Proton Magnetic Resonance Studies of Carbonic Anhydrase. III. Binding of Sulfonamides†

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ABSTRACT: Resonances of the histidine region of human carbonic anhydrase B have been studied by proton magnetic resonance spectroscopy in the presence of seven sulfonamide inhibitors. Results of difference spectroscopy and observation of the C-2 resonance of an additional titratable histidine in some of these spectra suggest a conformational change in the enzyme, while the large number of unaltered resonances indicates involvement of only a few residues. Inhibition of carbonic anhydrase by sulfonamides appears to involve: stabilization of an appropriately oriented initial complex by hydrophobic binding of the aromatic ring of the inhibitor to residues of the cavity forming the active site; ionization of the sulfonamido group, facilitated by its proximity to zinc; protonation and displacement of the high pH ligand to the metal controlling catalytic activity, thought here to be a histidine residue; and formation by the sulfonamido group of an ionic bond to zinc and a hydrogen bond to the hydroxyl group of serine or threonine. Diversity of spectra produced with various sulfonamides suggests that substituents on the ring and heteroatoms within the ring interact with additional groups at the active site. Increase in inhibitory potency appears to involve optimizing the number as well as the strength of these interactions. An upper limit for the dissociation rate constant of these complexes of 10 sec^{-1} was obtained.

Aromatic sulfonamides are potent and specific inhibitors of carbonic anhydrase used in the treatment of glaucoma and edema. An unsubstituted sulfonamido group directly bonded to an aromatic nucleus constitutes the minimal requirement for inhibitory activity (Mann and Keilin, 1940). Experiments with the catalytically active cobalt substituted enzyme (Lindskog, 1963, 1966) indicate direct interaction of the metal ion with bound inhibitors. X-Ray crystallographic studies (Lindskog *et al.*, 1971; Kannan *et al.*, 1971) show that sulfonamides bind within the first coordination sphere of the metal ion in the human C enzyme (HCA-C).[1] Studies of azide, using infrared spectroscopy (Riepe and Wang, 1968), and of chloride, using ^{35}Cl magnetic resonance spectroscopy (Ward, 1969), suggest binding of these anions to zinc and their displacement by sulfonamides. Proton magnetic relaxation experiments (Fabry *et al.*, 1970) suggest that both anions and sulfonamides displace water coordinated to the metal ion. Studies employing fluorescence spectroscopy (Chen and Kernohan, 1967) and ultraviolet difference spectroscopy (King and Burgen, 1970) confirm that sulfonamides bind to zinc as anions. Interaction of the aromatic ring of sulfonamides with hydrophobic residues at the active site appears to further stabilize these complexes (Chen and Kernohan, 1967). In this paper we have attempted to elucidate the structure–activity relationships governing interactions of sulfonamides with human carbonic anhydrase B (HCA-B). Such knowledge may help formulate new concepts to aid in the future design of pharmacologically active agents in this and other systems.

Methods and Materials

Benzenesulfonamide was purchased from Analabs. Benzolamide (2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide) and CL 13580 (2-(*o*-chloro)benzene-1,3,4-thiadiazole-5-sulfonamide) were generous gifts of Dr. Thomas Maren at the University of Florida at Gainsville. CL 5343 (2-amino-1,3,4-thiadiazole-5-sulfonamide) and CL 11312 (2-trifluoroacetamido-1,3,4-thiadiazole-5-sulfonamide) were made available through the courtesy of Dr. Selby Davis of Lederle Laboratories. Melting points and proton magnetic resonance (pmr) spectra of these compounds confirmed their purity (Pesando, 1973).

Samples were prepared as described in the first paper of this series except that the acidic inhibitors benzolamide and CL 11312 were dialyzed into the enzyme after 0.01 M sodium sulfate solutions containing the two compounds had been adjusted to pH* 7 (hereafter referred to simply as pH). Other materials and methods used have been described previously (preceding two papers).

Results

Sulfonamide–Enzyme Complexes. Six peaks are observed in pmr spectra of the histidine region of HCA-B in the presence of the three sulfonamides shown in Figure 1, and they possess comparable areas, chemical shifts, line widths, and spin–lattice relaxation times (Figure 2, Table I). The titratable histidines giving rise to peaks 2, 3, and 4 in the uninhibited enzyme are unaffected while that giving rise to resonance 1 (resonance 1′)[2] no longer appears to titrate, though it moves upfield with increasing pH in the

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[1] Abbreviations used are: HCA-C and HCA-B, human carbonic anhydrase C and human carbonic anhydrase B, respectively.

[2] Primed numbers indicate suspected identity of resonances in the free and inhibited states.

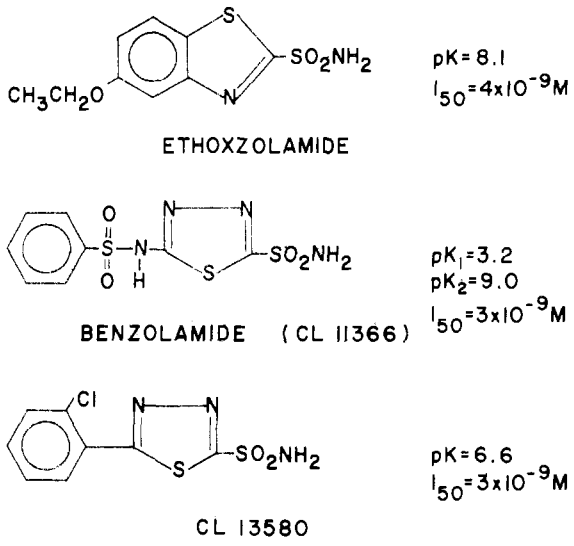


FIGURE 1: Three of the most potent known inhibitors of carbonic anhydrase. pK values of these compounds were taken from Maren *et al.* (1960). I_{50} values are the molar concentrations of drug required to inhibit 50% of enzymatic activity and are taken from Maren and Wiley (1968).

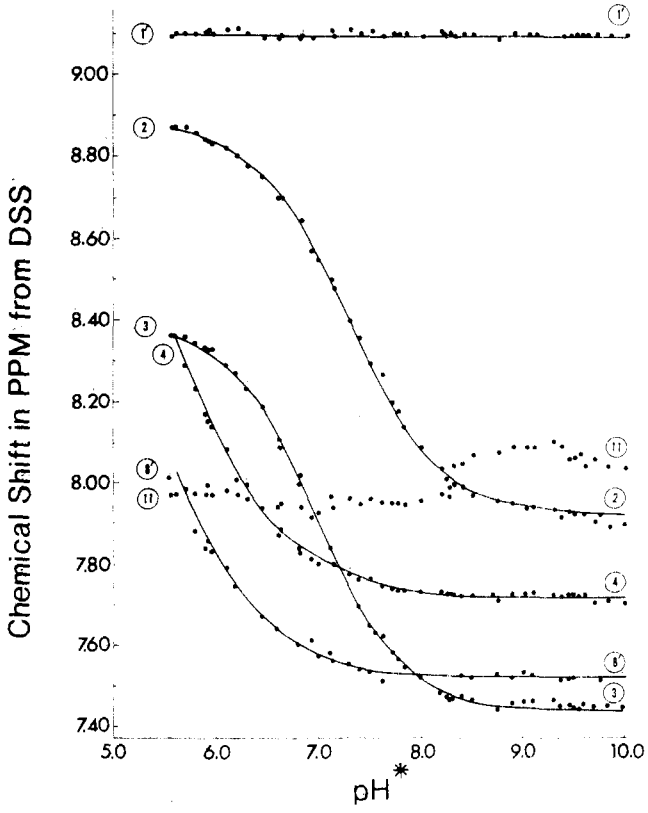


FIGURE 2: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by ethoxzolamide. Curves for resonances 2, 3, and 8′ were calculated using the Henderson–Hasselbalch equation and represent the best fit to the experimental points. The curve describing resonance 4 was hand drawn, and resonance 1 represents a nontitrating residue. The data for resonance 11 cannot be described by a regular curve. Figures showing the pH dependence of these resonances in HCA-B inhibited by benzolamide and CL 13580 were submitted to the reviewers for examination but were omitted from the final text at the request of the editor to conserve space. Copies will be furnished by the author to the interested reader.

presence of benzolamide. Peaks 5, 6, and 7 of the free enzyme are not observed. Two new and pH dependent resonances, designated 8′ and 11, appear with all three inhibitors. No drug resonances are detected on addition of the sparingly soluble inhibitor ethoxzolamide to the enzyme, probably because they are substantially broadened when the drug is bound. Drug resonances are observed with benzolamide and CL 13580 and appear to be those of the ring protons of free inhibitor.

Table I: Ionization Constants of Histidine Residues of HCA-B Inhibited by Sulfonamides.

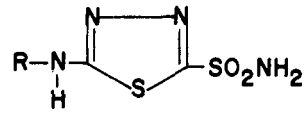
	Resonances					
	1	2	3	4	5	8
No Addition	8.2	7.23	6.98	6	8.24	NO[a]
Acetazolamide	NT[b]	7.26	7.00	6	NO	7.13
Ethoxzolamide	NT	7.25	7.00	6	NO	5.7
Benzolamide	NT	7.27	6.98	6	NO	9
CL 13580	NT	7.26	7.00	6	NO	6.5
CL 5343	NT	7.25	7.01	6	NO	NO
CL 11312	NT	7.28	6.98	6	NO	NO
Benzenesulfonamide	NT	7.25	6.97	6	NO	NO

[a] NO = Not observed. [b] NT = nontitrating.

The behavior of resonance 8′ is characteristic of the added inhibitor. With CL 13580, peak 8′ is observed only over a narrow low pH range and may reflect a residue titrating with a pK value between 6 and 7 (Table I). Resonance 8′ is not observed above pH 8 in the presence of benzolamide but may reflect a residue with a pK of approximately 9. Peak 8′ in the presence of ethoxzolamide (Figure 2) reflects a residue having a pK of about 5.7, and its pH dependence resembles that of resonance 10 of the metal-free and cadmium enzymes (Figures 5 and 6 of preceding paper). A second pH dependent resonance, representing a single proton, appears in the histidine spectral region of the enzyme in each of these three inhibited states but does not reflect a titratable residue. It is designated number 11, and its chemical shift is different with each inhibitor, tending to move downfield with increasing pH.

Two thiadiazole inhibitors closely related to acetazolamide (Figure 3) were also examined for their effects on the aromatic spectrum of HCA-B. Seven discrete C–H proton resonances are observed in the presence of all three inhibitors (Figure 4, and Figure 3 of preceding paper), and they possess comparable areas, line widths, and T_1 values. As with the other inhibitors studied, resonances 2, 3, and 4 are unaffected while resonance 1 appears to move downfield, losing its pH dependence. Resonances 5, 6, and 7 are not observed. Two new and pH-dependent resonances move downfield with increasing pH. However, unlike the spectra recorded with the inhibitors of Figure 1, neither of these two new peaks, designated numbers 11 and 12, appears to reflect a titratable residue. The spectra produced by addition of CL 5343 and CL 11312 to HCA-B are essentially identical and differ from those with acetazolamide in the presence and absence of resonances 11 and 8, respectively. Peak 12 is also not seen with acetazolamide, but there are additional unresolved resonances in its region of the spectrum. Resonances 2, 3, and 7′ are essentially identical with all three sulfonamides, but peak 1′ is farther downfield with acetazolamide.

With benzenesulfonamide, resonances of four single C–H protons are observed in addition to the omitted resonances



1. R=H CL 5343

2-Amino-1,3,4-Thiadiazole-5-Sulfonamide

$pK_1 = 7.8$
$pK_2 = 10.4$
$I_{50} = 6 \times 10^{-8}$ M

2. R=$CH_3-\overset{O}{\overset{\|}{C}}$ ACETAZOLAMIDE

2-Acetamido-1,3,4-Thiadiazole-5-Sulfonamide

$pK_1 = 7.4$
$pK_2 = 9.1$
$I_{50} = 2 \times 10^{-8}$ M

3. R=$CF_3-\overset{O}{\overset{\|}{C}}$ CL 11312

2-Trifluoroacetamido-1,3,4-Thiadiazole-5-Sulfonamide

$pK_1 = 3.8$
$pK_2 = 8.0$
$I_{50} = 4.5 \times 10^{-8}$ M

FIGURE 3: Single ring thiadiazole sulfonamides. pK values of compounds 1 and 2 are from Maren *et al.* (1960). That of number 3 was a personal communication from Dr. Barry Dvorchik working in the laboratory of Dr. Thomas H. Maren. I_{50} values are taken from Maren and Wiley (1968).

of the free drug molecule (Figure 5). Titratable histidines giving rise to peaks 2, 3, and 4 in the free enzyme are unaffected while resonance 1 moves downfield and becomes independent of pH. Resonances 5, 6, and 7 are missing, and there are no new peaks.

Difference Spectroscopy. Figure 6 shows the difference spectrum of HCA-B in the presence and absence of acetazolamide. Imperfect cancellation of the water resonance in the two original spectra produces the large signal at 4.67 ppm. Resonances indicated by 2 are those of the methyl group of free and bound acetazolamide. Identifiable peaks of single protons unique to the inhibited enzyme assist in calibration of the difference spectrum (1 of Figure 6). Roughly 30 of the approximately 1500 C–H protons appear to be affected by acetazolamide, but mutual cancellation of resonances in the difference spectrum probably makes this figure misleadingly low.

Dissociation Rates. Interaction of ethoxzolamide with HCA-B is slow on the pmr time scale, and the spectrum produced by addition of 0.5 equiv of inhibitor to the enzyme reveals two small peaks to low field with chemical shifts corresponding to those of resonance 1 in the free and ethoxzolamide-inhibited states. On addition of increasing amounts of inhibitor, the area of the more downfield peak increases at the expense of the other. An upper limit for the dissociation rate constant of the complex of approximately 10 sec^{-1} can be determined from the separation of the observed peaks in frequency units. Similar results are obtained for the other sulfonamides studied.

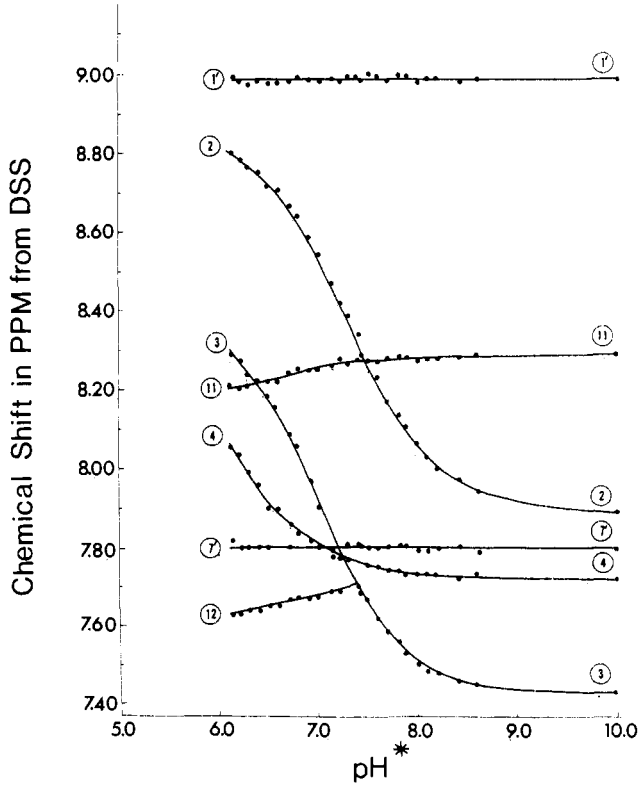


FIGURE 4: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by CL 5343. The data have been extrapolated between pH 8.6 and 10.0 where only the expected pH dependent changes appear to occur. Essentially identical results were obtained with CL 11312.

Discussion

Conformational Change. Crystallographic work on HCA-C reveals no change in its structure on addition of sulfonamides (Lindskog *et al.*, 1971; Kannan *et al.*, 1971; Liljas *et al.*, 1972). However, most of the inhibitors studied were dialyzed into crystals of the protein where the enzyme may not have sufficient freedom of motion to accommodate the structural changes normally induced. Under such conditions, any change in the conformation of the enzyme would tend to disrupt the structure of the crystal and occur only if the energy gained by binding of sulfonamides were sufficient to overcome the stabilization energy of the crystal lattice. Otherwise, the structure of the enzyme–inhibitor complex in the crystal may not reflect that which exists in solution. Studies of carbonic anhydrase in solution using both pmr (Cohen *et al.*, 1972; King and Roberts, 1971) and ultraviolet difference spectroscopy (King and Burgen, 1970) have been interpreted as indicating different conformations in the presence and absence of inhibitors.

The appearance of a new titratable group(s) in the presence of azide and acetazolamide (Figures 1 and 3 of preceding paper), *p*-carboxybenzenesulfonamide (King and Roberts, 1971), and three sulfonamides of Figure 1 suggests a conformational change in the enzyme with exposure of previously buried residue(s) and/or an increase in its freedom of motion. However, the invariance of the behavior of resonances 2, 3, and 4 in the free and inhibited enzymes (Table I) argues against changes in the immediate environments of their parent histidines. Since the residues giving rise to these peaks appear to be widely distributed over the surface of the molecule (Lindskog *et al.*, 1971; Kannan *et al.*, 1971; Liljas *et al.*, 1972; Andersson *et al.*, 1972; Liljas, 1971), any

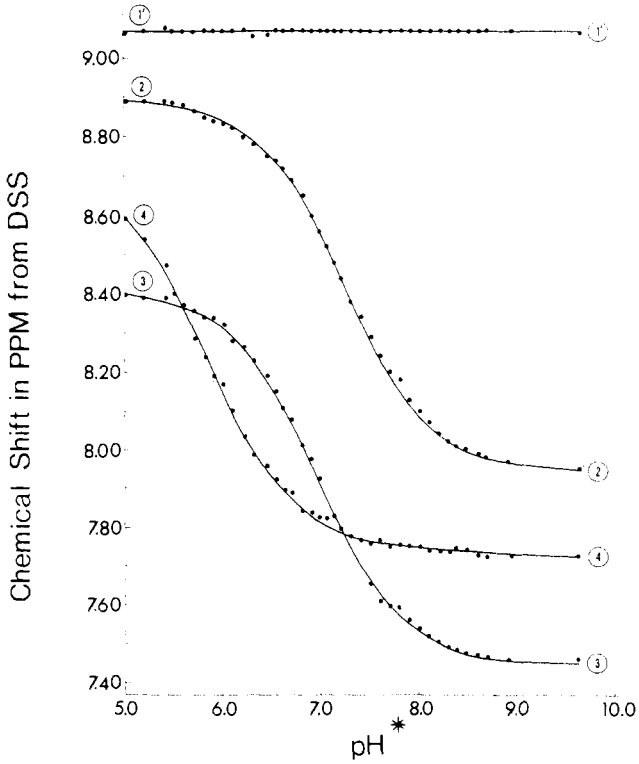


FIGURE 5: pH dependence of resonances in the histidine region of the pmr spectrum of HCA-B inhibited by benzenesulfonamide. Drug resonances centered at 7.96 and 7.63 ppm were omitted from the figure.

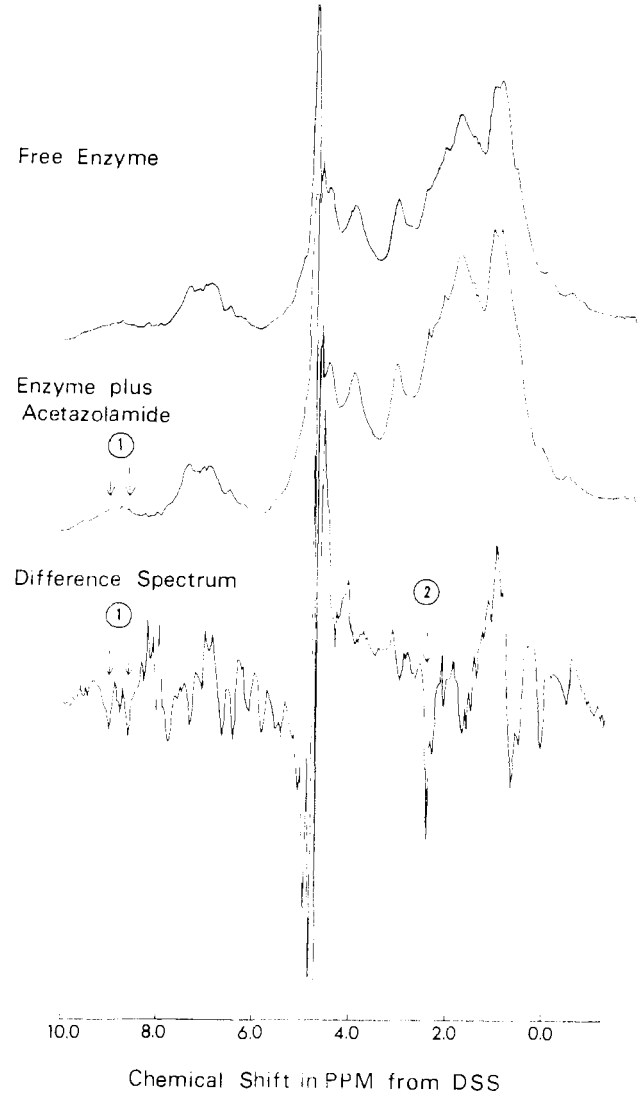


FIGURE 6: Pmr difference spectrum of HCA-B in the presence and absence of acetazolamide. pH of the sample decreased from 6.14 to 6.06 on addition of inhibitor.

conformational change of HCA-B on binding inhibitors that does not affect these resonances is probably limited in its scope.

Pmr difference spectroscopy provides a means of detecting possible conformational changes in the enzyme on binding inhibitors and estimating the number of protons whose magnetic and therefore chemical environments have been altered. The changes in the difference spectrum produced by addition of acetazolamide to HCA-B suggest a conformational change in the enzyme involving but a small number of residues (Figure 6). Small changes in the difference spectrum might, however, reflect gross changes in the disposition of a few groups. Most peaks in the difference spectrum are those of aromatic protons or of groups in the methyl region of the spectrum. Since X-ray data reveal no phenylalanine, tyrosine, or tryptophan residues in the immediate vicinity of bound sulfonamides (Lindskog *et al.*, 1971; Kannan *et al.*, 1971; Liljas, 1971), the observed aromatic protons should not be experiencing magnetic fields induced in the inhibitor and probably reflect indirect changes in their immediate environments. The methyl proton resonances in the difference spectrum might reflect location of their parent residues near bound acetazolamide.

Binding of Sulfonamides. HYDROPHOBIC INTERACTIONS. Although both anionic and hydrophobic interactions are thought to be essential for strong binding of sulfonamides to carbonic anhydrase (Lindskog *et al.*, 1971; Coleman, 1971), compounds such as benzenesulfonic acid are poor inhibitors. Taylor *et al.* (1970b) have explained this apparent discrepancy by arguing that sulfonamides must bind as neutral molecules to the alkaline form of the enzyme and that the sulfonamido group of the inhibitor then ionizes to donate a proton at the active site. Failure of benzenesulfonic acid to bind strongly as an anion to the acidic (and therefore protonated) form of native carbonic anhydrase (Taylor *et al.*, 1970b) suggests a role for the sulfonamido group in hydrophobic bonding. By masking the sulfonate group of benzenesulfonic acid, the neutral sulfonamido moiety of benzenesulfonamide may permit maximization of the initial hydrophobic interaction and optimal orientation of the inhibitor for subsequent interaction with the metal ion. The weak inhibition produced by aliphatic sulfonamides such as methanesulfonamide (Maren and Wiley, 1968) confirms that the aromatic ring system is necessary for an effective hydrophobic interaction.

HYDROGEN BONDING. Since molecules such as phenol and aniline which possess an ionizable functional group directly attached to an aromatic ring system and having an appropriate pK value, are weak inhibitors of carbonic anhydrase, the SO_2 portion of the sulfonamido group apparently plays an important role in stabilizing the enzyme–inhibitor complex. Crystallographic studies of HCA-C (Kannan *et al.*, 1971; Liljas, 1971) show that the SO_2 group of several bound inhibitors is hydrogen bonded to threonine-197. HCA-B is thought to possess a serine residue at the same postion in its chemical sequence (Andersson *et al.*, 1972; Laurent-Tabusse *et al.*, 1972), though this matter is now in

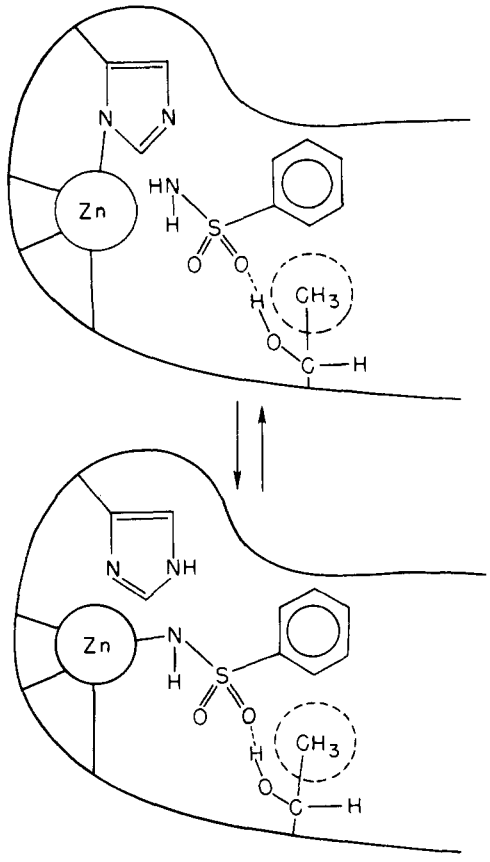


FIGURE 7: Schematic representation of binding of benzenesulfonamide to HCA-C. The sulfonamido group ionizes, donating a proton to a labile histidine that is displaced as a metal ligand. The complex is stabilized by an ionic bond to zinc, a hydrogen bond to the hydroxyl group of threonine, and hydrophobic interactions between the ring of the inhibitor and residues of the active site (not shown). A dashed circle suggests the approximate van der Waals radius of the methyl group of threonine. In HCA-B threonine is replaced by serine.

dispute (Lin and Deutsch, 1974). The hydroxyl groups of threonine and serine should be equally effective in forming hydrogen bonds to inhibitors, while the adjacent methyl group of the former might contribute to the unusual effect of ortho substituents on the binding of both substrates and inhibitors to HCA-C. Thus, Taylor *et al.* (1970a) reported a 5- to 50-fold increase in the dissociation rate of ortho-substituted sulfonamides bound to HCA-C when compared to molecules unsubstituted in that position, but observed no such phenomenon with HCA-B. Similarly, Verpoorte *et al.* (1967) observed a selective difference in the rate of hydrolysis of *o*- and *p*-nitrophenyl acetate by HCA-B and HCA-C. With its hydroxyl group hydrogen bonded to the SO_2 moiety of the inhibitor, the methyl group of threonine appears to be in a position to interfere sterically with any large substituent in the ortho position of the ring of the sulfonamide (Figure 7). Sulfonamide inhibitors and ester substrates thus appear to bind to the active site of the enzyme in similar orientations, and the hydroxyl group of serine or threonine may also act to orient water and/or substrate molecules during catalysis.

ADDITIONAL INTERACTIONS. The unique spectra of the low-field region of HCA-B in the presence of different sulfonamides indicate that, despite common bonding through the sulfonamido group, these molecules interact differently with residues of the protein at the active site to stabilize their respective complexes. In addition to substituents on the ring of the inhibitor, nitrogen and sulfur atoms within heterocyclic ring systems may be involved in these interactions. While derivatives of sulfonamides possessing heavy metal atoms might undergo unique interactions with the protein, crystallographic studies (Kannan *et al.*, 1971; Liljas, 1971) confirm that substituents of the ring of the inhibitor other than the sulfonamido moiety interact with groups on the protein.

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