in this field (8, 9) has also proved unable to differentiate between reaction schemes 1 and 2, although King and Burgen,[1] using homologous series of *para*-substituted benzenesulfonamides, have obtained several further examples of intrinsic association rate constants required to be in excess of 10^{10} M^{-1} sec^{-1} for scheme 2 to operate.

Assuming that reaction scheme 1 does apply for heterocyclic sulfonamide inhibitors, as seems likely for benzenesulfonamides, the values of the intrinsic association rate constant obtained for both these inhibitors are greater than any found for a series of benzenesulfonamides substituted on the aromatic ring (3). Both the association and dissociation rate constants are very similar for the two inhibitors, so that experimentally determined differences in the enzyme-inhibitor dissociation constant (1) are explained by the difference in the pK_a for ionization of the sulfonamide group. However, since the variation in dissociation rate constant of enzyme-sulfonamide complexes covers a fairly narrow range (2), the differences in intrinsic association rate constant between the variously substituted benzene- and heterocyclic sulfonamides preclude a generalized direct relationship between enzyme-inhibitor dissociation constant and the sulfonamide pK_a value, as was suggested by Miller *et al.* (10).

[1] R. W. King and A. S. V. Burgen, unpublished observations.

Curve fitting shows conclusively that the high-pH limb of the curve is controlled by the pK_a of the sulfonamide group, at least when this pK falls between 7.3 (methazolamide) and 10.0 [obtained by Taylor *et al.* (3) for dansylsulfonamide].

The pK_a value found for the enzymatic group, 7.5–7.6, is in very good agreement with the value reported previously for this ionization (3). The most recent observations on the hydration of CO_2 by human carbonic anhydrase B (11) indicate that at least one enzymatic function, with a minimum pK value of 7.6, is intimately involved in the conversion of the inactive to the active form of the enzyme.

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