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Influence of pH on the Kinetics of Complex Formation between Aromatic Sulfonamides and Human Carbonic Anhydrase*

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ABSTRACT: The kinetics of complex formation between aromatic sulfonamides and human carbonic anhydrases B and C have been examined over the pH range, 5.0–10.8 by stopped-flow kinetic measurements of fluorescence quenching. The apparent bimolecular association rate constant is highly pH dependent whereas the dissociation rate shows no variance with pH. The kinetic constants and their pH dependence are similar for the Zn and Co(II) forms of the enzyme. Correlation between the visible d–d absorption spectra of the Co(II) enzyme and the association kinetics suggest that the pH dependence of the association process is governed by a proton-dependent equilibrium between different coordination forms of the enzyme. Combination appears to occur between the neutral sulfonamide species and the coordination form of carbonic anhydrase which predominates at alkaline pH. The pH-insensitive dissociation rate is also consistent with spectroscopic evidence for a fixed coordination state of the sulfonamide complex. Carboxymethylation of a single histidine residue in human carbonic anhydrase B markedly affects the sulfonamide binding kinetics. The pH-independent dissociation rate is increased 45-fold while the pH dependence of association is shifted to higher pH with little change in the intrinsic rate constant.

Co(II) enzyme spectral changes upon carboxymethylation also show an alteration in the pH dependence of the equilibrium between coordination forms of the enzyme which is in good accord with the association rate–pH profile. A mechanism for sulfonamide complex formation involving its combination as a neutral species and subsequent transfer of the sulfonamido proton to a nucleophilic group on the enzyme is proposed.

Aromatic and heterocyclic sulfonamides form a group of specific inhibitors of the metalloenzyme, carbonic anhydrase, possessing a wide range of affinity constants (Maren, 1967; Taylor *et al.*, 1970). The crystal structure of a human carbonic anhydrase C–acetoxymercurisulfanilamide complex shows that the sulfonamido group resides in close vicinity to the coordination sphere of the metal (Fridborg *et al.*, 1967). Replacement of the single Zn atom in the enzyme by Co generate a visible d–d spectrum characteristic of the coordinated ligands and their geometric arrangement around the metal (Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1968). Changes in the Co(II) spectrum accompanying sulfonamide binding further support the concept that the sulfonamide may function as a metalloligand. Fluorescence enhancement observed with the sulfonamide, dansylamide, suggests that the essential aromatic ring of this inhibitor is bound in a relatively hydrophobic environment in the protein (Chen and Kernohan, 1967). Thus stabilization of the complex may be conferred through both ligand–metal bonding and hydrophobic interaction involving the unsaturated ring of the sulfonamide. Indeed, much of the specificity inherent in this reaction may be a consequence of the spatial arrangement of these interacting sites and the sequence of component steps involved in complex formation.

Studies employing rapid reaction techniques to examine the kinetics of ligand–metalloprotein complexes have largely been confined to small inorganic ligands and hemoproteins where advantage can be taken of the large spectral change accompanying ligand binding (Ellis and Dunford, 1968; Ver Ploeg and Alberty, 1968; Duffey *et al.*, 1966; Goldsack *et al.*, 1966). Studies of the pH–rate dependence have proved helpful in characterizing the ionizing groups affecting the reactivity of the protein. The sulfonamide–carbonic anhydrase system introduces the possibility of additional involvement of a hydrophobic interaction at a site distinct from the ligand–metal coordination sphere. A kinetic investigation of complex formation should help delineate the relative free-energy contributions arising from the various interactions stabilizing the complex.

Using stopped-flow instrumentation coupled with a fluorescence detection system, we have previously examined

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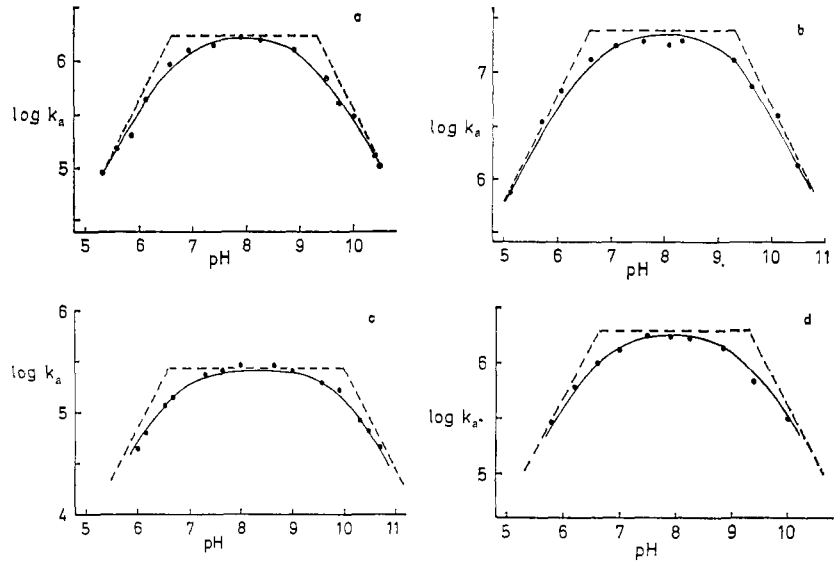

FIGURE 1: pH dependence of the association rate constants between various sulfonamides and human carbonic anhydrase C; (a) *p*-nitrobenzenesulfonamide and Zn isoenzyme C; (b) *p*-[salicyl-5-azo]benzenesulfonamide and Zn isoenzyme C; c, dansylamide and Zn isoenzyme C; (d) *p*-nitrobenzenesulfonamide and Co(II) isoenzyme C. The solid lines are calculated from reaction 1 in the Discussion section assuming pK_E to be 6.60, and pK_S to be 9.30 for *p*-nitrobenzenesulfonamide and salicylazobenzenesulfonamide and 10.00 for dansylamide. The intersection of the dotted lines with slopes of 1.0 and 0.0 also yield the respective pK values.

the relationship between structural variation of the sulfonamide and kinetics of complex formation (Taylor *et al.*, 1970). Development of a meaningful kinetic scheme also requires correlation with the ionizing groups influencing the conformation and reactivity of the macromolecule since kinetic studies alone seldom lead to an unambiguous reaction pathway or mechanism. The Co(II) enzyme provides a valuable intrinsic probe of the binding site as the energy of Co d–d transitions is sensitive to the nature of the ligands as well as to their symmetry and geometry around the central metal ion. Spectral differences can distinguish coordination forms of both free carbonic anhydrase and its complex, which may be involved in sulfonamide binding. Using the two isoenzymes of human carbonic anhydrase and a chemically modified derivative we have attempted to correlate structural information obtained from Co(II) spectra with the association and dissociation kinetics.

Experimental Section

Materials. The isoenzymes, carbonic anhydrases B and C, were isolated and purified from human erythrocytes according to the ion-exchange procedures of Armstrong *et al.* (1966) and stored as slurries in 60% $(NH_4)_2SO_4$ at 4°. Enzymatic activity was determined by the method described by these authors using *p*-nitrophenyl acetate as a substrate. Concentrated human carbonic anhydrase (hereafter simply called isoenzyme) was prepared for use daily by dilution with an equal volume of 0.1 M phosphate buffer (pH 7.0) and separation from $(NH_4)_2SO_4$ on Sephadex G-25 columns equilibrated with the buffer to be used for the series of reactions. Dansylamide and NBS[1] were obtained from British Drug Houses and K & K Laboratories; SABS was synthesized in the laboratory. The sulfonamides were further purified by repeated recrystallization and purity was checked by thin-layer chromatography (King and Burgen, 1970; Taylor *et al.*, 1970).

The apoenzyme and the Co(II) derivative of isoenzymes B and C were prepared as described by Lindskog (1963). For this work deionized H_2O was also treated with Chelex–Na resin (Bio-Rad Laboratories, Richmond, Calif.) to remove trace metal contaminants. Before addition of Co^{2+} the apoenzymes had 3–7% of the esterase activity that could be restored by the addition of Zn^{2+}. After addition of Co^{2+} the enzymes exhibited 95–125% of the esterase activity of the respective Zn forms.

Carboxymethylated isoenzyme B was made by reacting 0.1–0.4 mM Zn or Co(II) enzyme with 10 mM bromoacetate in 0.1 M phosphate buffer (pH 7.4). Bromoacetic acid was twice recrystallized from CCl_4. The reaction was allowed to proceed for 8 hr at 25°. It was terminated by passing the reactants over a Sephadex G-25 column and subsequent dialysis *vs.* 0.01 M buffer. The extent of carboxymethylation was ascertained by the reduction in esterase activity at pH 7.0. As found by Whitney *et al.* (1967) this was reduced to 4–6% of control activity after 8-hr reaction.

Spectroscopic Measurements. Molar extinction coefficients and Co(II) spectra were measured on a Zeiss PMQ II and a Jasco UV 5 recording spectrophotometer. Between 0.15 and 0.4 mM carbonic anhydrase was used in 2.0-cm cells. Samples were passed through a Millipore filter (PO 1300) before each recording. Correction for residual light scatter was made from absorption measurements below 440 mμ by extrapolation of an absorption *vs.* wavelength plot. The loss of transmission due to light scatter was 2–10% of the absorption reading in the wavelength of maximum absorption.

Isoenzyme concentrations were determined from absorp-

[1] The abbreviations used are: NBS, *p*-nitrobenzenesulfonamide; SABS, *p*-(salicyl-5-azo)benzenesulfonamide; dansylamide, 5-dimethylaminonaphthalene-1-sulfonamide.

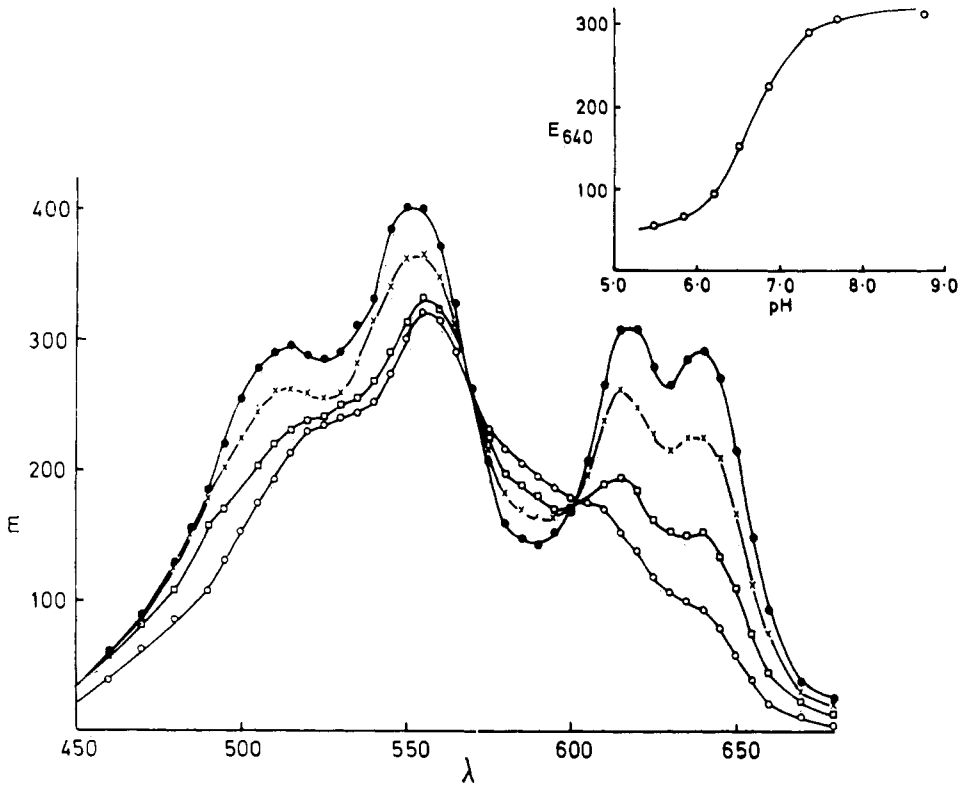

FIGURE 2: pH dependence of the Co(II) isoenzyme C d–d spectrum. 0.32 mM enzyme in 20 mM K_2SO_4 was pH adjusted with 0.05 N NaOH or 0.02 M H_2SO_4 and the spectra were recorded in 2.0-cm cells. (O—O) pH 6.19, (□—□) pH 6.50, (×—×) pH 6.86, and (●—●) pH 7.35. E is the molar extinction coefficient.

tion measurements at 280 mμ taking ϵ_{280} as 46.8 and 56.2 for isoenzymes B and C, respectively (Armstrong *et al.*, 1966). Dissociation constants for the sulfonamido proton of dansylamide and NBS were determined spectrophotometrically by following absorbance changes at 300 mμ. Hydrogen ion titration was also used to determine the dissociation constants of SABS and NBS.

Kinetic Studies. The procedure for following the reaction kinetics using tryptophan fluorescence quenching and the stopped-flow instrumentation have been described in detail (Taylor *et al.*, 1970). The association rates for NBS and dansylamide were determined under pseudo-first-order conditions using 0.625 μM isoenzyme and at least a 10-fold molar excess of sulfonamide. The slope of a plot of ln $(f_t - f_\infty)$ *vs.* time is $k_a(S_0) + k_d$, where k_a is the association rate constant, (S_0) the initial sulfonamide concentration and $f_t - f_\infty$ is the difference in fluorescence at time, t, and equilibrium. k_d, the dissociation rate constant, was measured independently. With the exception of the kinetic investigation of carboxymethylated isoenzyme the initial sulfonamide concentration was adjusted so that k_d was 1–10% of $k_a(S_0)$. To determine association rates for SABS equal concentrations of reactants were employed; the equation and method for determining the rate constants for this second-order-first-order reaction are described in the preceding study (Taylor *et al.*, 1970).

Dissociation rates were measured by reaction of a human carbonic anhydrase–sulfonamide complex with a 400–1000-fold excess of a second sulfonamide. The pair of sulfonamides are chosen to have different quenching capacities so that a maximum signal difference is obtained. It can be shown that the observed rate is limited by dissociation of the initial sulfonamide and is a measure of k_d (Taylor *et al.*, 1970). The reported rate constants are mean values obtained from three oscilloscope traces. The usual variation was ±5% for first-order kinetics and up to 10% for second-order kinetics. Equilibrium affinities were determined by fluorescence quenching titration (Taylor *et al.*, 1970). The temperature for the kinetic and equilibrium studies was 25.0 ± 0.2°. Over the pH range the following 20 mM buffers were employed: pH 5.0–5.5, succinate; pH 5.5–7.0, phosphate or imidazole; pH 7.0–9.0, Tris-SO_4; pH 9.0–10.2, 2-amino-2-methyl-1,3-propanediol; pH 10.2–10.8, triethylamine-HCl.

Results

The pH dependence of the association rate constant for isoenzyme C and NBS is shown in Figure 1a. The curve shows two pK's with values of 6.60 and 9.30, respectively. A similar pH dependence was found for SABS (Figure 1b), but in the case of dansylamide the second pK was shifted to 10.0 (Figure 1c). The higher of the two pK values corresponds closely with the ionization constant of the sulfonamido groups in the three inhibitors which were found to be 9.30, 9.35, and 9.95, respectively. There is no ionization in any of the sulfonamides corresponding to the pK of 6.60 in the association process.

By contrast, the dissociation rate of the complex was

TABLE I: Influence of pH on the Dissociation Rate of the Carbonic Anhydrase C–Sulfonamide Complex.

	pH	k_d (sec^{-1})[a]	pH	k_d (sec^{-1})[b]
A. *p*-Nitrobenzenesulfonamide				
			5.00	0.047
			5.50	0.046
			6.00	0.043
	6.50	0.049	6.50	0.040
	7.62	0.047	7.50	0.039
	8.48	0.053	9.00	0.045
	9.60	0.049	9.50	0.048
			10.00	0.043
		0.0495 ± 0.0013		0.0439 ± 0.0011
B. Salicylazobenzenesulfonamide	5.80	0.034		
	6.50	0.033		
	7.02	0.041		
	7.90	0.034		
	8.85	0.036		
		0.0356 ± 0.0015		

[a] Determined from kinetic measurements by the competition method. [b] Determined from the ratio of the measured association rate constant and equilibrium affinity.

totally insensitive to pH in the range examined, 5.0–10.0 (Table I). Direct kinetic determination of the dissociation rate was not possible over the complete range because of the low association rate at the extremes of pH. However, parallel determination of the equilibrium affinity constant enabled the dissociation constant to be evaluated from the ratio of the association rate constant and equilibrium affinity. Good agreement between the two methods was found.

Lindskog and Nyman (1964) have examined the pH dependence of the visible spectrum of Co(II) isoenzyme B. Distinct isosbestic points are evident at 568 and 602 mμ indicating an equilibrium between the two coordination forms of the enzyme with a pK of 7.3. Co(II) spectra of isoenzyme C are qualitatively similar, however this isoenzyme has a transition pK of 6.6 (Figure 2). The kinetic constants for sulfonamide association and dissociation with Zn and Co(II) isoenzyme C were found to be of similar magnitude (Table II). Furthermore, the first and second pK values for the sulfonamide association rate with Co(II) isoenzyme C were indistinguishable from those of the Zn enzyme (Figure 1d). Therefore we have spectroscopic evidence of a pK in the enzyme identical with that observed from the kinetics.

It would be useful if confirmation of this could be obtained with isoenzyme B whose association rate pK is nearly one pH unit higher than the C enzyme. Unfortunately, in our hands Co(II) isoenzyme B has proved unsuitable for sulfonamide kinetic studies at low pH so that a critical test has not been possible. Below pH 7.5 marked deviations from the predicted pseudo-first-order kinetics are evident. All that can be said is that partial titration curves for the association rate suggest a pK about 0.5–1.0 higher than for isoenzyme C. Nevertheless, a satisfactory correlation is found between the association rate of Zn isoenzyme B and the pK of its Co derivative determined spectroscopically (see below).

TABLE II: Kinetic Constants for Complex Formation between *p*-Nitrobenzenesulfonamide and Zn and Co(II) Human Carbonic Anhydrase C.

	k_a (M^{-1} sec^{-1})[a]	k_d (sec^{-1})	K (M^{-1})
Zn isoenzyme C	1.49×10^6	0.049	3.04×10^7
Co isoenzyme C	1.77×10^6	0.082	2.16×10^7

[a] Maximum observed association rate at pH 8.0.

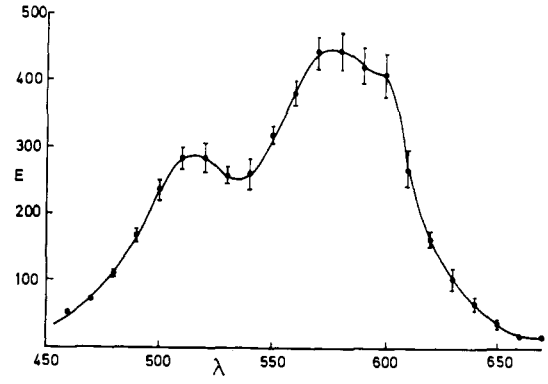

FIGURE 3: Co(II) spectra of isoenzyme C–*p*-nitrobenzenesulfonamide complex. A total of 1.5 ml of 1.0 mM sulfonamide was added to 6.0 ml of 0.22 mM isoenzyme. The recorded spectrum at pH 7.52 and variations from this for five spectra measured between pH 5.50 and 9.92 are shown.

TABLE III: Effect of Ionic Strength on the Association Rate between Human Carbonic Anhydrase C and *p*-Nitrobenzenesulfonamide.

	Ionic Strength (μ)	$(\mu)^{1/2}$	k_1 (M^{-1} sec^{-1})	log k_1
	A. 0.02 M Tris-Cl Buffer (pH 9.12), NaCl Added			
Total Cl^- Concn				
1.9 mM	0.0019	0.044	1.23×10^6	6.090
11.9 mM	0.0119	0.109	1.27×10^6	6.104
31.9 mM	0.0319	0.178	1.28×10^6	6.109
102 mM	0.102	0.320	1.23×10^6	6.090
302 mM	0.302	0.550	1.18×10^6	6.072
1.00 M	1.00	1.00	1.03×10^6	6.013
	B. 0.02 Tris-SO_4 Buffer (pH 7.6), K_2SO_4 Added			
Total SO_4^{2-} Concn				
7 mM	0.021	0.145	1.41×10^6	6.149
37 mM	0.111	0.334	1.31×10^6	6.117
107 mM	0.321	0.568	1.42×10^6	6.152
307 mM	0.921	0.975	1.47×10^6	6.167

TABLE IV: Effect of Carboxymethylation on Kinetics of Isoenzyme B Complex Formation with *p*-Nitrobenzenesulfonamide.

	k_a (M^{-1} sec^{-1})[a]	k_1 (M^{-1} sec^{-1})	k_d (sec^{-1})[b]	pK_E	pK_S
Isoenzyme B	2.7×10^6	3.5×10^6	0.049	7.5	9.3
Carboxymethyl isoenzyme B	9.0×10^5	3.0×10^6	2.18	9.1	9.3

[a] Maximum observed association rate constant at pH 8.5 for isoenzyme B and pH 9.2 for carboxymethyl isoenzyme B. [b] Calculated intrinsic association rate constant using reaction 1 in text and the above pK values.

The spectrum of Co(II) isoenzyme C complexed with NBS is different from that of either the acid or alkaline form of the enzyme and is entirely insensitive to pH in the range 6.0–10.0 (Figure 3). This shows, as did the pH insensitivity of the dissociation rate constant, that the complex does not undergo any protonation steps that influence either the dissociation of the ligand or its coordination.

By using a monovalent anion with a relatively low affinity for carbonic anhydrase at high pH or a divalent anion, specific anion binding to the enzyme may be ignored permitting an examination of solvent effects of ionic strength on the kinetics. As shown in Table III, the association kinetics are only slightly affected by ionic strength changes. At 1.0 M NaCl, pH 9.12, a 4% reduction in association rate can be estimated to be due to specific anion binding of Cl^-.[1]

Whitney *et al.* (1967) and Bradbury (1969a,b) have shown that 1–10 mM bromoacetate or iodoacetate will selectively and stoichiometrically alkylate a single histidine on isoenzyme B. At least 73% of the carboxymethyl groups can be detected on N-3′ of a particular histidine residue. This modification reduces the esterase activity and shifts its pH dependence to higher pH. Carboxymethylation can be prevented by the presence of sulfonamides or certain anions. Since the carboxymethyl group appears to be close to the active site, this chemical modification offers an interesting and well-defined derivative on which complex formation kinetics can be examined. As before, changes in the Co(II) spectra may provide a structural correlation for interpreting the kinetics, and any differences in the Co(II) spectra should be attended by an altered pH–rate profile.

Addition of 10 mM bromoacetate to Co(II) isoenzyme B causes an immediate reduction in the intensity of its spectrum (Figure 4a). For comparison the spectrum in the presence of 50 mM acetate is also shown. A similar spectrum is also obtained with 50 mM formate suggesting that each spectrum reflects carboxylate anion association with the metal. Many inhibitory inorganic anions have been shown by Lindskog (1966) to generate individually characteristic Co(II) spectra. After 8-hr reaction and subsequent removal of the noncovalently linked bromoacetate and reaction products, the spectrum is reintensified and looks strikingly similar to that of the unmodified enzyme (Figure 4b). A return to the previous spectra can be achieved by reintroducing acetate or bromoacetate. The above spectral change indicates that the covalently linked carboxymethyl group has assumed a different position with respect to the metal than the reversibly bound carboxylate anions. The specificity of the halo-

[1] This has been estimated from our measurements of Cl^- affinity at pH 7.6 and the pH dependence of anion inhibition. A complete study of anion binding kinetics using stopped-flow and nuclear magnetic resonance will appear in a forthcoming paper.

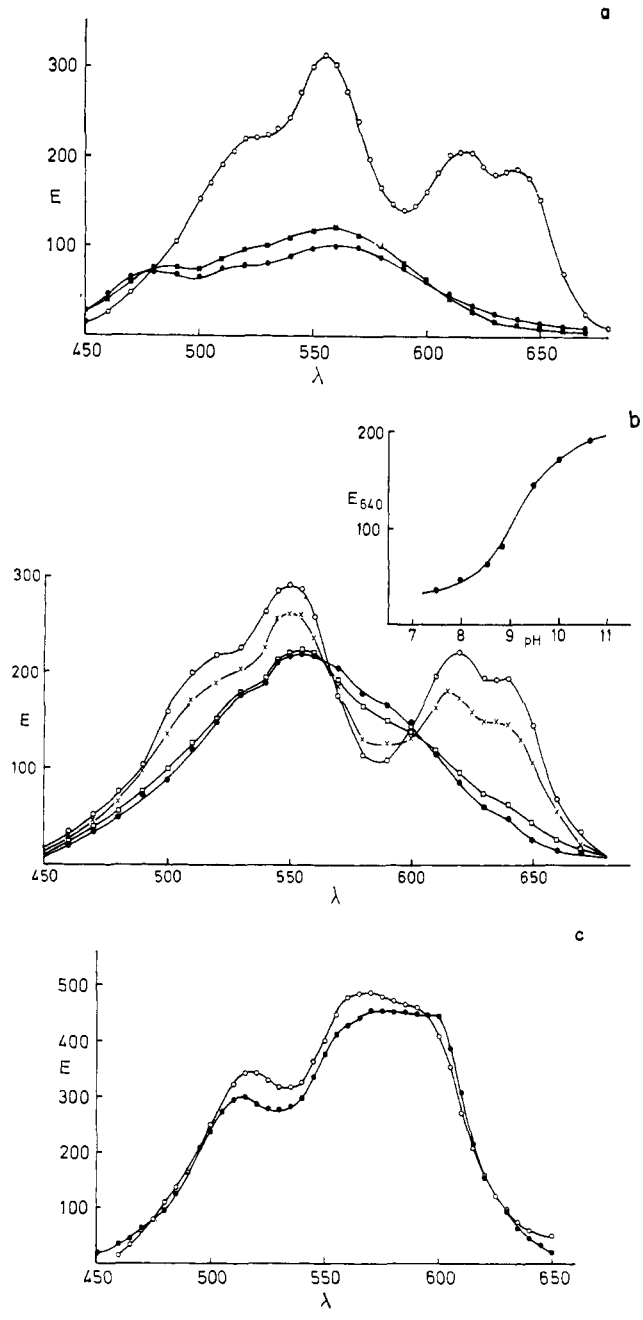

FIGURE 4: (a) Co(II) isoenzyme B spectra before and after addition of carboxylate anions; (O—O) 5.0 mM phosphate, pH 7.58; (■—■) the same enzyme solution after addition of 10 mM bromoacetate, pH 7.48; (●—●) after addition of 50 mM acetate, pH 7.60. (b) Carboxymethylated Co(II) isoenzyme B after removal of bromoacetate and reaction products; 0.194 mM isoenzyme was titrated with 50 mM NaOH and spectra were recorded in 2.0-cm cells. (●—●) pH 7.96, (□—□) pH 8.55, (×—×) pH 9.49, and (O—O) pH 10.65. (c) Co(II) spectra of *p*-nitrobenzenesulfonamide complexes of isoenzyme B (O—O) and carboxymethylated isoenzyme B (●—●).

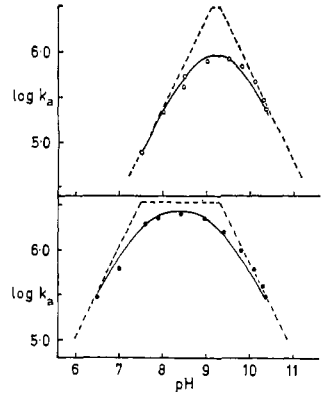

FIGURE 5: pH dependence of the association rate constants for complex formation between *p*-nitrobenzenesulfonamide and isoenzyme B (●—●); and its carboxymethylated derivative (O—O). The solid lines are calculated from reaction (1) in the Discussion section assuming pK_S to be 9.30 and pK_E to be 7.5 for isoenzyme B and 9.1 for the carboxymethylated enzyme.

acetates for one histidine, assumed to be near the active site, is presumably due to reversible binding of these anions to the metal giving a high concentration of reagent in this region (Bradbury, 1969a). However, after reaction the covalently linked carboxymethyl group is no longer liganded to the metal. The findings that anions still inhibit enzymatic activity (Bradbury, 1969a) and influence the Co(II) spectrum of the carboxymethylated enzyme tend to confirm this.

Upon titration to alkaline pH the typical absorption peaks at 620 and 640 reappear but the pK of the spectral change is found at pH 9.2 instead of 7.3 for the unmodified enzyme (Figure 4b). Thus insertion of this anionic covalently linked group yields qualitatively similar spectra and only appears to modify the H^+ dependence of the coordination change. The spectrum of the sulfonamide complex of carboxymethylated isoenzyme B is also virtually identical with the unalkylated complex (Figure 4c).

With carboxymethylation of Zn isoenzyme B the maximum observed association rate is depressed to $\simeq$30% of the unmodified enzyme rate with the pH optimum shifted to substantially higher pH (Figure 5). As ionization of the sulfonamide will remain the same, a unique value of the ionization constant on the enzyme, K_E, will control the pH profile. Although determination of this value becomes less accurate as the ionization constants on the enzyme and sulfonamide approach each other, pK_E is approximately 9.1 (Figure 5) and is in very close agreement with the pK observed from the Co(II) spectra. The intrinsic association rate constant, which can be calculated or determined from the horizontal dotted line, is only slightly affected by carboxymethylation (Table IV). The lower apparent association rate is thus due largely to an alteration in the relative populations of the sulfonamide-combining and noncombining forms of the enzyme. With carboxymethylation a comparatively smaller fraction of the combining form is present unless the pH greatly exceeds pK_E.

The dissociation rate constant was enhanced 48-fold (Table IV) so that reduction in affinity from carboxymethylation is primarily due to an increased dissociation rate. The presence of anions which reversibly bind to the enzyme does not appreciably affect sulfonamide dissociation making the selective increase in the rate unique to the covalently linked group. Smaller differences between association and dissociation rates preclude measuring k_d over a wide pH range by the competition method; however it can be estimated from observed association rates at different sulfonamide

concentrations (see Methods). Using this procedure we have been unable to find variation of k_d with pH between 7.6 and 10.4.

Discussion

The pH dependence of the association process appears to be due to: (a) the ionization equilibrium of the sulfonamido group which accounts for changes at the high pH and (b) an ionization dependent on the enzyme accounting for changes at lower pH. The quantitative correspondence of the pH dependence of the association reaction and the visible spectra of Co(II) isoenzyme C, Co(II) isoenzyme B, and Co(II) carboxymethyl isoenzyme B strongly suggest that the pH equilibria of the enzymes involve the coordination structure of the metal in the active site. Moreover, the pH-insensitive visible spectrum of the Co(II) enzyme–sulfonamide complex is consistent with the pH-invariant dissociation rate. In the complex neither component enters into an equilibrium with hydrogen ions and the ionization state is fixed. It seems likely that the pH-sensitive ligand has become displaced from the metal with sulfonamide association.

The possible reactions between the sulfonamide and enzyme may be expressed finally as follows

$$\begin{array}{lll} \overset{H^+}{\underset{+}{}}[CA] + \begin{cases} RSO_2NH_2 \xrightarrow{k_1} \\ RSO_2NH^- \xrightarrow{k_2} \end{cases} & & (1) \\ & & (2) \\ \Big\uparrow\downarrow K_E & & \\ [HCA]^+ + \begin{cases} RSO_2NH_2 \xrightarrow{k_3} \\ RSO_2NH^- \xrightarrow{k_4} \end{cases} & & (3) \\ & & (4) \end{array}$$

The pH dependence of the association reaction is compatible with either reaction 1 or 4, but not reaction 2 or 3. The reactions 1 and 4 are equivalent in their fit to the rate–pH dependence but yield different velocities. Sulfonamide association with bovine carbonic anhydrase B has previously been measured indirectly by following the rate of inhibition of catalytic activity (Kernohan, 1966; Lindskog and Thorslund, 1968; Lindskog, 1969). These studies have also led to the conclusion that single ionizing groups on the enzyme and sulfonamido moeity control the association rate–pH dependence. The pK of the group on the enzyme corresponds with the pK of the ionization which influences catalytic activity. The above authors have followed Kernohan's suggestion that the anionic sulfonamide species is the combining form and have favored mechanism 4 as the reaction path.

In the case of reaction 1, the experimentally determined value of k_a is related to its intrinsic rate constant by the relationship

$$k_{a,exptl} = k_1 \frac{1}{\left(1 + \frac{K_S}{(H^+)}\right)\left(1 + \frac{(H^+)}{K_E}\right)}$$

where K_S and K_E are the hydrogen ion equilibrium constants of the sulfonamide and enzyme, respectively. On the other hand, in the case of reaction 4, the equation becomes

$$k_{a,exptl} = k_4 \frac{K_S/K_E}{\left(1 + \frac{K_S}{(H^+)}\right)\left(1 + \frac{(H^+)}{K_E}\right)}$$

k_1 in reaction 1 is therefore smaller than k_4 in reaction 4 by the pH independent factor K_S/K_E. In the case of SABS and isoenzyme C this is equal to 2×10^{-3}, and in the case of dansylamide and isoenzyme C it is 4×10^{-4}.

If we calculate k_1 for the reaction of isoenzyme C and SABS, from reaction 1 we obtain a value of 2.23×10^7 M^{-1} sec^{-1} and from reaction 4 we obtain 1.12×10^{10} M^{-1} sec^{-1}. Both values satisfy the observed pH dependence, so that a decision as to which reaction path is correct must be made on other grounds. The most cogent of these is based on constraints imposed by diffusion-limited collision between the inhibitor and enzyme binding site. If the reactants are neutral molecules and the collision involves a site on the outer surface of the enzyme, the maximum rate constant permitted by diffusion is about 2×10^9 M^{-1} sec^{-1} at 25° (Alberty and Hammes, 1958). However, this crude collisional rate does not allow for geometric or orientation factors. X-Ray crystallographic data on the isoenzyme C–acetoxymercurisulfanilamide complex (Fridborg *et al.*, 1967) show that the sulfonamide is accommodated at the base of a relatively narrow cleft in the enzyme surface. This introduces a requirement for orientation and a restricted geometric angle of access for the attacking ligand. Both of these factors will considerably reduce the chance of complex formation from collision in random orientation. In fact, reaction velocities as high as 10^{10} M^{-1} sec^{-1} have been reported only for reactions between small oppositely charged ions (Caldin, 1964; Wilkins and Eigen, 1965); while rates greater than 2×10^8 M^{-1} sec^{-1} have not been observed for reactions involving biological macromolecules and ligands of comparable size and asymmetry to the azosulfonamides (Hammes, 1968). It is to be expected that a reaction between oppositely charged reactants might lead to an increase of up to threefold in the collision rate (Alberty and Hammes, 1958; Burgen, 1966), but this still falls short of k_4 calculated for reaction 4. The significance of charge acceleration is made even less likely by the failure of ionic strength changes to influence the reaction rate. Increasing ionic strength will shield the charges from each other and hence reduce the magnitude of the Coulombic attractive forces. Alberty and Hammes (1958) in considering diffusion limited reactions between oppositely charged species have used a Brønsted equation to account for salt effects at low ionic strength

$$\log k_a = \log k_0 + 1.02 z_a z_b \sqrt{\mu}$$

where k_0 and k_a are rate constants at infinite dilution and ionic strength, μ. z_a,z_b represent the effective charges on the combining ligand and its respective site of interaction of the macromolecule. For sulfonamide association a plot of log k_a *vs.* $(\mu)^{1/2}$ has a slope less than 0.1 (Table III). This is not consistent with the initial combination involving a direct interaction between a negatively charged group on the sulfonamide and an oppositely charged site on the enzyme (*e.g.*, the coordinated Zn). If the sulfonamide attacks as a neutral

species, subsequent unimolecular steps, which may involve an ionization, occur within the ion atmosphere of the protein and should proceed at rates independent of ionic strength.

It is also difficult to explain on the basis of diffusion-limited reactions why SABS despite its larger size has an association rate constant 13 times greater than NBS, a sulfonamide with the same pK. The respective dissociation rates of these two sulfonamides differ by a factor of only 1.4. Attempts to correlate sulfonamide affinities with the pK of the sulfonamido group have met with questionable success (see Lindskog, 1969).

Taken together, these arguments lead to a rejection of mechanism 4, and support for the neutral sulfonamide as the attacking species in mechanism 1. The previous studies with the bovine enzyme, which investigated fewer sulfonamides, did not find a complex where k_4 exceeded 10^9. Consequently, an important basis for distinguishing which of the alternative mechanisms is correct was lacking.

Ultraviolet difference spectroscopy, however, has recently shown the spectrum of the sulfonamide in the complex corresponds to that of the deprotonated sulfonamido anion (King and Burgen, 1970). The anionic ionization state in the complex remains fixed throughout the pH range of binding so that this species is protected from external pH changes just an dissociation of the complex and the Co(II) spectrum of the complex are. It appears, therefore, that the neutral sulfonamide must be deprotonated after the initial combination step. It would be satisfying if the spectrum of Co(II) enzyme–sulfonamide complex could be interpreted in terms of either selection of the acidic or alkaline coordination form of the free enzyme, however the spectrum of the complex shows unique features which indicate different ligand symmetry in the complex.

Our conclusion that the initial complex is formed from the neutral species with subsequent release of the sulfonamido proton requires complex formation to occur as a multistep process. In using low concentrations of reactants, which is necessary for the stopped-flow studies, the bimolecular step in the formation reaction may be expected to be rate limiting. Under these conditions intermediate species will not build up in detectable concentrations. However, other kinetic characteristics of this reaction make a multistep mechanism seem likely. For a series of sulfonamide complexes differences in their affinity are largely a consequence of variation in the respective association rates (Taylor *et al.*, 1970). This dependence of formation rate on ligand structure contrasts with simple ligand–metal substitution processes which show little sensitivity toward the nature of the incoming ligand; and, hence, variation in the stability of these coordination complexes is dissociation rate dependent (Wilkins and Eigen, 1965).

A probable explanation for this difference is that sulfonamide–enzyme complex formation proceeds through an initial preequilibrium complex which is stabilized by a hydrophobic interaction involving the aromatic sulfonamide ring. Formation of this rapidly associating and dissociating complex can control the concentration of sulfonamide correctly positioned for ligand–metal coordination. Coordination of the sulfonamido group with the metal would then occur as a subsequent unimolecular step; this step may well be coupled with the release of the sulfonamido proton.

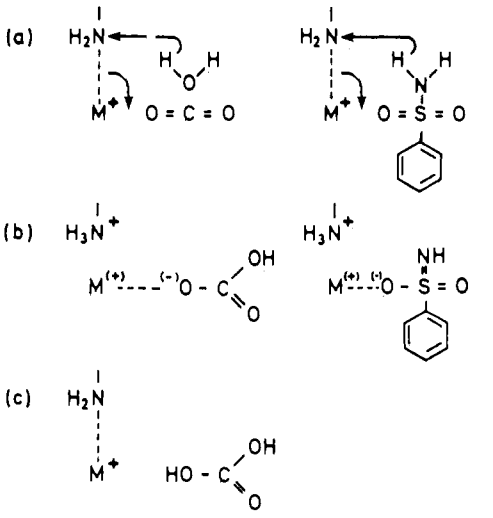

FIGURE 6

Since dissociation of the complex is clearly pH independent, the principle of microscopic reversibility requires that proton dissociation does not take place in a rate-limiting step. However, with an initial association complex, it is indeed possible for the proton to be transferred to a site on the enzyme. This type of proton transfer is completely compatible with a pH-insensitive dissociation rate as the proton is not released to or in contact with the solvent.

A mechanistic pathway for sulfonamide complex formation which incorporates these concepts is shown below (Figure 6) and may be compared with a parallel pathway for CO_2 hydration. Hydration of CO_2 has been shown to be catalyzed by the alkaline form of the enzyme (Kernohan, 1964, 1965); the same form we have proposed for sulfonamide combination. As shown in the initial sulfonamide association step (a), coordination of the metal is unaffected and the sulfonamido group occupies a position corresponding to the CO_2 and H_2O molecules. This initial complex is stabilized primarily through the aromatic sulfonamide ring. Subsequent uncoupling of the metal–protein amino bond is accompanied by transfer of the sulfonamido proton to the ligand and may well occur as a concerted process. Release of the sulfonamido proton is facilitated by the electrophilic influence of the neighboring metal which serves to effectively lower the pK of the sulfonamide. Although there is no direct evidence for coordination of the sulfonamide through its oxygen, the electronic state of this ligand may be represented by the canonical form in step b.

We must still explain why the final complex involves the sulfonamide anion yet this is not the preferred species in the initial step a. The most likely answer is that formation of the complex with the sulfonamido anion involves the obligate transfer of the freed proton to an acceptor in the enzyme site. This could be an underlying reason for the very large difference in affinity between aromatic sulfonamides and sulfonic acids for carbonic anhydrase. Reaction 4, therefore, may occur but it is quantitatively insignificant because of: (1) the low concentration of the product of charged reactants, (2) internal proton transfer is necessary for formation of a stable complex.

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Activity and Conformation of the Alkaline Form of δ-Chymotrypsin Studied by the Specific Acylation of Isoleucine-16*

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ABSTRACT: The N-terminal α-amino group of δ-chymotrypsin was specifically acetylated. During this acetylation, the enzyme showed a loss of activity and a decrease in the concentration of its active sites. The rate of loss of activity was the same as that of disappearance of free isoleucine, while the rate of loss of active sites was much lower. The derivative acetylated on Ile-16 was totally inactive toward specific ester and amide substrates, while it was still capable of hydrolyzing *p*-nitrophenylacetate. It was shown that this hydrolysis took place through a covalent intermediate, presumably an acyl-enzyme. The kinetic analysis of this reaction indicated that the acetylation of Ile-16 led to an appreciable decrease of the acylation rate constant and only to a slight reduction of the deacylation rate constant. Acetylation of Ile-16 leads to the disruption of the salt bridge Ile-16–Asp-194, in the same way as the deprotonation of Ile-16. The structural properties of these two proteins were very similar as observed by optical rotation, circular dichroism, and titration of exposed tyrosines. The catalytical properties observed for the acetylated enzyme are attributed to the high pH form of chymotrypsin, *i.e.*, a functional active site but no activity toward specific substrates. This absence of activity is probably due to a lack of binding, assigned to a distortion of the binding site. The zymogen was shown to be different from both the acetylated enzyme and the high pH form of chymotrypsin. A two-step activation mechanism is proposed, involving this high pH form as an intermediate between chymotrypsinogen and the active enzyme.

It is now well established that the decrease in the activity of chymotrypsin in the alkaline pH range is linked to the deprotonation of the amino group of its N-terminal residue, Ile-16 (Oppenheimer *et al.*, 1966; Himoe *et al.*, 1967; Ghélis *et al.*, 1967; Karibian *et al.*, 1968). The X-ray diffraction data describing the three-dimensional structure of the protein have shown that this group is involved in an electrostatic

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