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Kinetics of Complex Formation between Human Carbonic Anhydrases and Aromatic Sulfonamides*

P. W. Taylor, R. W. King, and A. S. V. Burgen

ABSTRACT: The kinetics of the reaction between human carbonic anhydrases B and C and aromatic sulfonamides have been followed using the stop-flow method by measuring changes in protein fluorescence associated with complex formation. Affinity constants calculated from the ratio of kinetic constants are in agreement with those measured by equilibrium titration for sulfonamides which quench the tryptophan fluorescence. By using a coupled reaction system it is possible to measure dissociation rates for both quenching and nonquenching sulfonamides. With corresponding equilibrium affinities, association rates for nonquenching inhibitors can also be evaluated. The two enzymes show marked differences in specificity for the series of sulfonamides. Thermodynamic parameters have been determined and the complex is stabilized primarily through a favorable enthalpy change. Rate and thermodynamic data both indicate that the association rate is not diffusion controlled but that the formation of the complex requires a distinct activation energy. In contrast to most ligand–metal substitution processes, differences in the thermodynamic stability of the sulfonamide complexes are largely a consequence of the variation in the association rate constants.

The aromatic and heterocyclic sulfonamides form a group of highly active inhibitors possessing unusual selectivity for the metalloenzyme, carbonic anhydrase (hereafter referred to as enzyme) (Mann and Keilin, 1940; Maren, 1967). Although an unsubstituted SO_2NH_2 group attached to a conjugated ring system is required for inhibition, substantial modifications of ring structure are possible with the retention of the characteristic high affinity. Sulfonamides thus present for interaction a relatively simple and rigid molecular structure to which a large number of ring substitutions can be made. The crystal structure of the human enzyme C–acetoxymercurisulfanilamide complex at 5.5 Å shows that this inhibitor binds within a cleft on the enzyme surface with the sulfonamido group closely positioned with respect to the coordination sphere of the Zn (Fridborg *et al.*, 1967). Replacement of Zn(II) by Co(II) gives visible Co spectra characteristic of ligand geometry and symmetry around the transition metal (Lindskog, 1963). Formation of the sulfonamide complex is accompanied by changes in the energy of the d–d transitions of the Co enzyme (Lindskog, 1963). This evidence supports the concept that the sulfonamide–carbonic anhydrase complex is coordinated through a ligand–metal bond. In addition, an enhancement of quantum yield obtained with the fluorescent probe, 5-dimethylaminonaphthalene-1-sulfonamide, upon binding indicates that the aromatic ring system of this inhibitor is associated with a hydrophobic region of the protein (Chen and Kernohan, 1967). The possibility that stabilization of this complex is conferred through both a ligand–metal bond and hydrophobic forces has stimulated us to examine the kinetics of this reaction. Kinetic investigations have been a useful approach in coordination chemistry for understanding the mechanism of ligand–metal substitutions and similar studies should help in delineating the role played by a ligand–metal bond in the formation and stabilization of this enzyme–inhibitor complex.

While sulfonamide inhibition constants of carbonic anhydrase catalytic activity have been extensively examined, rate constants for this interaction have been reported for only a few sulfonamides and were obtained indirectly by measuring the rate of inhibition of CO_2 hydration (Kernohan, 1966; Lindskog and Thorslund, 1969). We have been able to measure the kinetics of the reaction

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S$$

directly by the stop-flow method using fluorescence quenching

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of the tryptophan residues in the enzyme to follow the course of the reaction. Sulfonamides with spectral transitions overlapping the tryptophan fluorescence emission band are effective quenchers of the native protein fluorescence upon combination with carbonic anhydrase. The quenching arises from a dipolar coupling between the excited state of tryptophan and the sulfonamide acceptor resulting in radiationless energy transfer between donor and acceptor (Förster, 1959). This principle was first applied by Velick *et al.* (1960) to determine the affinity of certain hapten–antibody complexes.

Methods

Preparation and separation of human carbonic anhydrases B and C from erythrocytes were carried out according to the procedure of Armstrong *et al.* (1966) using DEAE-Sephadex. Enzymatic activity was determined by the esterase method using *p*-nitrophenyl acetate as a substrate (Armstrong *et al.*, 1966). Individual isoenzymes were stored at 4° as a slurry in 60% $(NH_4)_2SO_4$. Small portions were prepared for use daily by diluting an equal volume of the slurry with 0.02 M phosphate buffer (pH 7.0). The solution was then passed through a Sephadex G-25 column equilibrated with the buffer to be used for the kinetic or titration studies. Some of the sulfonamides were purchased from commercial sources while others were kindly provided by Imperial Chemical Industries; Lederle Laboratories, Pearl River, N. Y.; and by May and Baker Ltd. When sufficient material was available the sulfonamides were recrystallized two to three times from ethanol or ethanol–H_2O. Purity was checked by thin-layer chromatography on Kieselgel (Merck) using both methylene chloride–methanol (60:40, v/v) and diethyl ether solvent systems. Developed plates were stained using the chlorine–starch–iodide system of Rydon and Smith (1952).

A stop-flow fluid delivery and mixing system patterned closely after that described by Sturtevant (1964) was designed to fit in and above the cell compartment of a Farrand spectrophotofluorometer. A four-channel centrifugal mixing chamber was positioned directly above a quartz observation tube having an inside diameter of 1.0 mm. The quartz tube was fixed within a Farrand microcell adapter containing a converging lens system. The short path length within the observation tube was advantageous since it permitted measurements in the presence of relatively high concentrations of sulfonamide without excessive absorption of exciting radiation. Mixing efficiency was examined by the fluorescent indicators, 2-naphthol and salicylic acid. The overall dead time of the instrument is 2–3 msec.

The exciting wavelength was 290 mμ and emission was determined at 345 mμ. Photomultiplier output was measured on a Tektronix 502A oscilloscope. Experimental oscilloscope traces were recorded on 35-mm film and projected for measurement. Vertical displacements from equilibrium were measured at six to ten different horizontal time points and transposed to suitable coordinates. Photomultiplier voltage was adjusted so that 0.5 μM C gave an output of 300 mV. The complete stop-flow system was jacketed and temperature controlled from a circulating water bath. Over the range of 9–45°, the temperature of the reactant reservoirs, fluid delivery system, and the insulated compartment containing the mixing chamber and observation tube was uniform to ±0.2°. A collection syringe was employed to produce a slight resistance to flow. This reduced inertia effects of the drive system and gave greater consistency to each measurement.

Unless otherwise specified all measurements were made at 25° in 0.02 M phosphate buffer (pH 6.5) with no other anions present. The concentration of the enzyme determined from ϵ_{280} (Armstrong *et al.*, 1966) was 0.5–2.5 μM, and for the association reaction to give pseudo-first-order kinetics the sulfonamide was added in at least a 10-fold molar excess. When k_1 was greater than 10^6 M^{-1} sec^{-1}, equal concentrations of E and S gave the greatest sensitivity and accuracy. In this case the fractional approach to equilibrium was measured. For the dissociation rates the E–S complex was mixed with a 200–1000-fold excess of a second sulfonamide. The pair of inhibitors were selected to quench the anhydrase fluorescence to different extents making it possible to measure k_{-1} for quenching and nonquenching sulfonamides. Several different concentrations of the second sulfonamide were employed to ensure that the reaction was rate limited by dissociation of the initial complex.

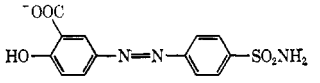
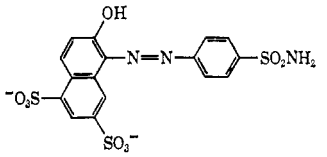
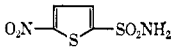
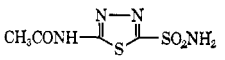
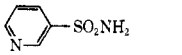
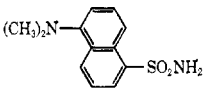
The stability of the fluorescence quantum yield of carbonic anhydrase in the light path of the stop-flow fluorometer was checked by using a shutter system on the light source. Free enzyme and most E–S complexes were quite stable in the presence of the exciting radiation showing fluorescence losses of less than 2%/min. However, four sulfonamides were found to cause a light-sensitive decrease of enzyme fluorescence. k_1 and k_{-1} were computed as means from three to four successive oscilloscope traces for each reaction variable. The coefficient of variation was less than 5% provided the pseudo-first-order rate constant was less than 20 sec^{-1}; above this value the precision decreased. In practice, the high sensitivity of the fluorescence method allows the initial concentration to be sufficiently dilute so that these rapid rates are avoided.

Equilibrium affinity constants for complex formation were determined from fluorescence titrations as previously described (Marlow *et al.*, 1969). Small relative volumes of sulfonamide were added from an Agla micrometer syringe to 2.0 ml of carbonic anhydrase solution, contained in a 1.0-cm^2 cuvet. To measure the affinity of a nonquenching sulfonamide a competitive back-titration against 4-[(5,7-disulfonic acid naphth-2-ol)-1-azo]benzene sulfonamide (**12**, Table I) was employed. The affinity of the nonquenching sulfonamide could be determined from the return of fluorescence and the affinity of the quenching sulfonamide. The enzyme concentration for these experiments was between 0.1 and 1.0 μM, a concentration at which internal quenching was negligible.

The kinetic analyses were carried out as follows: in the presence of excess sulfonamide (S) the slope of a plot of $\ln [(E_t) - (E_\infty)]$ against time is $k_1(S_0) + k_{-1}$. (E_t) and (E_∞) denote the fluorescence of enzyme at time, t, and at equilibbium and (S_0) is the initial sulfonamide concentration. In these experiments $k_{-1} \ll k_1(S_0)$ and was obtained separately from the dissociation rate experiments. When equimolar concentrations of E and S were used

$$\ln\left[\frac{(E_0)^2 - F(E_\infty)^2}{(E_0)^2 \quad (1 - F)}\right] = k_1\left(\frac{(E_0)^2 - (E_\infty)^2}{(E_0)}\right)t$$

TABLE I: Kinetic and Equilibrium Constants for Various Sulfonamides and Human Carbonic Anhydrase C, pH 6.5, 25°.

Compd	Sulfonamide	k_1 (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	k_1/k_{-1}	K_{equil} (M^{-1})[a]	% Quenching[b]
1	$C_6H_5SO_2NH_2$	1.06×10^5	0.164		6.48×10^5	
2	$CH_3C_6H_4SO_2NH_2$-*p*	1.17×10^5	0.060		1.95×10^6	10
3	$^-OOCC_6H_4SO_2NH_2$-*p*	2.71×10^5	0.073		3.71×10^6	
4	$O_2NC_6H_4SO_2NH_2$-*p*	7.37×10^5	0.048	1.53×10^7	1.60×10^7	67
5	$CH_3CONHC_6H_4SO_2NH_2$-*p*	1.12×10^5	0.070		1.60×10^6	
6	$ClC_6H_4SO_2NH_2$-*p*	5.13×10^5	0.062		8.27×10^6	
7	3,4-$Cl_2C_6H_3SO_2NH_2$	6.08×10^5	0.039		1.56×10^7	
8	$^-OOC(3,5\text{-}(NO_2)_2)C_6H_2SO_2NH_2$-*p*	1.26×10^6	0.098	1.29×10^7	1.40×10^7	62
9	$^-OOC(3\text{-}NO_2)C_6H_3SO_2NH_2$-*p*	5.75×10^5	0.061	9.42×10^6	1.02×10^7	64
10	$HO(3\text{-}NO_2)C_6H_3SO_2NH_2$-*p*	5.48×10^4	0.216	2.53×10^5	2.40×10^5	51
11		1.13×10^7	0.033	3.40×10^8	$>10^8$	88
12		5.81×10^5	0.075	7.74×10^6	6.90×10^6	90
13		8.60×10^6	0.036	2.38×10^8	$>10^8$	74
14		4.83×10^6	0.068		7.10×10^7	5
15			0.306		$<10^5$	
16	$Cl(3\text{-}NH_2)C_6H_3SO_2NH_2$-*p*	2.04×10^5	0.050	4.08×10^6	7.95×10^6	55
17	$^-OOC(3\text{-}NH_2)C_6H_3SO_2NH_2$-*p*	7.56×10^4	0.087	8.69×10^5	1.18×10^6	70
18	$CH_3(2\text{-}Cl,5\text{-}NH_2)C_6H_2SO_2NH_2$-*p*	1.78×10^5	0.191	9.42×10^5	9.53×10^5	43
19	2,4,5-$Cl_3C_6H_2SO_2NH_2$	1.75×10^6	0.258		6.80×10^6	
20	2,4,6-$Cl_3C_6H_2SO_2NH_2$	6.58×10^5	0.144		4.57×10^6	
21	$Cl(2\text{-}NO_2)C_6H_3SO_2NH_2$-*p*	1.96×10^6	1.55	1.26×10^6	2.17×10^6	54
22	$NC(2\text{-}NO_2)C_6H_3SO_2NH_2$-*p*	2.50×10^6	2.71	9.2×10^5	1.21×10^6	47
23	$O_2N(2\text{-}^-OOC)C_6H_3SO_2NH_2$-*p*				$<10^4$	
24		2.40×10^5	0.390	6.15×10^5	5.80×10^5	70

[a] Determined by affinity titration. [b] Maximal quenching at complete saturation of carbonic anhydrase.

where (E_0) and (E_∞) are the initial and equilibrium concentrations of anhydrase. F, the fractional approach to equilibbium, equals

$$\left[\frac{(E_0) - (E_t)}{(E_0) - (E_\infty)}\right]$$

and is obtained from the respective vertical distances on the oscilloscope trace. (E_∞) was calculated from equilibrium affinity measurements or approximated from k_{-1} and an estimate of k_1 from the initial rate. If the latter procedure was used it was necessary to employ two to three successive reiterations using a new value of (E_∞) obtained from the previous estimation of k_1/k_{-1}. Each adjustment produced an improved fit to the above equation.

The dissociation reaction involves the relationship

$$E\text{-}S_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + S_1 + S_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}S_2$$

Provided that $k_{-2} \simeq k_{-1}$, $k_2(S_2) \gg k_1(S_1)$, $k_2(S_2) \gg k_{-1}$, the reaction kinetics will be first order and independent of (S_2). The observed rate constant should then be an accurate reflection of k_{-1}.

Results

The azo sulfonamides, dansylamide,[1] most nitro- and aminosulfonamides quench the fluorescence arising from the

[1] Abbreviation used is: 5-dimethylaminonaphthalene-1-sulfonamide.

seven tryptophans in human enzyme C with a high degree of efficiency (Table I). These compounds also show significant absorption in the region between 320 and 380 mμ and thus satisfy a necessary criterion for the Förster dipolar energy transfer (Förster, 1959). Of the 24 sulfonamides whose kinetics of complex formation were examined all apparently confirmed the postulates of reversible bimolecular–unimolecular reaction. Figures 1 and 2 show examples of the experimental results for both the association and dissociation reactions and the kinetic cases on which this conclusion is based. Association rate constants as high as 1.13×10^7 M^{-1} sec^{-1} were observed.[2]

In Table I for the series of quenching sulfonamides the affinity constants obtained from the ratio $k_1:k_{-1}$ (column 5) are in good agreement with those determined independently by equilibrium titration (column 6). The correspondence between values determined from the kinetic and equilibrium methods justifies the calculation of k_1 from the product $(k_{-1}K_{equil})$ for sulfonamides which quench the enzyme fluorescence less than 20%. The kinetic method also allows the evaluation of affinity constants for firmly bound complexes where only a lower limit can be determined by equilibrium titration owing to the presence of extremely small amounts of free species (**11** and **13**, Table I).

Certain sulfonamides (**16**, **17**, **18**, and **19**, Table I) were found to cause a decrease in enzyme fluorescence due to a light-activated species of the sulfonamide. This effect which caused deviations from the predicted kinetics could be minimized by working at light levels of minimal intensity and duration and by the addition of 10^{-3} M dithioerythritol.

With the series of sulfonamides studied a 240-fold range in k_1 values was found. This was considerably greater than the range of k_{-1} which was only 80-fold (Table I). The greater dependence of K_{equil} on k_1 is more systematically illustrated in Figure 3. When the *ortho*-substituted compounds are considered separately the slope of a logarithmic plot of k_1 *vs.* K_{equil} is approximately five times the corresponding slope for k_{-1}. The deviations from the relationship observed for those inhibitors with a substituent *ortho* to the sulfonamide group reflect a large k_{-1}. Too few of these sulfonamides have been investigated to ascertain whether these effects depend on the nature of the *ortho* substituent. However, from Table I it appears that the relatively large *ortho*-nitro substituent increases the dissociation rate 30–50-fold (compare **6** with **21** and **22**) while *ortho*-chloro substitution increases k_{-1} about fivefold (compare **7** with **19**, **20**; **16** with **18**). A relatively large k_{-1} is observed for dansylamide (**24**) where the second aromatic ring of the naphthalene may be regarded as having a steric equivalence to an *ortho* substituent.

A smaller number of determinations on human enzyme B revealed distinct differences in the specificity between the two isoenzymes (Table II). Human enzymes B and C have a number of common physical properties such as zinc content, sedimentation coefficient, molecular weight, and optical rota-

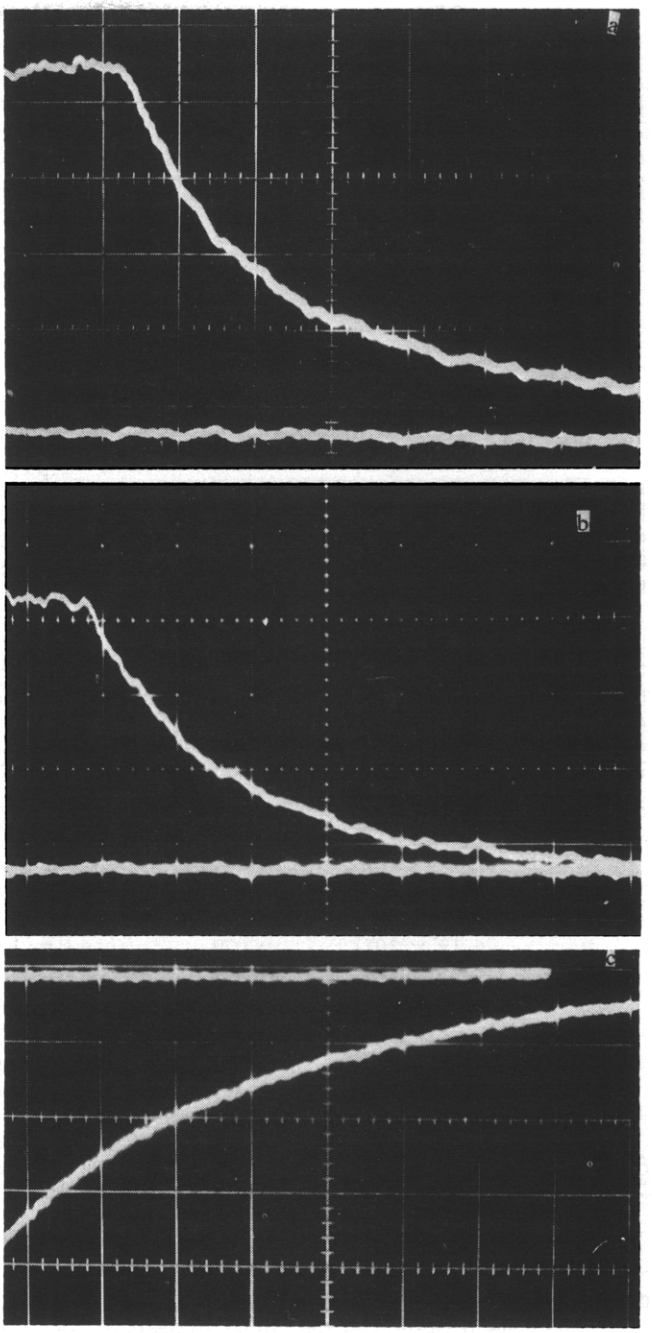


FIGURE 1: Oscilloscope traces recording changes in enzyme fluorescence upon combination or dissociation of a quenching sulfonamide. The time dependence of fluorescence is monitored during and after cessation of flow. (a) Association reaction between 1.25 μM human enzyme C and 1.25 μM salicylazobenzenesulfonamide. The lower horizontal line was triggered about 10 sec after the initiation of the reaction and represents the fluorescence at equilibrium. Each large horizontal increment corresponds to 50 msec. (b) Association reaction between 0.625 μM human enzyme C and 14.0 μM *p*-nitrobenzenesulfonamide. Each horizontal increment corresponds to 50 msec. (c) Dissociation of 1.25 μM *p*-nitrobenzenesulfonamide from 1.25 μM human enzyme C. The above complex was reacted with 5×10^{-4} M acetazolamide. Each large horizontal increment represents a time of 5 sec.

[2] A maximum association rate of 2.04×10^7 M^{-1} sec^{-1} is observed for salicylazobenzenesulfonamide between pH 7.5 and 8.0. A complete analysis of the pH dependence of association and dissociation kinetics for the Zn(II) and Co(II) enzymes will appear in a forthcoming publication (P. W. Taylor, R. W. King, and A. S. V. Burgen, in preparation). It appears that the sulfonamide with an un-ionized SO_2NH_2 group is the attacking species. As the pK_A's of the sulfonamido protons are usually in the range of 8.5–10.8, at pH 6.5 the sulfonamides will be almost completely in this un-ionized form. The inflection appearing near pH 6.5 for C is a consequence of an ionizing group on the enzyme and has been found to be invariant with the sulfonamides examined.

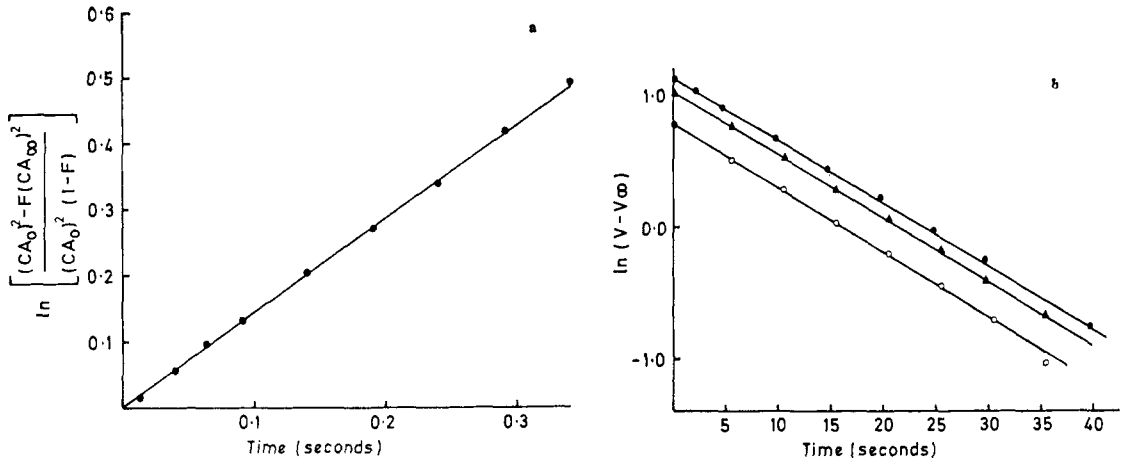


FIGURE 2: (a) Plot of $\ln((E_0)^2 - F(E_\infty)^2/(1-F)(E_0)^2)$ *vs.* t for a second-order association–first-order dissociation reaction between salicylazobenzenesulfonamide and human enzyme C. The concentration of reactants is the same as the upper portion of Figure 1. (b) Dissociation of *p*-nitrobenzenesulfonamide by acetazolamide. A mixture of 1.25 μM human enzyme C and 1.25 μM sulfonamide in one drive syringe was reacted with the specified concentration of acetazolamide; (○—○) 5×10^{-4} M, (△—△) 1.5×10^{-4} M, (●—●) 5×10^{-5} M, at pH 6.5 in 0.02 M phosphate buffer. $V - V_\infty$ represents the vertical distance on the trace between t and at equilibrium.

TABLE II: Kinetic and Equilibrium Constants for Complex Formation between Various Sulfonamides and Human Carbonic Anhydrase B, pH 6.5, 25°.

Compd	k_1 (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	k_1/k_{-1}	K_{equil}	k_1 (C/B)[b]	k_{-1} (C/B)[c]
4	2.86×10^5	0.049	5.84×10^6		2.54	0.98
6		0.127				0.47
11	6.8×10^6	0.016	4.25×10^8		1.66	2.36
12	9.7×10^5	0.018	5.39×10^7		0.500	4.17
9	5.12×10^5	0.079	6.4×10^6		1.12	0.70
13	2.52×10^6	0.024	1.05×10^8		3.41	1.50
21	7.7×10^5	0.013	7.5×10^6		2.55	15.0
22	1.8×10^6	0.052	3.4×10^7		1.39	52.1
24	1.34×10^5	0.030	4.46×10^6	4.0×10^6	1.8	12.6
	1.29×10^5[a]	0.031[a]	4.16×10^6			

[a] Determined by changes in dansylamide fluorescence at 468 mμ with excitation at 335 mμ. [b] Ratio of association rate constants for the two isoenzymes, carbonic anhydrases C and B. [c] Ratio of dissociation rate constants.

tory dispersion spectrum Armstrong *et al.*, 1966; Rickli *et al.*, 1964). The general features of the visible absorption spectra of their Co(II) derivatives are nearly identical suggesting equivalent ligand coordination and geometry (Lindskog and Nyman, 1964). The two isoenzymes, however, show a substantial variation in amino acid composition (Armstrong *et al.*, 1966). This may account for the lack of order observed when association or dissociation rates for the two enzymes are compared (Table II). The most striking contrast is the lack of effect of *ortho* substitution on the dissociation rates of the human enzyme B complexes (**21** and **22**). In agreement with this dansylamide (**24**) has a higher affinity for human enzyme B than human enzyme C due mainly to a reduced k_{-1}. The difference suggests that there is less steric restriction in the neighborhood of the *ortho* group on the sulfonamide in the human enzyme B binding site as compared with that in human enzyme C.

Verpoorte *et al.* (1967) in studying the hydrolysis rates of *o*-nitro and *p*-nitrophenyl acetate also observed a selective difference between the human B and C enzymes for these substrates. The ratio of hydrolysis rates for the *para* to *ortho* compounds was 7 to 1 for human enzyme C and 0.57 to 1 for human enzyme B. Separation of the kinetic constants indicated that this was due largely to differences in k_{cat} and not K_m. A space-filling *ortho* substituent if sterically restricted may force a slightly different positioning of the aromatic ring with respect to the protein surface. This positioning could influence both esterase activity and sulfonamide affinity since each is believed to be dependent on the orientation of an interacting group with respect to the Zn in this metalloenzyme (Pocker and Storm, 1968).

For dansylamide (**24**) rate constants could be determined by following either sulfonamide fluorescence enhancement at 468 mμ with excitation at 335 mμ or by quenching of the protein tryptophan fluorescence. The results obtained by these two techniques were found to agree closely (Table II).

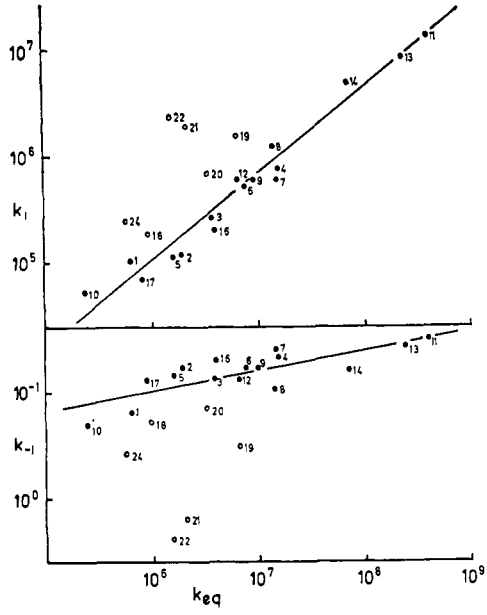


FIGURE 3: Upper: relationship between the rate constant of complex formation, k_1, and the affinity of the complex, K_{equil}, for various sulfonamides and human enzyme C. The sulfonamides are numbered according to Table I; (○) *ortho* substituted, (●) non-*ortho* substituted. The diagnonal line has a slope of 0.83 and was calculated from a least-squares analysis by considering only non-*ortho*-substituted sulfonamides. Lower: relationship between the dissociation rate of the complex, k_{-1}, and K_{equil}. (○) *ortho*-substituted sulfonamides, (●) non-*ortho* substituted. For the non-*ortho*-substituted compounds a regression line with a slope of 0.18 was calculated.

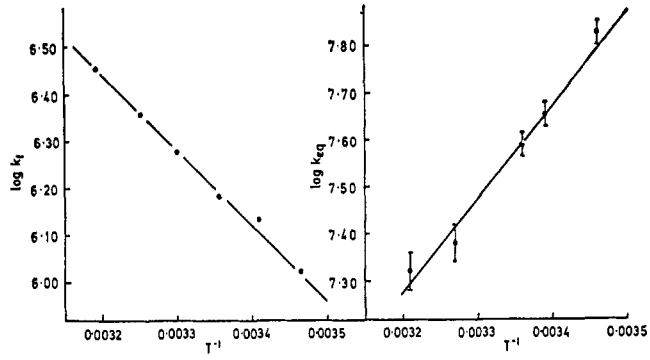


FIGURE 4: Arrhenius plots for the association rate constant, k_1, and the affinity of the complex, K_{equil}, as a function of $1/T°K$ for the *p*-nitrobenzenesulfonamide–human enzyme C complex.

Tryptophan fluorescence quenching is the more suitable general method because of the larger number of sulfonamides whose complex formation kinetics can be directly measured.

With *p*-nitrobenzene sulfonamide thermodynamic parameters of complex formation were obtained from rate and equilibrium measurements between 15 and 42°. The Arrhenius law is followed for k_1 and K_{equil} permitting ΔH and ΔS to be evaluated (Figure 4 and Table III). The free energy of complex formation is largely a consequence of a favorable enthalpy change.

Discussion

The simplest physical model for the kinetics of formation of a complex between enzyme and sulfonamide is a diffusion limited one in which the rate is determined by the frequency of collison between the ligand and binding site. Because of the relative size of the ligand it can be regarded as diffusing more rapidly than the macromolecule and the latter can be considered to be effectively immobile. If it is assumed that the ligand and its combining site have similar dimensions, the rate of collision is approximately 2×10^9 M^{-1} sec^{-1} at 25° (Alberty and Hammes, 1958). These considerations for diffusion limitation of a bimolecular reaction are based on radial diffusion of the smaller ligand into a convex hemispherical site on the outer surface of the macromolecule. For the E–S complex the highest observed association rate is two orders of magnitude smaller than this diffusion limitation and the lowest differs by more than four orders of magnitude (Table I). Furthermore, in a diffusion-limited complex it is to be expected that the affinity constants for a series of ligands will be closely dependent on their relative dissociation rates, but largely independent of their association rates. This is the opposite of what has been found as the range of variation of k_1 for the sulfonamide ligands is considerably greater than k_{-1}. Finally in a diffusion-controlled reaction the enthalpy of association should be that of diffusion in water (*i.e.*, 2.5–4 kcal/mole) rather than the 6.6 kcal/mole found (Table III).

Lower association rates would result from the combining site being geometrically restricted reducing the solid angle of approach through which the ligand molecules could gain access to this site. There is indeed evidence from X-ray crystallographic studies of human enzyme that the combining site is within a rather narrow cleft (Fridborg *et al.*, 1967). However, this factor cannot account for the greater dependence of the affinities of the ligands on their relative association rates nor can it explain the relationship between the size of the sulfonamides and their association rates. Since diffusion-controlled complex formation should show a dependence on both translational and rotational diffusion it would be predicted that the unsubstituted sulfonamides would be least hindered and hence have the highest association rate. However, almost all substituents lead to a greater association rate when compared with the parent compound, benzene sulfonamide (**1**). Salicylazobenzene sulfonamide

TABLE III: Thermodynamic Parameters for Complex Formation between *p*-Nitrobenzenesulfonamide and Human Carbonic Anhydrase C.[a]

	k_1	k_{-1}	K_{equil}
ΔF (kcal/mole)	9.0	19.3	−10.3
ΔH (kcal/mole)	6.6	16.1	−9.5
ΔS (cal/mole deg)	−7.9	−10.9	3.0

[a] Rate and equilibrium determinations were made in 0.02 M phosphate buffer (pH 7.6) at temperatures between 15.5 and 40.2°. Datum temperature 25°. At pH 7.6 both the association rate and the affinity of *p*-nitrobenzenesulfonamide are maximal.

(**11**) is much bulkier and yet has an association rate more than two orders of magnitude greater than benzene sulfonamide. The *ortho*-substituted compounds (**18–22**) also illustrate this when compared with their unsubstituted congeners. From a thermodynamic point of view, one would expect that geometric limitations on access should be reflected in greater negative entropy of formation rather than the observed larger enthalpy. The entropy term in absolute rate theory corresponds with the Arrhenius equation frequency factor which is derived from collision probabilities.

Since complex formation requires an activation energy distinct from and greater than that of diffusion the potential contribution from the ligand–metal bond must be considered. The relatively large net enthalpy change, ΔH_{equil} (Table III), is more in character with a complex stabilized through a dominant ligand–metal bond rather than hydrophobic interactions (Kauzmann, 1959). Kinetic investigations of structural variation of combining and existing ligands of the complex offer an important operational test of mechanism in coordination chemistry. In simple ligand–metal coordination complexes the kinetics of association are controlled by dissociation of the leaving group (*e.g.*, a water molecule) from the coordination sphere of the metal ion and show little sensitivity toward the incoming ligand (Eigen and Wilkens, 1965; Wilkens, 1962; Sutin, 1966). The thermodynamic stability of these coordination complexes thus is governed largely by the variance in their dissociation rate constants.

In the series of sulfonamides the 400-fold variation in affinity, K_{equil}, is largely accounted for by differences in the association rate constant, k_1, as illustrated by the logarithmic plot in Figure 3. The log of an equilibrium constant is proportional to the standard free-energy change accompanying the reaction ($\Delta F°$)

$$\log K_{equil} = \frac{-\Delta F°}{2.3RT}$$

Also, according to absolute rate theory (Laidler, 1958; Eyring, 1935) a specific rate constant can be expressed in terms of a standard free energy of activation ($\Delta F_1^{\ddagger}$)

$$\log k_1 = \log \left(\frac{RT}{Nh}\right) - \frac{\Delta F_1^{\ddagger}}{2.3RT}$$

The solid line in Figure 3 has a slope of 0.83 and thus yields the relationship: $\Delta F_1^{\ddagger} = 0.83\Delta F° + C$, where C is constant at constant temperature.

This close correlation of $\Delta F°$ with $\Delta F_1^{\ddagger}$, which contrasts with most ligand–metal coordination complexes, illustrates that substituent variation on the incoming ligand influences the energy of the transition state. This dependence of the formation rate on structure suggests an associative type of mechanism involving the participation of the incoming ligand (Landford and Gray, 1965).

The visible spectrum of neither Co(II) human enzyme nor its sulfonamide complex correspond precisely to that found in any simple Co(II) complexes making the coordinate geometry in the enzyme and the changes occurring in forming the complex uncertain. However, the spectral changes induced in Co(II) human enzyme by several sulfonamides appear to be identical suggesting that the coordinate structure is the same for all these complexes. This makes it difficult to see how the coordination step can exert a differential effect on association and therefore we must also consider other interacting forces in the activation barrier. In addition to a hydrophobic interaction involving the essential unsaturated ring structure of the sulfonamide a change in macromolecule conformation will contribute to the energetics of the reaction. Ultraviolet difference spectra of carbonic anhydrase characteristic of perturbation of the environment of tryptophan and tyrosine residues have been observed when sulfonamides combine (R. W. King and A. S. V. Burgen, submitted for publication). The consequent alteration in *intramolecular* bonding forces clearly must contribute to any net energy change and could influence the activation energy of the formation reaction. At present there is no evidence that the energetics of the conformational change are dependent on ligand structure. Thus it is difficult from only an examination of structural variation of the combining sulfonamide to partition the relative free-energy contributions between the ligand–metal bond, hydrophobic interactions, and the conformational change.

Since stabilization of this complex appears to be conferred through more than a single type of interaction, one must consider the possibility of a multistep formation pathway. Here, the formation of the complex is described in terms of a rapid preequilibrium association involving at least one less stable intermediate as shown

$$E + S \underset{k_b}{\overset{k_a}{\rightleftharpoons}} (ES)_1 \underset{k_d}{\overset{k_c}{\rightleftharpoons}} (ES)_2$$

The formation of initial complex, $(ES)_1$, can modify the apparent reaction kinetics even though it may not necessarily be present in detectable concentrations. As an example, the initial complex may only involve the hydrophobic interaction of the aromatic ring. Its formation is bimolecular and it would be expected to contribute to fluorescence quenching only under conditions of high initial concentrations of sulfonamide ($k_a(S) > k_b + k_c$). Thus, with the relatively dilute solutions necessary for stop-flow experiments, fluorescence quenching should only measure the formation of the final complex, $(ES)_2$, and biphasic reaction kinetics would not be detected. The second step is effectively unimolecular. If this step were to involve ligand–metal bonding the preformation of the initial complex would give a statistical advantage to this process.

This concept is similar to the outersphere complex or ion-pairing mechanisms proposed for many coordination complexes (Eigen and Wilkens, 1965). The formation of the initial complex through hydrophobic bonding, however, can accommodate the observed dependence of the association rate on ligand structure. Thus although k_c may be insensitive to the nature of the incoming ligand the concentration of $(ES)_1$ can be affected by the substituent groups on the sulfonamide. A further test of this possibility will come from comparative kinetic studies with enzyme and inorganic anions which are believed to interact solely through ligand–metal coordination. These studies will be reported in a subsequent publication.

We have concentrated our interest on human enzyme C because of the recent developments on its crystal structure. The eventual description of the three-dimensional structure

at high resolution will provide a unique opportunity to make rather stringent tests of structure and fit in relation to the kinetics and stabilization energy of complex formation.

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