Structure–Activity Relationships of Sulfonamide Drugs and Human Carbonic Anhydrase C: Modeling of Inhibitor Molecules into the Receptor Site of the Enzyme with an Interactive Computer Graphics Display[1]

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Abstract □ We have analyzed the molecular interaction of 28 sulfonamide inhibitors with human carbonic anhydrase C (HCAC) using an interactive computer graphic display. Small aromatic sulfonamides gain most of their inhibitory power towards HCAC from the interaction of hydrogen bond acceptors at the *para* or *meta* positions with hydrophilic residues of the enzyme. Additional coordinated water molecules stabilize the complexes of heterocyclic sulfonamides (*i.e.*, thiophenes, 1,3-thiazoles, and 1,3,4-thiadiazoles) with HCAC. We propose two different modes of binding of the heterocyclic ring with respect to the active site cleft: the heterocyclic sulfur atom of a 3,4-unsubstituted thiophene approaches the oxygen atom of a coordinated water molecule (sulfur "out"), whereas in 3,4-unsubstituted 1,3,4-thiadiazoles, the sulfur is in contact with a hydrophobic part of the receptor site (sulfur "in"). This proposal is consistent with crystallographic evidence. Sulfonamides with two aromatic or heterocyclic rings also interact with a hydrophobic pocket of the enzyme located >10 Å away from the active site metal Zn^{2+}. We also discuss the possibility that the relative inactivity of thiazide diuretics is due to the steric interaction of the *ortho* chlorine atom with the enzyme receptor cavity.

Keyphrases □ Sulfonamides—structure–activity relationships with human carbonic anhydrase C, interactive computer graphic display □ Human carbonic anhydrase C—structure–activity relationships with sulfonamides, interactive computer graphic display □ Structure–activity relationships—enzyme–inhibitor complexes, modeling by interactive computer graphic display, sulfonamides with human carbonic anhydrase C

The isoenzymes of human carbonic anhydrase (HCAB, HCAC) reversibly catalyze the decomposition of carbonic acid into carbon dioxide and water at a remarkably fast rate (up to 1×10^6 s^{-1}). These zinc-containing enzymes consist of 260 amino acids with a molecular mass of ~30 kDa. The active site of human carbonic anhydrase contains a four-coordinated zinc ion: three nitrogen atoms (N—ϵ_2 of His 94, N—ϵ_2 of His 96, and N—δ of His 119) and one oxygen atom (coordinated water molecule, at higher pH values a hydroxide anion) leading to distorted tetrahedral metal coordination (1).

Aromatic and heterocyclic sulfonamides are very specific inhibitors of carbonic anhydrase when the sulfonamido group is unsubstituted. The inhibition of carbonic anhydrase was first reported by Keilin and Mann (2). Kinetic studies have shown sulfonamides to be competitive to the dehydration of carbonic acid as well as the hydration of carbon dioxide (3).

The detailed interaction of sulfanilamide, acetazolamide, and acetoxymercury sulfanilamide with HCAB and HCAC has been reported by Kannan *et al.* (4, 5). The sulfonamido group binds the metal ion at the fourth coordination site (replacing the water molecule from the native enzyme) through the nitrogen or oxygen atom of the sulfonamido moiety, probably through the amide nitrogen atom. This atom is also hydrogen-bonded to the hydroxyl group of Thr 199 in the same way as the fourth coordinated water molecule in the native enzyme. One of the two oxygen atoms is liganded to a fifth coordination site of the zinc. The sulfur atom (or carbon atoms) from the heterocyclic (or aromatic) ring interact with Ala 121 in HCAB and with Val 121 in HCAC, respectively.

Little attention has been paid thus far to enzyme–inhibitor interactions removed from the active site. Nevertheless, these interactions with protein side chains may contribute significantly to the stability of the enzyme–sulfonamide complex. While 4-methylbenzenesulfonamide forms an enzyme–inhibitor complex with a K value of 1.95×10^6 mol^{-1}, the complex formation constant of *p*-(salicyl-5-azo)-benzenesulfonamide is 2.38×10^8 mol^{-1}, two orders of magnitude greater. This is only explainable by the interaction of the salicyl group with side chains of the enzyme located >10 Å from the active site metal (*i.e.*, Ile 91, Trp 123, and Phe 131 in HCAC). We have, therefore, investigated sulfonamide–carbonic anhydrase (HCAC) interactions in an extended environment of the active site using original crystallographic coordinates and an interactive computer graphic display.

THEORETICAL

Several experimental and computational methods are now sufficiently developed to provide insight to the interaction of a small molecule with a macromolecule. Interactive computer graphic techniques are very useful for the study of molecular interactions characterized as recognition, binding, enzyme catalysis, and inhibition.

Basic adjustments of the model (*i.e.*, inhibitor molecule) to the receptor structure (*i.e.*, enzyme) on a 3-dimensional display are possible in real time (no intermediate keyboard commands) by means of the program FIT (6):

1. Model: (*a*) complete or subunit model translations relative to the receptor; (*b*) individual atom translation; (*c*) complete or subunit model rotation; (*d*) complete subunit rotation about a given bond; and (*e*) dihedral rotation before or after any selected bond.
2. Receptor: in principle the same as for the model, but due to the complexity of the protein only reorientations of amino acid side chains have been performed so far by dihedral rotation about single bonds.

In addition to the above functions, which are basic to any model-fitting method, several utility functions are furnished including (*a*) picture scale; (*b*) stereo image separation (right–left or top–bottom); (*c*) global picture translation and rotation; (*d*) receptor–model relative intensities; and (*e*) I/O functions to exchange or modify the receptor structure, exchange models, print any geometric information about the model (*i.e.*, atom identification, bond length, bond angles, dihedral angles), save the current picture and output the current model coordinates, calculate distances of enzyme–inhibitor interactions, and facilitate still or motion-picture photography.

[1] Dedicated to Professor J. D. Dunitz mentor and friend in honor of his 60th birthday.

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Table I—Enzyme–Inhibitor Complex Formation Constants K (mol^{-1}) for Sulfonamides with Human Carbonic Anhydrase

Inhibitor Molecule	Taylor *et al.* (9)[a] Stop Flow	Taylor *et al.* (9)[a] Affinity Titration	Taylor *et al.* (9)[a] Relative[d]	Kakeya *et al.* (10)[b] Calorimetry	Kakeya *et al.* (10)[b] Relative[d]	Sprague (11)[c] (Relative)[d]
Aromatic Sulfonamides						
Benzenesulfonamide Substituent						
4-H-	6.46×10^5	6.48×10^5	1.00	1.33×10^5	1.00	1.00
4-CH_3-	1.95×10^6	1.95×10^6	3.01	3.13×10^5	2.35	2.13
4-NH_2-				4.00×10^4	0.30	0.25
4-CO_2-	3.71×10^6	3.71×10^6	5.73			2.50
4-NO_2-	1.53×10^7	1.60×10^7	24.3	1.82×10^6	13.7	5.75
4-Cl-	8.27×10^6	8.27×10^6	12.8	9.09×10^5	6.83	4.50
4-N≡C-				1.54×10^6	11.6	3.75
4-SO_2NH_2-						10.0
3,4-di Cl-	1.56×10^7	1.56×10^7	24.1	3.33×10^6	25.0	14.0
3-SO_2NH_2, 4-Cl-						23.3
2,4,5-tri Cl-	6.78×10^6	6.80×10^6	10.5			
2,4,6-tri Cl-	4.57×10^6	4.57×10^6	7.05			
2,5-di NO_2, 4-Cl-	1.29×10^7	1.40×10^7	20.8			
3-SO_2NH_2, 4,5-di Cl-						43.8
3-SO_2NH_2, 4-Cl-6-NH_2-						0.93
H_3C—CO—NH—	1.60×10^6	1.60×10^6	2.47			3.75
Heterocyclic Sulfonamides						
Sulfonamide Substituent						
2-H-thiophene-						0.90
2-NO_2-thiophene	2.38×10^8	$>10^8$	368			
5-H-1,3-thiazole-2-						30.0
2-NH_2-1,3-thiazole-5-						0.75
4-CH_3-1,3-thiazole-2-						12.5
2-Acetamido-1,3-thiazole-5-						3.75
2-H-1,3,4-thiadiazole-5-						19.8
2-NH_2-1,3,4-thiadiazole-5-				1.85×10^6	13.9	
2-Acetamido-1,3,4-thiadiazole-5-	7.10×10^7	7.10×10^7	110	8.33×10^6	62.2	82.5
2-Propamido-1,3,4-thiadiazole-5-						99.0
2-Butamido-1,3,4-thiadiazole-5-						99.0
2-Acetimido-3-CH_3-1,3,4-thiadiazole-5-				1.00×10^7	75.2	132
Complex Sulfonamide						
p-(Salicyl-5-azo)-benzenesulfonamide	3.48×10^8	$>10^8$	526			
2-Benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide						421
2-(*p*-Chloro)-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide						314
4-Chloro-*N*-furfuryl-5-sulfamoylanthranylic acid (furosemide)						0.3
5-Dimethylaminonaphthalene-1-sulfonamide	6.15×10^5	5.80×10^5	0.92			
2-(4-Sulfamylphenylazo)-1-hydroxonaphthalene-4,6-disulfonate	7.74×10^6	6.90×10^6	11.3			
6-Ethoxybenzothiazole-2-sulfonamide (ethoxzolamide)[e]		6.25×10^8				

[a] Using HCAC, pH 6.5, 25 °C. [b] Using HCAB and HCAC, pH 7.0 15 °C. [c] Using HCAB and HCAC, pH 5.5, 25 °C (50% inhibition). [d] Relative to benzenesulfonamide (H substituted at the 4-position). [e] See Ref. 13.

Thus, a flexible substrate may be fit into a relatively rigid receptor site. While potentially limiting, this assumption is reasonably valid for "lock and key"-type enzymes. These methods are entirely general in terms of the size and functionality of the receptor site and ligand. A number of advantages of graphically generated models (reproducibility, instant quantification of geometric parameters, and accessibility to refinement procedures) make this approach superior to desk-top modelling approaches.

EXPERIMENTAL[2]

Except for modified enzyme side-chain positions, the crystal structure parameters of the native human isoenzyme C were used [HCAC; Protein Data Bank 1976, 2.0-Å resolution (7)]. Individual side chains in the active site were repositioned, if necessary, by appropriate torsional rotations. The molecular coordinates (sulfonamides) were taken from crystal structure determinations or generated by the program PAD (8). To obtain a more realistic fitting, the side chains of the enzyme were not restricted to their native conformation. These dihedral rotations were also performed with the program FIT.

The starting coordinates for the sulfonamido group in the fitting routine were taken from the crystal structure determination of the HCAC–acetazolamide enzyme–inhibitor complex (5). The potency of some inhibitors can only be explained if one takes into account their interaction with additional coordinated water molecules; these were generated by PAD and inserted with FIT.

[2] The following standard designations for amino acids are used throughout this paper: (Ala) alanine; (Asn) asparagine; (Gln) glutamine; (Gly) glycine; (His) histidine; (Ile) isoleucine; (Leu) leucine; (Phe) phenylalanine; (Pro) proline; (Ser) serine; (Thr) threonine; (Trp) tryptophan; (Val) valine.

RESULTS AND DISCUSSION

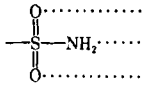
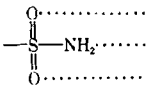
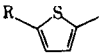
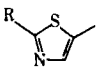
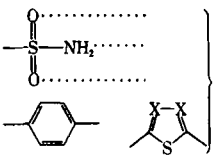
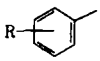
A large number of sulfonamides have been examined by kinetic and thermoanalytical methods by Taylor *et al.* (9), Kakeya *et al.* (10), and Sprague (11); an abstract of these data is given in Table I. The general features of sulfonamide interactions with HCAC are presented in Table II.

Aromatic Sulfonamides—In addition to the interaction of the sulfonamido group with the enzyme, two additional types of interactions are observed for aromatic sulfonamides:

1. Hydrophobic interactions of the benzene ring and aliphatic substituents with nonpolar residues of the enzyme.
2. Hydrogen bonding involving hydrophilic substituents and polar residues of the enzyme.

The orientation of the benzene ring is very sensitive to the number and type of hydrogen bond interactions of its substituents and may be constrained in a less favorable position with respect to its hydrophobic contacts and *vice versa*. For example, 4-aminobenzenesulfonamide shows a threefold smaller inhibition constant than the unsubstituted 4-*H*-benzenesulfonamide (*cf.* Table I or Ref. 10). The crystal structure determination of the 4-aminobenzenesulfonamide–HCAC complex (5) shows that the interaction of the benzene ring with the hydrophobic part of the enzyme forces the amino group in the *para* position to a repulsive

Table II—General Features of Sulfonamide Interaction with HCAC

Functional Group of Inhibitor	Interaction with HCAC Residue	Type of Interaction
	Aromatic Sulfonamides	
	Zn^{2+}, (His 119)	coulombic[a]
	Zn^{2+}, Thr 199	coulombic, hydrogen bond[a]
	(NH of Thr 199 after small reorientation of the backbone of the enzyme)	
	Val 121, Leu 141 Val 143, Leu 198 Val 207	hydrophobic[a]
Ring Substituents		
para: aliphatic	Phe 131, (Ile 91)	hydrophobic
charged or hydrophilic	Gln 92, (Asn 67) solvent	hydrogen bonds[a]
meta: aliphatic	Val 121, (Pro 200, 201)	hydrophobic
charged or hydrophilic	His 64, Gln 92, O=C< of Pro 201, (Asn 67) solvent	hydrogen bonds
ortho: aliphatic	Leu 198, Val 121 Leu 141, Val 143 Val 207	hydrophobic
charged or hydrophilic	not very favorable	
	Heterocyclic Sulfonamides	
	as above[b]	
 (S pointing out)	ring C: Val 121, Leu 141 ring S: H_2O...O=C< of Thr 200 and Pro 201	hydrophobic[c] hydrogen bonds[c]
 (S pointing in)	ring C, N: H_2O...O=C< of Thr 200 or Pro 201 ring S: Val 121	repulsive ?, hydrogen bonds hydrophobic
 (S pointing out)	ring C, N: Val 121, Leu 141 ring S: H_2O...O=C< of Thr 200 and Pro 201	hydrophobic
 (S pointing in)	ring S: Val 121 ring N: H_2O...O=C< of Thr 200 or Pro 201	hydrophobic[d] hydrogen bonds[d]
R: aliphatic	Phe 131, Ile 91 (Trp 123)	hydrophobic[b]
charged or hydrophilic	Gln 92, (Asn 67), His 64, solvent	hydrogen bonds[e]
	Complex Sulfonamides	
	as above[a,b]	
—N=N—	Gln 92, solvent	hydrogen bonds[f]
—CO—NH—	Gln 92, solvent	hydrogen bonds
—CH_2—NH—	Gln 92, Phe 131	hydrogen bonds, hydrophobic[g]
—SO_2—NH—	Gln 92, His 64, Asn 67, solvent	hydrogen bonds
—S—NH—	Gln 92, solvent	hydrogen bonds
	Phe 131, Ile 91 (Trp 123)	hydrophobic[f,g]
R = small aliphatic	Phe 131, Ile 91 (Trp 123)	hydrophobic
charged or hydrophilic	solvent	hydrogen bonds[f]

[a] See Fig. 1. [b] See Figs. 3–5. [c] See Fig. 3. [d] See Fig. 4. [e] See Figs. 3–6. [f] See Fig. 6. [g] See Fig. 7.

interaction with the amido group of Gln 92. (A reorientation of the amido moiety is not very probable, since its carbonyl oxygen atom is also hydrogen-bonded to the N—H of His 94).

Hydrogen bond acceptors are favorably oriented with respect to the *para* and *meta* positions, since more hydrophilic residues of HCAC are located in this region and the active site funnel is open to the solvent (hydrogen bonds to Asn 67 and Pro 201 through coordinated water molecules). 3-Sulfamoyl-4,5-dichlorobenzenesulfonamide is a very potent inhibitor of HCAC. The *ortho* position is not very favorable for hydrophilic or bulky hydrophobic substituents; steric hindrance with Leu 198 requires a rearrangement of the backbone of the enzyme. 4-Nitro-2-carboxybenzenesulfonamide inhibits HCAC three orders of magnitude less than 4-nitro-2*H*-benzenesulfonamide [$<10^4$ *versus* 1.60×10^7 (9)].

As an example, *p*-nitrobenzenesulfonamide will be discussed in detail (Fig. 1). The sulfonamido group has discrete interactions with the enzyme:

1. The amino group (2.14% ionized in free solution at pH 7.38) occupies the fourth coordination site at the Zn^{2+} ion (d = 2.5 Å) and is hydrogen-bonded to Thr 199 (d = 2.8 Å). This position is identical with the coordinated water molecule in the native enzyme.
2. O-1 occupies the fifth coordination site at the Zn^{2+} ion (d = 3.0 Å).

Table III—Main Interactions of Various Sulfonamides with HCAC[a]

Functional Group of Inhibitor	Atom	Enzyme Residue[b]	Atom	Distance, Å[c]
		p-Nitrobenzenesulfonamide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.5
		Thr 199	O-γ	2.8
	O-1	Het 301	Zn^{2+}	3.0
	O-2	Leu 198	C-α	3.1
		Thr 199	NH	3.7
Benzene ring	C-2	Val 121	C-γ	4.1
			C-γ_2	3.6
	C-3	Val 121	C-γ	4.1
			C-γ_2	4.0
NO_2	O-2	Gln 92	N-ϵ_2	2.9
		2-Nitrothiophene-5-sulfonamide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.4
		Thr 199	O-γ	3.4
	O-1	Het 301	Zn^{2+}	2.9
	O-2	Val 143	C-γ_2	3.4
Thiophene ring	S-1	Water	O	3.8
	C-3	Val 121	C-γ	3.2
		Leu 141	C-δ_2	3.5
	C-4	Val 121	C-γ	3.4
			C-γ_2	3.4
		Leu 141	C-δ_2	3.5
Nitro group	O-2	Gln 92	N-ϵ_2	2.8
		Acetazolamide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.8 (2.5)
		Thr 199	O-γ	3.2 (3.2)
	O-1	Het 301	Zn^{2+}	3.0 (3.1)
	O-2	Val 143	C-γ_2	3.1 (3.0)
Thiadiazole ring	S-1	Val 121	C-γ	3.2 (3.4)
			C-γ_2	3.1 (2.9)
	N-3	Water	O	2.9
	N-4	Water	O	2.9
Acetamido group	N	Phe 131	C-ϵ_2	3.2 (3.0)
	O	Gln 92	N-ϵ_2	2.6 (1.7)
	C	Phe 131	C-ϵ_2	3.2 (3.1)
		Metazolamide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.5
		Thr 199	O-γ	3.4
	O-1	Het 301	Zn^{2+}	3.0
	O-2	Val 143	C-γ_2	3.5
Thiadiazole ring	S-1	Val 121	C-γ	3.3
			C-γ_2	3.0
	Ring-CH_3	Phe 131	C-ϵ_2	4.1
		Leu 141	C-δ_2	3.9
		Leu 198	C-γ	3.8
Acetimido group	O	Gln 92	N-ϵ_2	2.7
	C	Ile 91	C-γ_2	3.6
		Phe 131	C-γ	3.1
			C-ϵ_2	2.7
			C-δ_2	2.7
		p-(Salicyl-5-azo)-benzenesulfonamide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.7
		Thr 199	O-γ	3.4
	O-1	Het 301	Zn^{2+}	2.7
	O-2	Val 143	C-γ_2	3.4
Benzene ring	C-2	Val 121	C-γ	3.2
			C-γ_2	3.1
	C-3	Val 121	C-γ	2.5
	C-4	Val 121	C-γ	3.6
Azo bridge	N-1	Phe 131	C-ϵ_2	3.1
		Gln 92	N-ϵ_2	3.6
	N-2	Phe 131	C-ϵ_2	2.8
Salicyl ring	Since no relaxation of the backbone of HCAC was performed, these hydrophobic contacts with Ile 91 and Phe 131 turn out to be a little too short (<2 and 3 Å).			
		Furosemide		
SO_2NH_2	N	Het 301	Zn^{2+}	2.7
		Thr 199	O-γ	3.2

continued

Table III—*Continued*

Functional Group of Inhibitor	Atom	Enzyme Residue[b]	Atom	Distance, Å[c]
	O-1	Het 301	Zn^{2+}	3.4
	O-2	Val 143	C-γ_2	3.0
Benzene ring	C-2	Leu 141	C-δ_2	3.5
Ring substituents	CO_2:O-1	Gln 92	N-ϵ_2	2.6
	Cl	Leu 141	C-δ_2	2.5
Furfuryl group	C-3	Phe 131	C-ϵ_2	3.0
	C-4	Ile 91	C-δ	2.9
		Phe 131	C-γ	2.8
	C-5	Ile 91	C-δ	2.8
		Phe 131	C-γ	3.1

[a] Atomic coordinates for the inhibitors in the proposed model fits are available from the author on request. [b] Het = Protein Data Bank notation for any hetero atom or group. [c] Values in parentheses are calculated using atomic coordinates from the crystal structure determination of the HCAC–acetazolamide complex; taken from Ref. 5.

Because the dehydration of carbonic acid requires both the fourth and fifth coordination sites at the zinc, sulfonamides are competitive inhibitors to this reaction.

3. For O-2 no strong interaction could be found using the native conformation of the backbone of HCAC. However, it seems possible that a small reorientation of residues 196–200 would allow a better interaction of this oxygen atom with Thr 199 (NH of the backbone) and increase the distance to Leu 198 (Table III).

The benzene ring interacts with the hydrophobic part of the enzyme: C-2 and C-3 approach Val 121 at d = 3.6 Å and d = 4.0 Å, respectively. A hydrogen bond between the O-2 of the nitro group towards Gln 92 is present, d = 2.9 Å. Additional solvent molecules (H_2O) could be expected to bridge the oxygens of the nitro group with the carbonyl groups of Pro 201 and Asn 67. Since the most powerful aromatic inhibitors (*cf.* Table I) have good hydrogen bond acceptors in the *para* or *meta* position, these additional bridging water molecules may contribute significantly to the enzyme–inhibitor complex stability.

Heterocyclic Sulfonamides—*Participation of Solvent Molecules*—A 1,3,4-thiadiazole ring attached to the sulfonamido group facilitates sulfonamide–HCAC complex formation. Acetazolamide (2-acetamido-1,3,4-thiadiazole-2-sulfonamide) shows a 45-fold greater inhibition constant than 4-acetamidobenzenesulfonamide ($K = 7.1 \times 10^7$ *versus* 1.6×10^6 mol^{-1}). A crystal structure study of the acetazolamide–HCAC complex shows the heterocyclic sulfur atom of the 1,3,4-thiadiazole ring pointing towards Val 121. This interaction was reported to be the main reason why acetazolamide binds 10 times stronger to HCAC than to HCAB, where the ring sulfur atom approaches Ala 121.

We propose that in the acetazolamide–HCAC complex an additional significant contribution to the complex stability can be attributed to the interaction of the two heterocyclic nitrogen atoms through a coordinated water molecule with the backbone of Thr 200 or Pro 201 (Fig. 2). The structural rigidity of the portion of the receptor cavity occupied by Pro 201 and *cis*-Pro 202 should be noticed. The fixed backbone carbonyl groups, nevertheless, are well positioned for hydrogen bonding. Because this part of the enzyme is open to the solvent, the water molecule might be dynamically exchanged making it undetectable by X-ray diffraction analysis at ambient temperatures. In the receptor site of HCAB this coordinated water would interact in an unfavorable manner with His 200 (HCAC has a Thr residue at this position) and, thus, would be less probable.

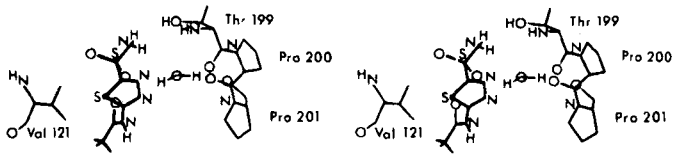


Figure 2—*H_2O molecule bridging the heterocyclic nitrogen atoms of the 1,3,4-thiadiazole ring of acetazolamide and the carbonyl group of Thr 200 or Pro 201.*

Such a coordinated water molecule is very probably present in all heterocyclic sulfonamides with a 3,4-unsubstituted 1,3,4-thiadiazole ring interacting with HCAC, as well as with thiophene ring systems (Fig. 3). Another water molecule may be expected to bridge the amino terminus of Asn 67 with the carbonyl function(s) of inhibitor molecules. Because no good interaction of O-4 of the sulfonamido group could be found with HCAC complexes, one might expect an additional water molecule between O-4 and an hydrophilic residue or the backbone in the vicinity (*i.e.*, Gly 196, Ser 197, Leu 198, Thr 199, Thr 200). With the coordinates of the native enzyme, however, no good site could be detected within the limits of the rigid receptor assumption.

Orientation of the Heterocyclic Ring—2-Nitrothiophene-5-sulfonamide is a very strong inhibitor for HCAC [$K = 2.38 \times 10^8$ *versus* 7.10×10^7 for acetazolamide (11)]. Assuming the same orientation as found in acetazolamide, the ring sulfur atom would point toward Val 121 and the 3,4-ring carbon atoms outward into the solvent. This would not contribute favorably to the binding energy of the enzyme–inhibitor complex and could not explain the large binding constant.

We therefore propose that 3,4-unsubstituted thiophenesulfonamides show an inverted binding to the active site cleft with respect to the orientation of the ring sulfur atom. 2-Nitrothiophenesulfonamide would thus achieve very good interactions of the ring carbon atoms with Val 121

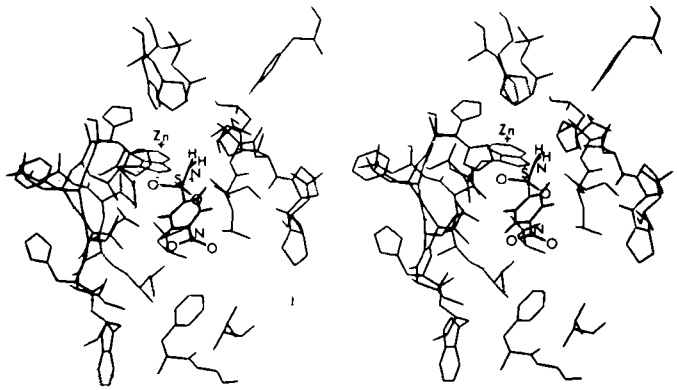


Figure 1—*Stereoprojection of the model fit of* p*-nitrobenzenesulfonamide in the receptor site of HCAC. The hydrogen atoms of the enzyme are not shown; the Zn^{2+} ion is represented by a cross (+).*

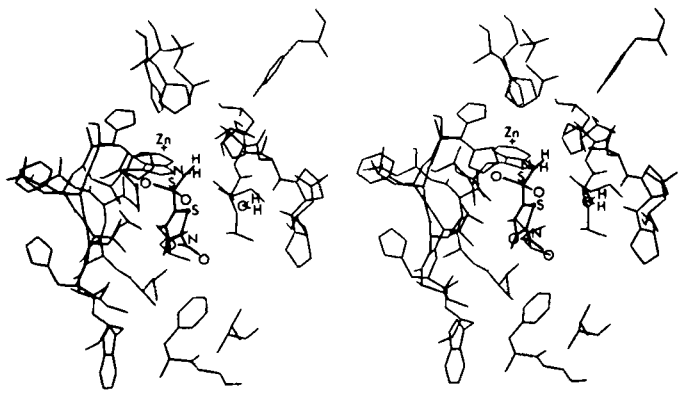


Figure 3—*Stereoprojection of the model fit of 2-nitrothiophene-5-sulfonamide into the receptor site of HCAC, with the bridging H_2O molecule to the right of the ring S atom.*

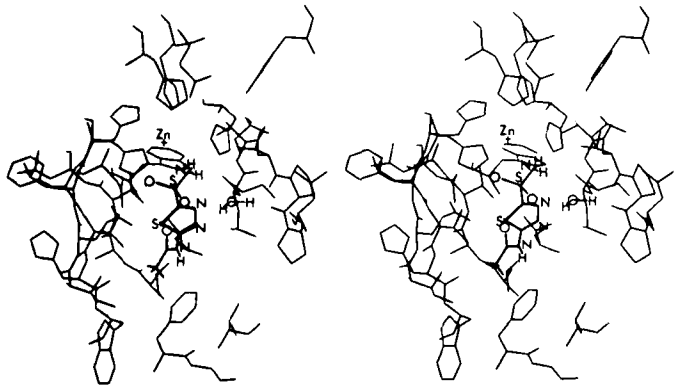


Figure 4—*Stereoprojection of the model fit of 2-acetamido-1,3,4-thiadiazole-5-sulfonamide (acetazolamide) into the receptor site of HCAC, with the bridging H_2O to the right of the ring N atoms.*

and Leu 141, and the sulfur atom would approach the oxygen atom of the coordinated water molecule (Fig. 3, Table III).

For 3,4-unsubstituted 1,3-thiazolesulfonamides, both orientations of the heterocyclic ring seem possible (Table II). This could also explain the unusually strong activity of 5-*H*-1,3-thiazole-2-sulfonamide *versus* 2-acetamido-1,3-thiazole-5-sulfonamide (Table I). For 3,4-unsubstituted 1,3,4-thiadiazolesulfonamides, an inverted binding of the heterocycle is very improbable, since the 3,4-ring nitrogen atoms would point into a hydrophobic part of the enzyme (*i.e.*, Val 121 and Leu 141).

The sulfonamido group of these inhibitors interacts with HCAC in the same manner as described for the aromatic sulfonamides. The main difference is that due to the lower pK value, heterocyclic sulfonamides may bind in the ionized form. At pH 7.38 (pH 6.50) acetazolamide is 49.9% (11.2%) deprotonated and metazolamide is 54.6% (13.7%) deprotonated.

With the inverted binding of the thiophene ring, 4-nitrothiophenesulfonamide achieves the following interactions with HCAC. The ring carbon atoms C-3 and C-4 are in contact with Val 121 and Leu 141 (d = 3.2 Å, d = 3.4 Å; d = 3.4 Å, d - 3.5 Å), whereas the ring sulfur atom approaches the oxygen atom of the postulated coordinated water molecule (d = 3.8 Å) which would be hydrogen bonded to the carbonyl group of Thr 200 (d = 3.0 Å) and Pro 201 (d = 2.7 Å). The nitro group would be hydrogen bonded to Gln 92 (d = 2.8 Å) and probably to the open solvent (Fig. 3, Table III).

The fit of acetazolamide, disregarding the additional water molecule bridging the heterocyclic ring with Thr 200 or Pro 201, is derived from crystal structure data reported by Kannan (5). Interactions include the heterocyclic sulfur atom with Val 121 (d = 3.1 Å and d = 3.2 Å), the acetamido oxygen with Gln 92 (d = 2.6 Å), and the methyl group with Phe 131 (d = 3.2 Å). The acetamido group is therefore tilted 74° out of the ring plane. In the crystal structure of molecular 2-acetamido-1,3,4-thiadiazole-5-sulfonamide (acetazolamide), this group is reported to be almost coplanar (4.9°) with the 1,3,4-thiadiazole ring, which allows an intramolecular S(ring)· · ·O(acetamido) contact of 2.75 Å (12). The two ring nitrogen atoms are located 2.9 Å from the water molecule (Fig. 4, and Table III), which is hydrogen bonded to Thr 200 (d = 3.4 Å) or Pro 201 (d = 3.1 Å).

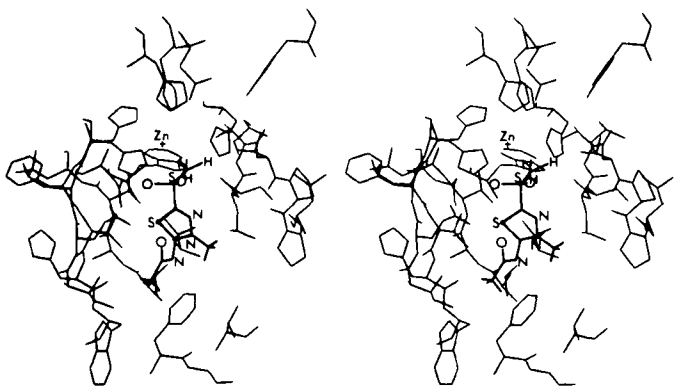


Figure 5—*Stereoprojection of the model fit of 2-acetamido-3-methyl-1,3,4-thiadiazole-5-sulfonamide (metazolamide) into the receptor site of HCAC.*

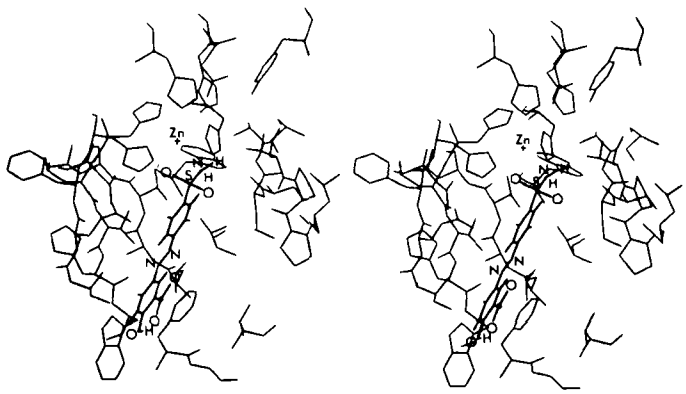


Figure 6—*Stereoprojection of the model fit of* p-*(salicyl-5-azo)-benzenesulfonamide into the receptor site of HCAC.*

The inhibitory power of metazolamide (2-acetamido-3-methyl-1,3,4-thiadiazole-5-sulfonamide) is reported to be 60% higher than in acetazolamide (11). This may be attributed to an additional interaction of either the 3-methyl group or the imido nitrogen atom of metazolamide with HCAC (Fig. 5). The heterocyclic sulfur atom is in contact with Val 121 (d = 3.0 Å, d = 3.3 Å). The ring methyl group is located in a hydrophobic pocket: Phe 131 (d = 4.1 Å), Leu 141 (d = 3.9 Å) and Leu 198 (d = 3.8 Å). The acetamido oxygen is hydrogen bonded to Gln 92 (d = 2.7 Å), whereas the methyl group is in contact with Ile 91 (d = 3.6 Å) and Phe 131 (d = 2.7 Å). The carbonyl group is tilted 35° out of the ring plane, and the intramolecular O(carbonyl)· · ·S(thiadiazole) distance is 3.27 Å.

Complex Sulfonamides—The interactions of *p*-(salicyl-5-azo)-benzenesulfonamide and furosemide (4-chloro-*N*-furfuryl-5-sulfamoylanthranylic acid) with HCAC will be discussed in detail. The benzene ring of *p*-(salicyl-5-azo)-benzenesulfonamide has close contacts to Val 121 (d = 2.5 Å, d = 3.1 Å, d = 3.2 Å, and d = 3.6 Å) (Fig. 6). The azo nitrogen atoms are 2.8 Å and 3.1 Å away from Phe 131, respectively. The amido nitrogen atom of Gln 92 interacts with the π-system of N-1 (d = 3.6 Å). The apparently short contacts of the salicyl group to Ile 91 and Phe 131 are not unrealistic if one takes into account that the backbone of the enzyme is not rigid and will relax to a more favorable conformation. (This additional degree of freedom is one of the subjects of our future investigations using force-field refinement.) The hydroxyl and carboxyl groups form hydrogen bonds with the surrounding solvent, which probably bridges these groups to the carbonyl group of Asn 67. A fit of the salicyl group into the hydrophobic pocket between Phe 131 and Val 135 was also investigated, but leads to less favorable contacts of the benzene ring.

Earlier investigations on aromatic sulfonamides indicated an enhancing effect of certain substituents (*i.e.*, halogens, trifluoromethyl, nitro, and methyl) *ortho* to the sulfonamido group with respect to diuretic activity. This structural alteration has decreased enzyme inhibitory activity and shifted the excretion pattern to a saluretic response. This separation of the two biological activities has been maintained and extended in the thiazide diuretics (11).

We therefore have investigated the steric interactions of such a sulfonamide drug, furosemide, with HCAC. With the native conformation of HCAC no favorable fit of furosemide could be obtained (Cl· · ·Leu 141: d = 2.5 Å). The chlorine in the *ortho* position of the benzene ring hinders

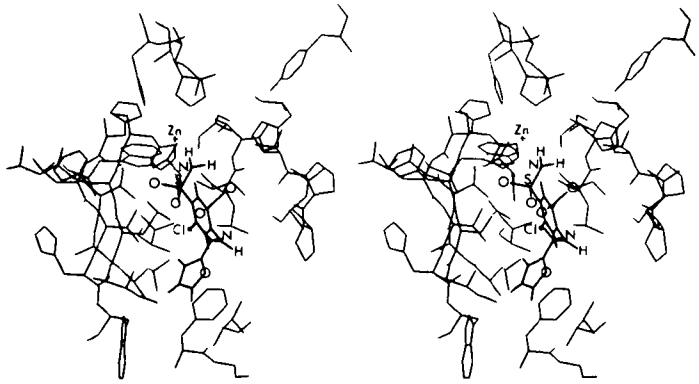


Figure 7—*Stereoprojection of the model fit of furosemide into the receptor site of HCAC.*

the molecule from entering deeply enough into the active site cleft to form good contacts (*i.e.*, sulfonamido group with Zn^{2+} and Thr 199, CO_2^- with Gln 92, furyl ring with Ile 91 and Phe 131). More drastic rearrangements of the hydrophobic part of the receptor site would be necessary in order to form good interactions. Therefore, simply for steric reasons, furosemide is expected to be a rather poor inhibitor of carbonic anhydrase; this is confirmed by *in vitro* measurements (11).

Figure 7 and Table III show that furosemide (without a chlorine atom in the *ortho* position) would be a good inhibitor of HCAC. The carboxyl group could hydrogen-bond to Gln 92 (d = 2.6 Å) and probably through a coordinated water molecule to the carbonyl groups of Pro 201. The furfuryl group would be located in the hydrophobic pocket between Ile 91 and Phe 131 (d = 2.8 Å through $d \approx 3.3$ Å), its oxygen atom pointing out into the solvent.

CONCLUSIONS

We have investigated 28 sulfonamide–HCAC complexes with interactive computer graphics in order to obtain a realistic starting conformation for force-field refinement. Aromatic sulfonamides gain most of their inhibitory power toward HCAC from the interaction of hydrogen bond acceptors in the *para* and *meta* positions with Gln 92 and Pro 201 and (*via* a coordinated water molecule) with Asn 67. No significant rearrangement of the backbone *versus* the conformation of native HCAC is expected, so long as the benzene ring is not substituted with bulky or multiple ligands.

In general, heterocyclic sulfonamides (*i.e.*, thiophenes, 1,3-thiazoles, and 1,3,4-thiadiazoles) are more potent inhibitors of HCAC than aromatic sulfonamides. This is due to the interaction of their heterocyclic ring with a hydrophobic part of the receptor site (Val 121$\cdots$S) and with Thr 200 and Pro 201 *via* a coordinated water molecule (S,N$\cdots H_2O\cdots$O=C$\langle$). We propose two different orientations of the heterocyclic ring with respect to the active site cleft:

1. The ring sulfur atom is in contact with Val 121, whereas the ring nitrogen atoms build hydrogen bonds to Thr 200 and Pro 201 (sulfur "in") for 3,4-unsubstituted 1,3,4-thiadiazoles. This is confirmed by the crystal structure determination of the acetazolamide–HCAC complex (5).
2. The ring sulfur atom is in contact with the oxygen atom of a coordinated water molecule, whereas the ring carbon atoms are located in a hydrophobic part of the receptor site such as Val 121 and Leu 143 (sulfur "out") for 3,4-unsubstituted thiophenes.

In addition to those interactions mentioned above, complex sulfonamides (two or more aromatic or heterocyclic rings) also show interactions of the "bridge" between the two ring systems (*e.g.*, —N=N—, —CO—NH—) with Gln 92 and the solvent. A significant contribution to the complex stability is derived from the interaction of aliphatic and aromatic substituents with the hydrophobic pocket around Ile 91, Trp 123, and Phe 131. Such sulfonamides, as well as aromatic or heterocyclic sulfonamides with larger substituents, may require a rearrangement of parts of the enzyme in order to bind. The hydrophobic pocket (Ile 91, Trp 123, Phe 131) for bulky aromatic or aliphatic parts of the inhibitor and the backbone around Leu 198 may need to be rearranged in order to obtain better interactions between the second sulfonamide oxygen atom and the enzyme.

Bulky ligands in the *ortho* position of the aromatic ring are not very favorable to inhibitory activity. Steric hindrance with Leu 141 and Leu 198 is the main reason why thiazide diuretics (Cl in the *ortho* position) are rather poor inhibitors of HCAC.

While crystallographic studies offer definitive information on the locus of binding and type of coordination of the sulfonamides with HCAB and HCAC, individual details cannot always be elucidated at the atomic level. By surveying the literature and applying known rules of stereochemistry and weak interactions, we have been able to propose alternative modes of binding of these small-molecule inhibitors that are consistent within the limits of the crystallographic studies and offer a better explanation for kinetic values. Interactive computer graphics, therefore, is a useful tool in surveying a broad literature data base to seek detailed correlations of structure and function. Such methods, when aided by the developing discipline of force-field refinement, offer the potential for detailed structure–activity studies. They may be extended by the intuitive chemist to include the prediction and design of novel drugs, inhibitors, and transition state analogues.

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