CHEMICAL REACTIONS OF SULFONAMIDES WITH CARBONIC ANHYDRASE

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Carbonic anhydrase from bovine erythrocytes was the first Zn(II) metalloenzyme to be discovered. Although the nutritional role of Zn(II) in mammals had been known since the middle of the 19th century, it was not until Keilin & Mann reported their work on carbonic anhydrase in 1940 that Zn(II) was shown for the first time to be a tightly incorporated and essential cofactor for an enzyme (1). Keilin & Mann showed the enzyme to contain 0.33% zinc and to be inhibited by monodentate metal-binding anions like cyanide, azide, and sulfide (1). Homogeneous crystalline mammalian erythrocyte carbonic anhydrase obtained from many species have all been shown to contain 1 atom of Zn(II) per monomer of mol wt ~30,000 (2).

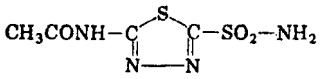
In addition to the inhibition by metal-binding anions Mann & Keilin (3) also demonstrated that sulfonamides were potent inhibitors of the enzyme. While the enzymes of the pathway utilizing *p*-aminobenzoic acid in bacteria (the classic metabolic pathway inhibited by sulfonamides) have not been purified, carbonic anhydrase remains the one crystalline enzyme for which sulfonamides are potent inhibitors. Excellent reviews of earlier work on carbonic anhydrase-sulfonamide interactions are available (4, 5).

Sulfonamides are not prominent metal complexing agents. It is therefore somewhat surprising to find sulfonamides joining the list of metal-complexing anions like CN^-, SH^-, N_3^-, and ^-OCN as potent inhibitors of carbonic anhydrase. Overwhelming evidence now indicates, however, that sulfonamides coordinate the metal ion at the active site. Moreover, the sulfonamide group appears to bind the metal ion in the anion form, $-SO_2NH^-$. Thus a proton dissociates from the complex if it is formed at pH below the pK_a of the sulfonamide group. Because of their highly

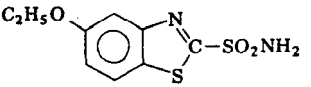
specific 1:1 interaction with the active center of carbonic anhydrase, sulfonamides have assumed a prominent place in physicochemical studies of the enzyme. The physicochemical studies predominantly employ equilibrium methods and are discussed first followed by a summary of the extensive kinetic studies on the mechanism of sulfonamide inhibition. Erythrocytes of several mammalian species contain two isozymes (2). Most physicochemical studies of carbonic anhydrase have utilized the human B isozyme, the human C isozyme, or the bovine B isozyme. While there are some subtle differences in the interaction of sulfonamides with the various species and isozyme variants, for the most part all the general physicochemical properties to be described here apply to all mammalian carbonic anhydrases. While plant carbonic anhydrases now appear to be inhibited by sulfonamides, studies of this inhibition are not extensive. The plant enzymes are Zn(II) enzymes containing one Zn(II) per polypeptide chain of mol wt 29,000 (6, 7).

CHEMICAL NATURE OF SULFONAMIDE-CARBONIC ANHYDRASE BINDING

It has been shown by measuring the optical absorption of the bound sulfonamide chromophore or bound [^{3}H]-acetazolamide (I) in equilibrium dialysis experiments that sulfonamide binding to the enzyme in solution is metal-ion-dependent (8,9). The apoenzyme shows four orders of magnitude less affinity for [^{3}H]-acetazolamide (9). Co(II) is the only other divalent metal ion among the first transition



I Acetazolamide



II Ethoxzolamide

and IIB metal ions that induces high affinity sulfonamide binding ($K_s \sim = 10^{-7}$ M) when added to the apoenzyme. The Mn(II) and Cu(II) enzymes bind acetazolamide with much lower affinity (9) (see below). Co(II) is also the only metal of the first transition and IIB group besides Zn(II) that restores significant activity to the apoenzyme (2). This must relate to some subtle feature of the coordination geometry shared by Zn(II) and Co(II) (perhaps bond angles of monodentate ligands from solution) which is also reflected in the dissociation constants of the sulfonamide complexes. It is difficult to explain the very small dissociation constants for the sulfonamide-carbonic anhydrase complexes [$\sim 10^{-9} M$ for ethoxzolamide (II)] purely on the basis of monodentate liganding to the metal ion. Indeed it has been noted since the early studies of inhibition by the sulfonamides that the large aromatic or heterocyclic ring is necessary for high affinity of sulfonamides for the enzyme. The structure of the crystalline sulfonamide-enzyme complex to be discussed below suggests that the ring system does interact strongly via hydrogen bonding, hydrophobic and van der Waals contacts with the protein groups lining the active site cavity.

X-Ray Structure of Carbonic Anhydrase-Sulfonamide Complexes

The basic structural chemistry involved in sulfonamide binding to carbonic anhydrase has been revealed by the crystal structure of the human C isozyme and its sulfonamide complexes at 2Å resolution (10-14). The protein molecule is roughly spherical but rather irregular in shape with dimensions approximately 40 X 40 X 55 Å measured between the extreme points of the peptide backbone. The zinc atom is located near the center of the molecule coordinated to three amino acid side chains of the protein, His-93, His-95, and a third residue believed to be the histidyl residue at position 118 in the sequence of the C enzyme, 119 in the sequence of the B enzyme (15, 16), and originally called His-117 in the X-ray structure (12). The identity of these ligands is supported by recent electron spin resonance studies using ligand superhyperfine structure on the ESR signals of the CN^- complexes of the Co(II) and Cu(II) enzymes to identify the chemical nature of the ligand nuclei (17). Superhyperfine coupling to three nitrogen ligands is observed. The residues immediately surrounding the Zn(II) are shown in stereoprojection in Figure 1. A fourth coordination position is occupied by solvent H_2O or ^-OH to complete a considerably distorted tetrahedral geometry around the Zn(II). The most recent detailed analysis of the electron density map suggests a hydrogen bond between the coordinated solvent molecule and Thr-197.

Other general features of the protein molecule include a broad twisted sheet of parallel and antiparallel β-pleated sheet running through the center of the molecule which accounts for about 37% of the residues. A few widely scattered sections of helix account for 20% of the remaining residues. Very few residues in these helical

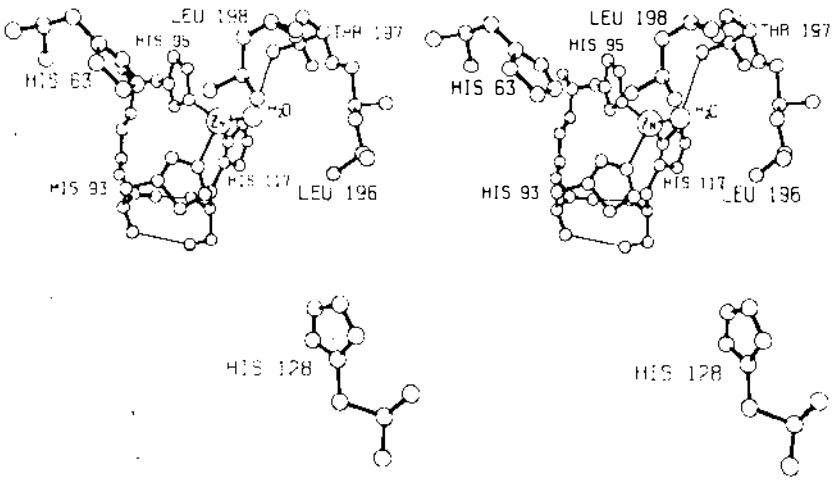


Figure 1 Stereoscopic view of the residues around the zinc ion in human carbonic anhydrase C (12). Residue 198 has recently been reidentified as Thr rather than Leu (see Figure 3). Residue 128 is Phe rather than His and is residue 129 in the sequence.

segments actually belong to the classical Pauling α-helix. The three ligands to the Zn(II) ion all come from the center section of the pleated sheet structure and place the zinc ion at about the center of the molecule at the bottom of a funnel-shaped cavity ~15 Å deep. The upper part of the molecule forms part of this cavity and the β-structure forms the bottom and part of the funnel-shaped mouth of the cavity. The lining of the cavity contains both polar and nonpolar residues including His-63 Phe-129, Thr 197, and Thr 198, besides those binding the zinc. The funnel-shaped cavity with the Zn(II) ion at the bottom clearly represents the active center of the molecule. Only the three histidyls and the water molecule are close enough to interact directly with the metal ion. Others like His-63, Asn-66, Phe-129, Thr-197, Thr-198, and Thr-196 are 4 to 8 Å away (Figure 1).

The electron density maps of the sulfonamide complexes show the sulfonamide to occupy the active center cavity (12, 14). The sulfonamide group itself binds to the Zn(II) ion probably through coordination of the nitrogen of the sulfonamide group (but oxygen coordination is not ruled out) (Figure 2). This is in agreement with earlier solution data showing that the metal is necessary for sulfonamide binding. Spectral data (18) also show that the bound form of the sulfonamide is the anion, $-SO_2NH^-$, in complete analogy to formation of a simple metal-ligand complex with a protonatable ligand. The H_2O or OH^- in the fourth coordination position of the Zn(II) is displaced (11, 12). Other anion inhibitors also appear to occupy the coordination position normally occupied by solvent, but the data are not as complete. Solution data already show that at high pH the binding of an anionic ligand like CN^- is accompanied by H^+ uptake or ^-OH release while at low pH the binding of a species like HCN is accompanied by release of an H^+ (19) (see below). The one anion complex structure that has been determined by X-ray methods is the iodide complex (13). This structure shows the center of gravity of the iodide ion to be 3.5 to 3.7 Å from the Zn(II), somewhat large for a direct coordination bond, but it appears to displace the solvent from the Zn(II) coordination site. This has led to some speculation that the inhibitory anions do not directely coordinate the metal ion. ESR data, however, show that at least for small anions like CN^-, bond forma-

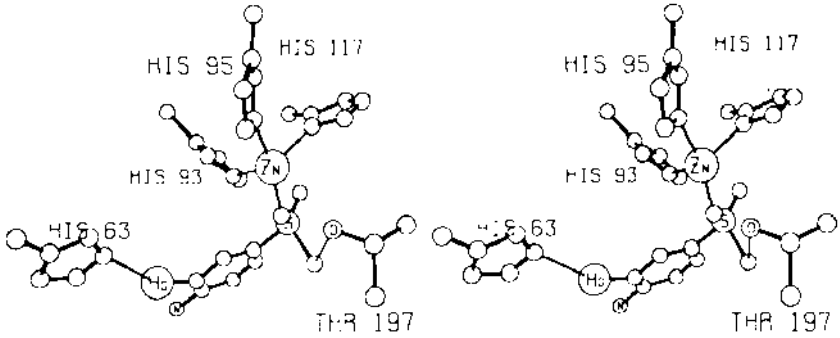


Figure 2 Stereoview of the complex of 3-acetoxymercuri-4-aminobenzenesulfonamide with the active site of human carbonic anhydrase C (12).

tion is directly with the metal ion which is coordinated to the carbon atom of CN^- (17, 20).

The structures of three human carbonic anhydrase C-sulfonamide complexes have been determined by X-ray diffraction; the complex with salamide (IV) (4-amino-6-chlorobenzene-1,3-disulfonamide, by projection only), the complex with 3-acetoxymercurisulfanilamide (AMSulf) by 3-dimensional structure determination at 2Å resolution, and the complex with acetazolamide (I) (Diamox ®) by 3-dimensional structure determination at 2.5 Å resolution (12-14). All three complexes show the sulfonamide group 2.9 to 3.0 Å from the zinc ion. Salamide binds with one sulfonamide group coordinating the Zn(II) ion and the other sulfonamide group in contact with His-63. In the complex with AMSulf, the sulfonamide group occupies almost the same position (within 0.1 Å) and the benzene ring stretches toward Gln-91 and Phe-129. Thus the side chain occupies a position somewhat different from that of salamide. The substituted Hg ion approaches His-63 (Figure 2).

In the Diamox® complex the sulfonamide group is coordinated to the Zn(II) (Figure 3). The heterocyclic ring of acetazolamide stretches out toward the opening of the active site cavity in almost the same direction as in the AMSulf complex. The acetylamido group is in van der Waals contact with Phe-129, and the oxygen of the same group is within hydrogen bonding distance of the ϵ-N of Gln-91. In all the complexes the sulfonamide groups in addition to being coordinated to the zinc atom are in a position such that the two uncoordinated atoms (O, O or N, O) can form hydrogen bonds with Thr-197 and Thr-198 (see Figure 3 for details).

There are a variety of chemical studies in solution that bear on the mechanism of sulfonamide binding. Most support the model derived from the electron density map of the crystalline complexes (Figures 2 and 3).

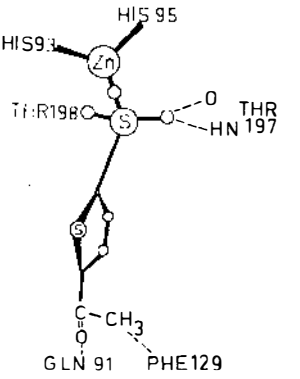
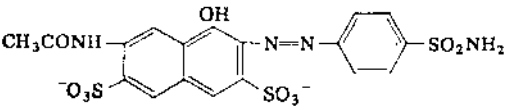


Figure 3 Schematic of the electron density map of the acetazolamide complex of human carbonic anhydrase C (14). The sulfonamide group is bound to the Zn(II), via O or N. One of the remaining two atoms of the sulfonamide group is within hydrogen bonding distance of Thr-197 (either the γ-O or the NH group), and the other is within hydrogen bonding distance of the γ-O of Thr-198. The planar ring of the Diamox is positioned such that one side is exposed to the hydrophilic and the other side to the hydrophobic side of the active site cavity.

Absorption, Fluorescence, and Phosphorescence Spectroscopy of Carbonic Anhydrase-Sulfonamide Complexes

Co(II) carbonic anhydrase shows absorption bands at 520 ($\epsilon = 205$), 555 ($\epsilon = 340$), 615 ($\epsilon = 230$), 640 nm ($\epsilon = 240$), 900 ($\epsilon \sim 25$), and 1250 nm ($\epsilon = 95$) representing the d–d transitions of Co(II) (21, 25). Binding of acetazolamide to the Co(II) derivative intensifies the spectrum and moves the higher energy bands to 520 ($\epsilon = 350$), 570 ($\epsilon = 550$), and 600 nm ($\epsilon = 500$). Analyses of the intensities, the change in the band energies on sulfonamide binding, and the magneto circular dichroism of these bands in the presence and absence of sulfonamide suggest that the geometry around the metal ion is initially that of a distorted tetrahedron and that the binding of the sulfonamide shifts the geometry to a more regular tetrahedral symmetry (21, 24). These spectral changes initially suggested that the sulfonamide was adding a ligand to the inner coordination sphere of the metal ion (21, 23).

Many sulfonamides have near ultraviolet absorption bands and occasionally intense visible bands, e.g. derivatives containing phenylazo groups (III). The visible bands of the bound azosulfonamide group show hypochromic and bathochromic



III 2-(-4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate

shifts not unlike those that occur when these chromophores are dissolved in nonpolar solvents suggesting that the environment of the binding site on carbonic anhydrase is hydrophobic (26).

A number of sulfonamides show characteristic changes in ultraviolet absorption spectra as a function of the $SO_2NH_2 \rightleftharpoons SO_2NH^- + H^+$ ionization. An extensive study of the ultraviolet spectra of several sulfonamides in the bound and unbound form show that the absorption spectrum of the bound sulfonamide is typical of the anion form (18). Both the near ultraviolet and visible absorption bands of the optically inactive symmetrical sulfonamides are rendered intensely optically active by binding to the active center of carbonic anhydrase emphasizing the dissymmetry of the surrounding protein potential field contributed by the protein groups lining the binding cavity (22, 23, 26).

Fluorescence of Sulfonamide Complexes

Bovine carbonic anhydrase forms a highly fluorescent complex with 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) (27). The fluorescence of the free sulfonamide has a peak emission at 580 nm and a quantum yield of 0.055, but the bound compound has an emission maximum at 468 nm and a quantum yield of 0.84. The large blue shift in the emission maximum can be adequately explained if it is assumed that the binding pocket is hydrophobic and that the $-SO_2NH_2$ group of the ligand loses a proton upon binding to the enzyme (27).

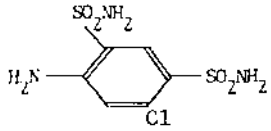
Calculation of the energy transfer efficiency gives the surprising result that 85% of the photons absorbed by the 7-tryptophan residues are transferred to the single bound DNSA molecule. The transfer efficiency is much higher than hitherto observed for a protein having only one 5-dimethylaminonaphthalene-1-sulfonyl group. Although the diameter of the protein is roughly 45 Å, the bound DNSA group is probably within the critical transfer distance R_o (=21.3 Å) of all tryptophans. These findings suggest that the sulfonamide binding site and the tryptophans are in the interior of the molecule, a suggestion borne out by the X-ray findings, although these apply only insofar as the structure of the human C enzyme will be found analogous to the bovine B enzyme upon which the fluorescence and phosphorescence (see below) studies were carried out.

Phosphorescence of Sulfonamide Complexes

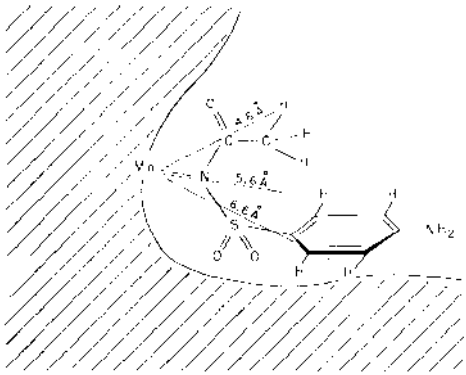
Electronic excitation energy can be transferred between singlet states of chromophores separated by distances of the order of 30 Å. On the other hand triplet-triplet energy transfer requires a much closer approach and can measure distances on the order of 12 Å. If the singlet state of a triplet acceptor is at a higher energy than the singlet state of the triplet donor the triplet donor can be excited by light of a wavelength not absorbed by the triplet acceptor. Under these conditions, phosphorescence of the triplet acceptor is observed only if there is triplet-triplet energy transfer. Galley & Stryer (28) obtained this arrangement in the complex of bovine carbonic anhydrase with *m*-acetylbenzenesulfonamide (MABS). Tryptophan phosphorescence of carbonic anhydrase is excited by light of wavelength 280 nm. The sulfonamide phosphorescence is excited by light of wavelength 330 nm, while the enzyme shows no phosphorescence if 330 nm light is used. If the MABS carbonic anhydrase complex is excited at 330 nm, the phosphorescence observed was that of tryptophan rather than MABS. Thus a tryptophanyl residue appears to be located very near the sulfonamide binding site, perhaps lining the cavity. In the structure of the human C isozyme determined by X-ray diffraction Try-207 does lie in the active site cavity and is partially buried in the wall.

NMR, EPR, and Magnetic Susceptibility Studies of Carbonic Anhydrase-Sulfonamide Complexes

The dissociation constants of sulfonamides bound to Mn(II) carbonic anhydrase are large enough that the bound sulfonamide exchanges rapidly with free sulfonamide in solution. NMR experiments focusing on the protons of the inhibitor in solution are not exchange limited, and chemical shifts or paramagnetic broadening of these resonances by Mn(II) in the bound form of the inhibitor can be observed in the presence of a large excess of inhibitor due to rapid exchange of the bound with the free sulfonamide. Distance calculations from the Mn(II) to various protons on sulfacetamide calculated from the paramagnetic effect of Mn(II) on the relaxation of these protons are shown in V (29). The data are compatible with a model in which the nitrogen of the substituted sulfonamide group is directly coordinated to the



IV Salamide



V Mn(II) Carbonic Anhydrase-sulfacetamide

Mn(II) ion, although this is not unique; an oxygen of the sulfonamide could also be the coordinating nucleus.

At alkaline pH both Co(II) and Mn(II) carbonic anhydrase relax the protons of bulk solvent water showing that solvent protons are in rapid exchange with a solvent (H_2O or ^-OH) occupied coordination site. This exchange may represent rapid proton exchange with a metal-hydroxide species. Binding of sulfonamides completely abolishes this relaxation enhancement (30, 31). Thus the sulfonamide closes off the access of solvent to the metal ion.

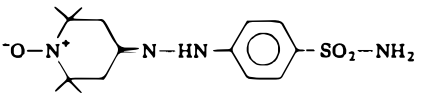
The binding of a spin-labeled sulfonamide (VI) to several isozymes of carbonic anhydrase has been studied. In the case of bovine carbonic anhydrase the spin-label is highly immobilized and has a rotational correlation time equal to that of carbonic anhydrase calculated from Stoke's law considering the enzyme as a rigid sphere of radius ~20 Å and 30,000 mol wt (27, 32). Thus the sulfonamide appears to have no motion in the cavity of the bovine enzyme relative to the protein. The spin-labeled sulfonamide is slightly less immobilized in the cavity of several other isozymes (32). Several other spin-labeled sulfonamides of different structure are also less immobilized in the bound state and must have some additional motion relative to the protein (33). As the side chain gets longer, this motion appears to increase systematically and has been used to estimate the depth of the cavity as ~14 Å (34). Tight binding of the spin-labeled sulfonamide is induced only by Zn(II) and Co(II) confirming the metal-ion-dependent nature of the binding (32). Studies of denaturation of the protein by following dissociation of the spin-labeled sulfonamide shows that the sulfonamide appears to protect the protein from denaturation by guanidine HCl, but not from denaturation by urea (32).

Magnetic susceptibility studies have shown that cobalt carbonic anhydrase contains high-spin Co(II) and it remains high-spin in the sulfanilamide complex (25). Small changes in susceptibility on formation of the sulfanilamide complex are not currently interpretable. An NMR study measuring the broadening of the resonances of several sulfonamide protons caused by binding of the sulfonamides to bovine

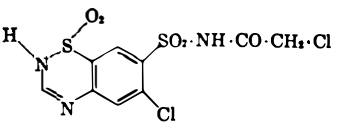
carbonic anhydrase B has been carried out using sulfonamides which exchange relatively rapidly between the complex and free ligand (35). The sulfonamides used were N^1-acetylsulfanilamide, *p*-toluenesulfonamide, and sulfanilamide. The rapid exchange allowed observation of broadening of the sulfonamide resonances in a 0.02 *M* solution of sulfonamide induced by $1.5 \times 10^{-3} M$ enzyme. The broadening mechanism appears to be a dipolar relaxation mechanism rather than a chemical shift (35). The dissociation rates for these inhibitors calculated from the NMR data were found to be greater than 200 sec^{-1}. The rotational motion of the aromatic rings of the bound inhibitors was found to be close to that of the whole protein molecule, while that of the methyl groups was found to be much faster.

CHEMICAL MODIFICATION OF CARBONIC ANHYDRASE SULFONAMIDE COMPLEXES

Chemical modification of the sulfonamide group, e.g. acylation, drastically reduces the affinity of any sulfonamide for carbonic anhydrase (4, 36). However, N-chloroacetylchlorothiazide (VII) does bind to human carbonic anhydrase B and the



VI 2,2,6,6-tetramethyl-4-piperidone-1-oxyl-*p*-sulfamylphenylhydrazone



VII N-Chloroacetylchlorothiazide

N-chloroacetyl group acylates a histidine residue in the enzyme shown by sequence studies to be His-67 (11) (labeled His-66 in the high resolution structure of the B enzyme) (37). The N-3' of His 66 is 8.9 Å from the zinc ion, and this residue is not present in the C enzyme where it is replaced by Asn-66 (16). N-chloroacetylcyclothiazide does not modify the C enzyme. Because His-66 is rather distant from the active site, the conformation of the B enzyme around the active site cavity suggests that the sulfonamide must initially bind to the metal ion via the $-SO_2NH^-$ function in the heterocyclic ring which would place the substituted sulfonamide in a position to alkylate His-66.

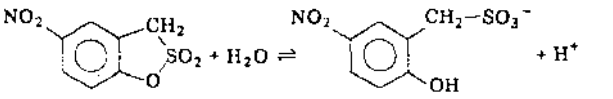
From the NMR studies of the Mn(II) enzyme cited above it does appear that the acylated sulfonamide group of sulfacetamide coordinates the metal ion, although some caution should be exercised in interpreting the binding of sulfonamides in which the sulfonamide group itself is modified. When acetazolamide brominated on the carbon of the acetamido group is bound at the active site it alkylates His-63 (38, 39) compatible with the location of this group near the lip of the active site cavity. This also agrees with the X-ray structure determination of the positioning of acetazolamide in the active site cavity (Figure 3).

Carboxymethylation of His-198, located 5.6 Å from the Zn(II) ion in the human B isozyme, (37) does not prevent the binding of sulfonamide VI, although it increases the dissociation constant of the complex with VI (32). If His-198 is alkylated

with a spin-labeled derivative of bromosuccinic acid, then access of sulfonamides to the active site cavity appears to be blocked (32). Carboxymethylation of His-63 in the C isozyme by bromopyruvate is more difficult in the presence of salamide (IV) (13). This part of the sulfonamide molecule clearly binds very near His-63.

SULFONAMIDE-LIKE ESTER AS A SUBSTRATE FOR CARBONIC ANHYDRASE

In recent years carbonic anhydrase has been shown to have considerable catalytic activity in the hydrolysis of a number of esters, e.g. *p*-nitrophenylacetate. Kaiser & Lo (40) synthesized a cyclic sultone (2-hydroxy-5-nitro-α-toluenesulfonic acid sultone) (Equation 1) which has structural analogies to the sulfonamides. The hydrolysis of this compound is rapidly catalyzed by carbonic anhydrase with a turnover number for some isozymes of ~20,000 mol/min per mole of enzyme (2). While not as rapid as CO_2 hydration the rate exceeds that for other esters by



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manyfold. The rapid catalysis probably relates to specific sulfonamide-like interactions with the active center.

KINETICS AND EQUILIBRIA OF SULFONAMIDE BINDING: pH-DEPENDENCE, PROTON EQUILIBRIA

The pH-dependence of carbonic anhydrase–catalyzed reactions can be summarized by the statement that the activity depends on a group in the enzyme with pK_a of ~7, the basic form, E, being required for hydration of CO_2, while the acid form, EH^+, appears to be required for dehydration of HCO_3^- (41). The identity of this group has given rise to extensive discussion (31, 41–43). One hypothesis is that this group represents the pK_a of a Zn-coordinated water molecule, while other hypotheses suggest that it represents the pK_a of an amino acid side chain near the active site. The detailed electron density map of the active center shows that there is a solvent-occupied coordination site on the Zn(II) and that Thr-197 appears to form a hydrogen bond with this group (Figure 1). Depending on the donors and acceptors this hydrogen bond might represent a stabilization of the Zn^{++}–OH^- and lower the pK_a for the dissociation of the first proton on the Zn-coordinated water. In any event the ionization governing activity is closely coupled to the metal ion as reflected in the pH-dependence of the absorption spectrum of Co(II) carbonic anhydrase (8, 19), the pH-dependence of solvent relaxation by Co(II) and Mn(II) carbonic anhydrase (31, 44), the pH-dependence of the nuclear quadrupole relaxation of coordinated $^{81}Br^-$ or $^{35}Cl^-$ ions by Zn(II) carbonic anhydrase (45, 46), and the pH-dependency of binding of anions including sulfonamides (47–51). All show sigmoid pH-dependencies. As is discussed below, the pH-dependency for many of

these phenomena, including sulfonamide binding, is actually bell-shaped reflecting both acid and alkaline ionization processes involved in binding. The pK_a of the enzyme group involved in activity appears to be a component of all pH dependencies of binding for both anions and sulfonamides. Just how it enters is discussed below.

The equilibrium constants for sulfonamide binding as a function of pH have been measured by equilibrium dialysis and by kinetic means measuring the pH dependency of the inhibition constant K_i (9, 47, 48). These are plotted together for several sulfonamides and for several simple anionic inhibitors of carbonic anhydrase in Figure 4. All show bell-shaped pH-dependencies with the exception of NCO^- (see below). The equilibrium data for binding of [^{3}H]-acetazolamide agree well with the kinetic data. The spread between the acid and alkaline arms of these binding functions varies considerably and appears to depend on several factors including the pK_a and pK_i of the inhibitors.

The second order rate constants, k_a, for the association of sulfonamides with the enzyme were initially measured for several sulfonamides by stopped-flow methods monitoring the onset of the inhibition of CO_2 hydration (47, 48). Determination of the magnitudes of the k_a values as a function of pH showed that the second order association constants vary according to bell-shaped curves similar to those followed by the K_i values or equilibrium constants (Figure 4). The dissociation rate constant, k_d, appeared to be independent of pH (48). For bovine Zn(II) carbonic anhydrase and sulfanilamide, $k_a = 3.9 \times 10^4 M^{-1}$ sec^{-1} (25°, 0.1 ionic strength, pH 8.0) and for bovine Co(II) carbonic anhydrase and sulfanilamide, $k_a = 7 \times 10^4 M^{-1}$ sec^{-1} (pH

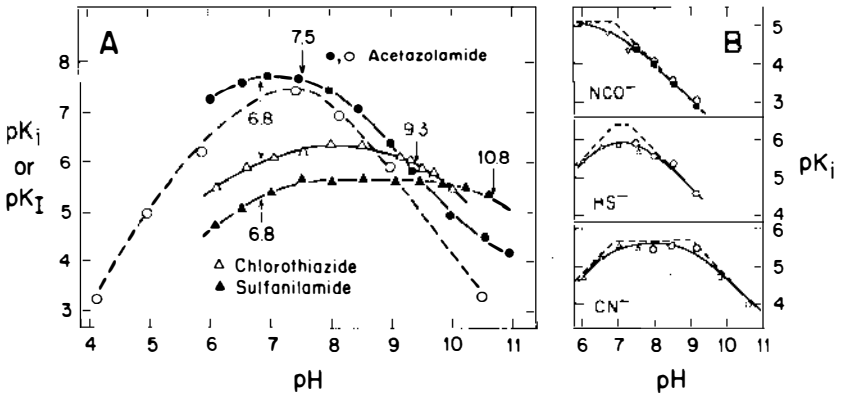


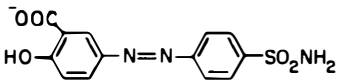
Figure 4 *A* pH-dependence of K_i for the inhibition of bovine carbonic anhydrase by acetazolamide (●), chlorothiazide (Δ), and sulfanilamide (▲). *p*H-dependence of the dissociation constant, K_I, for the ^{3}H-acetazolamide complex of human carbonic anhydrase B(○). Data replotted from references 9 and 47. *B* pH-dependence of K_i for the inhibition of bovine carbonic anhydrase by cyanate (*top*) sulfide (*middle*) and cyanide (*bottom*). The dotted lines have slopes of +1, 0, and −1 and intersect at the apparent pK_a values required to describe the curves. Data replotted from reference 49.

7.9, 25°) (47, 48). For the combination of benzene sulfonamide with the bovine enzyme, $k_a = 2.1 \times 10^5 M^{-1}$ sec^{-1} (pH 8.0) (52). For ethoxzolamide the k_a is much higher, $2.9 \times 10^7 M^{-1}$sec^{-1}, and for the combination of acetazolamide with the human B enzyme, $k_a = 6 \times 10^6 M^{-1}$ sec^{-1} (48). The magnitude of these rate constants were interpreted as compatible with a simple mechanism in which RSO_2NH^- combines directly with the acidic form of the active site in an almost diffusion-controlled reaction. The complexity of the sulfonamide interaction suggests, however, that there may be many microscopic steps involved (see below).

More recently extensive direct measurements of k_a, k_d, and the equilibrium constants, K_I, for a great variety of sulfonamides combining with human carbonic anhydrase C have been made using stopped-flow techniques (50, 51, 53). The method takes advantage of the fact that the absorption bands of the sulfonamide chromophores overlap the spectral region of the fluorescence emission of the tryptophan chromophores of the protein. Thus the binding of sulfonamides (represented by k_a) can be monitored by the quenching of the protein fluorescence caused by energy transfer to the bound sulfonamide chromophore. Dissociation constants, k_d, can be measured by using one sulfonamide to displace another, a process controlled by the dissociation rate of the first sulfonamide (53). The sulfonamide pairs are chosen so they show a very different degree of quenching of the protein fluorescence, and hence the displacement reaction is accompanied by large changes in quenching.

The results of the detailed direct measurements amply confirm the earlier conclusions. The pH-dependency of K_I follows curves like those in Figure 4. The pH-dependency of K_I is accounted for solely by the pH-dependency of the association rate constant, k_a. The dissociation rate constant, k_d, is pH-independent (50). The pH-independence of k_d is compatible with observations that the d-d absorption spectrum of the Co(II) enzyme-sulfonamide complex is pH-independent, once the complex is formed (50), a fact also pointed out for the complexes with anions like CN^- which also show pH-independent spectra with the Co(II) enzyme once the complex is formed (19).

While the rates of association for a series of 24 sulfonamides are fast ($k_a = 5.48 \times 10^4 M^{-1}$ sec^{-1} for HO (3–NO_2) $C_6H_3SO_2NH_2$–p to $1.13\ 10^7\ M^{-1}$ sec^{-1} for VIII), the fastest rate is at least two orders of magnitude less than that



VIII *p*-(salicyl-5-azo) benzenesulfonamide

expected for a diffusion-controlled reaction between a small ligand and a macromolecule under these conditions. The association constant, k_a, for such a reaction would be expected to be $\sim 2 \times 10^9 M^{-1}$ sec^{-1} (53).

The dissociation constants, k_d, for carbonic anhydrase complexes with sulfonamides of widely variant structure vary from 0.036 sec^{-1} to 2.71 sec^{-1}, a range of 80-fold. On the other hand, k_a varies by 240-fold. Hence the variation in the affinity

of the various sulfonamides for the active center is influenced predominantly by the rate of the "on" reaction. This is in marked contrast to diffusion-limited complex formation involving small molecules where the magnitude of the equilibrium constants depend largely on the relative dissociation rates of the ligands, the association rates being fast and of similar magnitude.

It could be argued that the slow "on" rates reflect the geometrically restricted binding cavity. This would not appear to be a major factor, however, because many sulfonamides with large bulky ring structures and several side chains combine much faster than simple unsubstituted sulfonamides which would be expected to be the least hindered by rotational and translational diffusion in a geometrically restricted binding site. Arrhenius plots of the association rate constant, k_a, for the *p*-nitrobenzene-sulfonamide-human enzyme C complex show that the complex is stabilized by a large favorable enthalpy change ($\Delta H = 6.6$ kcal/mol) (53). This is large for a diffusion-controlled reaction where the enthalpy of association should be that of diffusion in water (2.5–4 kcal/mol) (53). The large net enthalpy change is perhaps more like a complex stabilized through a dominant metal-ligand bond than through a hydrophobic interaction. However, if the metal-ligand bond were the predominant force, one would not expect the stability of the complex to be controlled primarily by the association reaction. The relative stabilities of model metal-ligand complexes are governed largely by the variation in their dissociation rate constants, and thus the rates of ligand exchange reactions are determined primarily by the dissociation rate of the leaving ligand. The kinetics are relatively uninfluenced by the nature of the incoming ligand. However, this may not be the case in carbonic-anhydrase even for simple metal-binding anions, since ligand exchange takes place at the bottom of a deep and highly restricted cavity (see below).

Finally, if geometrical restriction at the site were primarily responsible for the slow and variable "on" rates of sulfonamides one would expect that formation of the complexes would be accompanied by a large negative entropy of formation rather than a large enthalpy change. It would thus appear that the sulfonamide binding involves a combination of forces including metal-sulfonamide bond formation as well as hydrophobic interactions, hydrogen-bond formation, and van der Waals interactions with various protein groups lining the binding cavity. All of these interactions are suggested by the X-ray structures of the complexes reviewed above. Such multiple interactions suggest that formation of the sulfonamide-carbonic anhydrase complexes could involve a multistep mechanism. Under the conditions of most experiments, however, concentrations of the intermediates would be expected to be small, to form in rapid preequilibrium steps, and not to be detected by the spectroscopic techniques employed thus far, because most of these techniques appear to monitor only the accumulation of the final complex.

The kinetic and equilibrium data describing the combination of sulfonamides with human carbonic anhydrase B are qualitatively similar to those described above for the C isozyme. There are, however, some striking quantitative differences in association and dissociation rates for various sulfonamides with the two enzymes. For example, orthosubstitution of the sulfonamide ring decreases the affinity of the sulfonamide for the C isozyme, while it has little effect on the affinity of the

sulfonamide for the B isozyme. This is primarily due to a large change in k_d for the C enzyme complex. This effect is illustrated by the data for the combination of dansylamide with the two isozymes (isozyme C: $k_a = 2.40 \times 10^5 M^{-1}\text{sec}^{-1}$, $k_d = 0.390\ \text{sec}^{-1}$; isozyme B: $k_a = 1.34 \times 10^5 M^{-1}\ \text{sec}^{-1}$, $k_d = 0.030\ \text{sec}^{-1}$). These effects must reflect specific steric differences between the lining of the active site cavities produced by alterations in the primary amino acid sequence.

The bell-shaped pH dependencies of the equilibrium constants and the association rate constants for the carbonic anhydrase-sulfonamide complexes have generally been described by the statement that the pH-dependency of binding depends on two ionizations. One, with an apparent pK_a of 6.6 to 6.8, appears to be relatively invariant as a function of sulfonamide structure, while the second higher pK_a varies between pH 7 and pH 11 depending on sulfonamide structure (Figure 4). Because the latter variable pK_a seems to relate to the pK_a of the $-SO_2NH_2$ group on the sulfonamide and is in general similar in magnitude to the pK_a values of the free sulfonamides, the higher pK_a has been assigned to the $-SO_2NH_2 \rightleftharpoons -SO_2NH^- + H^+$ ionization. The lower relatively invariant pK_a does not correspond to pK_a values of protonatable groups on the sulfonamide inhibitors and has been assigned to the enzyme group whose ionization is coupled to the metal ion and involved in activity (47,50). The apparent pK_a values observed in the pH-functions describing binding (Figure 4), however, may not represent simple unperturbed pK_a's, but may be more closely related to formation curves as observed for simple chelate systems where the position of the apparent midpoint of the pH-dependent formation function represents a combination of the pK_a of the ligand, the intrinsic affinity of the ligand donor atom for the metal, and the presence of competing ligands for the metal ion, e.g. ^-OH.

The reaction between carbonic anhydrase and the sulfonamides can be written in four possible ways (equation 2*a, b, c, d*) involving the different ionization states of the enzyme group involved and the sulfonamide. The *p*H-dependency of k_a (identical with the equilibrium data in Figure 4) is compatible

$$\begin{aligned} &(a)\ [HCA]^+ + RSO_2NH_2 \xrightarrow{k_1} \\ &(b)\ [HCA]^+ + RSO_2NH^- \xrightarrow{k_2} \\ &(c)\ [CA] + RSO_2NH^- \xrightarrow{k_3} \\ &(d)\ [CA] + RSO_2NH_2 \xrightarrow{k_4} \end{aligned} \qquad RSO_2NH^-\text{–}ZnCA \qquad 2.$$

$$\begin{aligned} &(a)\ [HCA]^+ + HCN \xrightarrow{k_1} \\ &(b)\ [HCA]^+ + CN^- \xrightarrow{k_2} \\ &(c)\ [CA] + CN^- \xrightarrow{k_3} \\ &(d)\ [CA] + HCN \xrightarrow{k_4} \end{aligned} \qquad NC^-\text{–}ZnCA \qquad 3.$$

with either reaction *b* (the combination of the anionic form of the sulfonamide with the protonated form of the enzyme) or reaction *d* (the combination of the netural

sulfonamide with the deprotonated or basic form of the enzyme). Kinetic studies cannot distinguish these mechanisms. Based on the very high association rate for sulfonamide VIII, Taylor et al (53) suggest that the neutral sulfonamide combines with the alkaline form of the enzyme (equation 2*d*), because reaction 2*b* would require k_2 to be faster than observed in most diffusion-controlled reactions. This argument has also been made for the binding of pentafluorobenzenesulfonamide to bovine carbonic anhydrase B, based on a pK_a for the sulfonamide of 3.1 (54). This has since been shown to be in error; the pK_a of the sulfonamide group of pentafluorobenzenesulfonamide is 8.05 (55). The association rate constants using this pK_a value are well below the diffusion limits assuming either mechanism. It is clear that the final form of the sulfonamide complex is one in which the protonatable group of the enzyme has been displaced and the sulfonamide is in the anion form (18, 27, 48). Exactly the same set of equations may be written for anions like CN^- (equation 3*a, b, c, d*) which also show a bell-shaped pH-dependency for binding with the significant exception of some of the more weakly binding anions like NCO^- (Figure 4), SCN^-, and Cl^- which show only a single phase, sigmoid to low pH (see discussion below). These formal kinetic analyses assume that the alternate form of the enzyme (either E or EH^+ depending on choice of mechanism) has no affinity for the sulfonamide or that there is no shift in the mechanism as a function of pH. These may be oversimplifications.

It is instructive to consider sulfonamide binding in light of the ligand exchange reactions that may be involved. The simple anions are discussed first, because the binding is not complicated by the additional interactions of the side chain with the active site cavity as is the case with the sulfonamides. If the ionizing group on the enzyme involved in activity is assigned to a Zn-coordinated water molecule the reactions of cyanide with the enzyme throughout the pH-range can be described by the equilibria in equation 4.

$$\begin{array}{lcccl} (a) & CAZn \cdot H_2O & + & HCN \rightleftharpoons CAZn \cdot CN^- + H_2O + H^+ & \\ & \Big\| \; (-H^+) & & (-H^+) \; \Big\| & \\ & pK_{a_{enz}} = 8 & & pK_{I_a} = 9.3 & \quad 4. \\ & \Big\| & & \Big\| & \\ (b) & CAZn \cdot {}^-OH & + & CN^- \rightleftharpoons CAZn \cdot CN^- + OH^- & \end{array}$$

$K_I = \frac{[CAZn][CN^-]}{[CAZn{\cdot}CN^-]}$ = the intrinsic dissociation constant of the inhibitor-metal complex

$K_{I_a} = \frac{[CN^-][H^+]}{[HCN]}$ = the acid dissociation constant of the inhibitor

$K_{enz} = \frac{[E][H^+]}{[EH^+]}$, EH^+ = acid form of the enzyme ($CAZn \cdot H_2O$, E = basic or "active" form of the enzyme ($CAZn{\cdot}OH$).

By direct measurement of proton and hydroxide ion release between pH 6 and 10, the equilibria in equation 4 have been shown to hold for the combination of both cyanide and sulfide with human carbonic anhydrase B (19). The data generate the

correct pK_{I_a} values for the inhibitors, 6.9 for H_2S, 9.3 for HCN, and an enzyme pK_{enz} of ~8, similar to the midpoint of the pH-rate profile of esterase activity of the human B enzyme under the same conditions (19). If the equilibria in equation 4 are treated as ligand exchange reactions, the pH-stability of $CAZn{\cdot}CN^-$ (the inhibited species) will depend on the intrinsic affinity of the anion for the zinc site, K_I, the affinity of protons for the anion (K_{I_a}), the concentration of OH^-, and the pK_{enz} of the enzyme ($CAZn{\cdot}H_2O \rightleftharpoons CAZn{\cdot}OH^- + H^+$), because the latter determines the pH at which OH^- begins to compete with the anion. It has been shown by measuring the pH-dependency of enzyme activity in the presence of anions and also by measuring the transition between the acid and alkaline form of Co(II) enzyme (as followed by the pH-dependency of the absorption spectrum of the d–d transitions in the presence of anions) that the sigmoid pH-dependency of the enzyme activity is preserved, but offset proportionately toward higher pH as a function of increasing binding affinity of the anion for the enzyme active site in the order $F^- < Cl^- < Br^- << I^- < NO_3^- << NCO^-$ (56). This is compatible with competition between the inhibiting anion and OH^-. The greater the affinity of the anion, the higher the $[OH^-]$ required to displace the anion and generate the active alkaline form of the enzyme.

The anions of strong acids like Cl^-, Br^-, and NCO^- do not have pK_a values within the pH range used to study carbonic anhydrase (pH 5.5 to 12); the pK_a values are all at very acid pH, reflecting their relatively weak affinity for H^+ and for Zn^{2+} as well. For the anions that do not protonate within the pH range of study, the acid arm of the binding curve is missing, e.g. NCO^- in Figure 4*B*. This has led Taylor & Burgen (51) to propose that the mechanism of anion binding, in contrast to sulfonamide binding, involves the combination of the anion form with the acid or protonated form of the enzyme. This distinction is artificial, because for high affinity anions like SH^- (pK_a for $H_2S \approx 6.9$) or CN^- (pK_a for HCN = 9.3) which do protonate within the range used to study carbonic anhydrase, the pH-dependencies of binding are bell-shaped and completely analogous to sulfonamide binding (Figure 4*B*). At pH values much above the pK_a of the enzyme, the $Zn{-}OH^-$ species tends to be the one favored at equilibrium (perhaps because of stabilization of the hydroxide species by a hydrogen bond) although the high affinity anions like cyanide compete successfully up to a relatively high pH (Figure 4). In the case of HCN and H_2S, the binding mechanism must involve a proton transfer from the acid form of the anion to the solvent or to a group on the enzyme. The latter is analogous to the sulfonamides, because most are protonated except in the relatively high pH range.

It is generally agreed that the alkaline arm (sigmoid to low pH) of the pH-binding function for anions to carbonic anhydrase is related to pK_{enz}, compatible with the ligand competition with OH^- discussed above (31, 41, 45, 49, 51). For those anions protonated within the region of study, e.g. ^-SH and CN^-, the acid arm of the pH-binding function has then been related to the pK_a of the inhibitor (51) and the decrease in binding affinity at low pH is related to the favorable equilibrium for the formation of HI (equation 4). This disregards of course any pH-dependent change in protein conformation associated with the protonation of the enzyme group that

might alter the affinity of EH^+ for the anion. Anion binding may also involve some electrostatic and other binding forces at the bottom of the active site cavity other than the metal-anion coordination bond.

If one compares the intrinsic binding constants of the anions for the zinc site of carbonic anhydrase and the affinity of protons for the anions as reflected by the pK_a of IH, then the midpoints of all the acid arms of all the binding curves (this may well include sulfonamides) fall roughly in the range pH 6 to 8, even though their pK_a's and intrinsic binding constants K_I vary by orders of magnitude. This occurs because agents with the higher pK_{I_a} values generally have higher intrinsic binding constants for the zinc ion. While the total affinity of a sulfonamide for the enzyme is clearly modified by the diverse interactions of the side chain with the protein, it may be that the pH-stability of the complex is primarily determined by the affinity of the $^-SO_2NH^-$ group for the metal ion.

Similar ligand exchanges can be considered to apply to the sulfonamide binding curves pictured in Figure 4*A*, if sulfonamides are considered as simple anions binding the metal ion, as the sulfonamide group alone appears to be. This is clearly an oversimplification, but equilibria like those in equation 5 can be formulated and may represent a description of sulfonamide binding to a first approximation. The nitrogen of the sulfonamide group has been chosen as the coordinating nucleus.

$$\begin{array}{lcccl} (a) & CAZn \cdot H_2O & + & RSO_2NH_2 \rightleftharpoons CAZn \cdot {}^-NHSO_2R + H_2O + H^+ & \\ & pK_{a_{enz}} = 8 \;\Big\uparrow\Big\downarrow\; (-H^+) & & (-H^+) \;\Big\uparrow\Big\downarrow\; pK_{I_a} = 7.5\text{–}10.8 & 5. \\ (b) & CAZn \cdot {}^-OH & + & RSO_2NH^- \rightleftharpoons CAZn \cdot {}^-NHSO_2R + {}^-OH & \end{array}$$

As discussed above, the apparent pK_a of the acid arm of the sulfonamide pH-binding function has been related to the pK_{enz}, while the apparent pK_a of the alkaline arm has been assigned to the sulfonamide group. The opposite assignments for the apparent pK_a's of the pH-dependency of sulfonamide binding could be made by assuming that the acid arm represents the protonation of the inhibitor as discussed above for cyanide binding. This would be analogous to a metal-ligand formation curve in which the dissociation of the proton from a ligand group with a high pK_a is induced at much lower pH by complex formation with the metal. There is no compelling evidence that the major processes involved in the pH-dependency of binding of RSO_2NH_2 and RSO_2NH^- (as opposed to the absolute magnitude of the dissociation constant) are any different from those involved in the binding of HCN and CN^-. Significant movement or disappearance of the acid arm of the pH-dependency of binding as a function of sulfonamide pK_a would require the use of sulfonamides with a far greater range of pK_a values than presently available, e.g. sulfonamides with differences in pK_a comparable to HCN (9.3) and HCNO (3.5) or analogous to a strong acid like HBr or HCl.

Under the above interpretation the alkaline arm of the binding function would represent competition between RSO_2NH^- and ^-OH for the Zn(II) site, much as

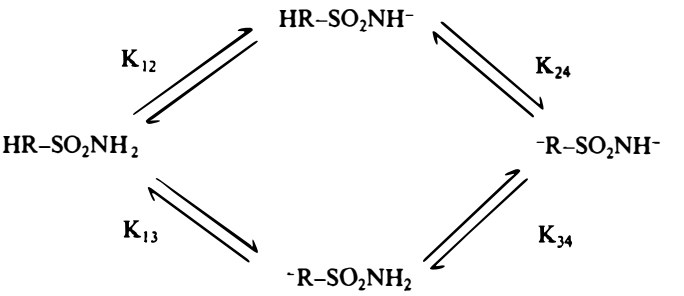
anions shift the apparent pK_a of the enzyme. The affinity of RSO_2NH^- for the Zn(II) ion may be expected to be roughly proportional to the pK_a and the alkaline arm would be expected to move up or down with the pK_a as observed (Figure 4). It may be that the sulfonamide binding mechanism involves the initial formation of a labile complex more or less independent of the ionization states of the sulfonamide and the metal-linked group on the enzyme. The rate-limiting step may then be the final formation of the $R–SO_2NH^-Zn(II)$ bond following a rapid redistribution of protons (11). If pK_{enz} is not the pK_a of a Zn-coordinated water molecule, but that of an adjacent protein side chain, then formulations of the mechanisms of anion and sulfonamide binding become more complex (41). Alternate hypotheses must account for the close coupling of this pK_a to the metal ion and its influence on anion and sulfonamide binding.

The sulfonamide group itself is expected to be a very poor ligand for the metal, certainly not with an intrinsic affinity constant over 10^2. On the other hand, the aromatic or heterocyclic side chain itself has little intrinsic affinity for the cavity as shown by its failure to bind to the apoenzyme or to metallocarbonic anhydrases other Zn(II) and Co(II) except with vastly reduced affinity (9). Yet the combination of the right metal at the active site and binding of the side chain to the pocket produces a dissociation constant for the complex with ethoxzolamide (II) of 10^{-9} *M*. Thus, there appears to be a "chelation" contribution to the binding energy produced by the dual binding to an inorganic metal-ligand site and the binding of the organic side chain to the protein. This may render simple interpretation of the pH-binding curves a little risky.

It should also be kept in mind that a number of sulfonamides contain additional ionizing groups on the side chain. Titration of acetazolamide, for example, yields two pK_a's, 7.2 and 8.8 (48). The two protons are derived from the nitrogen atoms of the sulfonamide and the acetylamido groups respectively. Thus the complete ionization of this sulfonamide is described by four microscopic ionization constants (equation 6). It cannot be assumed that $HR–SO_2NH_2$ and $^-R–SO_2NH_2$ or the corresponding pair with the sulfonamide group ionized have identical affinities for the enzyme. Lindskog (48) has analyzed this case in some detail, and while the ionization of the sulfonamide appears to be the major ionization involved in binding, effects of the ionization of the side chain appear to be present. The pH-dependence of binding of chloroacetazolamide (side chain $pK_a = 5.7$, sulfonamide $pK_a = 8.4$) is complex and shows that ionization of the side chain appears to decrease the binding affinity of the compound for the enzyme (48). The rapid loss of binding affinity of acetazolamide above pH 9 may also be partially contributed to by the ionization of the acetylamido group (Figure 3).

Interactions of Sulfonamides and Anions with Carboxymethylated Human Carbonic Anhydrase B

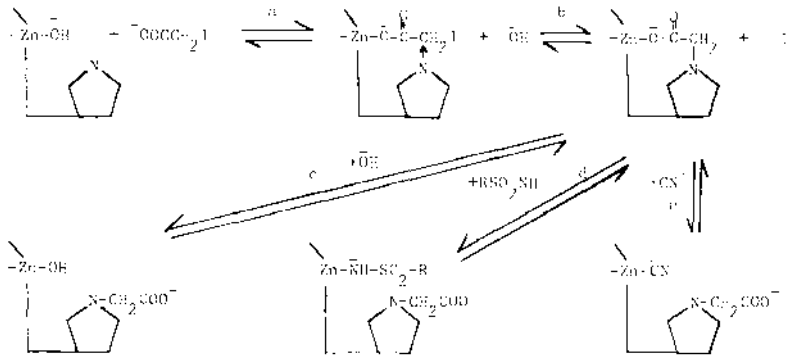
Reaction of the human B isozyme with iodo- or bromoacetate alkylates the 3'–N of a histidyl residue near the active center (57, 58). This residue was originally designated as 204 in the sequence, but revision of the sequence and fitting of the



6.

For acetazolamide: $pK_1 = 7.20$, $pK_2 = 8.80$, $pK_{12} = 7.46$, $pK_{13} = 7.55$; $pK_{24} = 8.54$, $pK_{34} = 8.45$; $K_1 = K_{12} + K_{13}$; $1/K_2 = 1/K_{24} + 1/K_{34}$.

polypeptide backbone to the electron density map of the B isozyme at high resolution has resulted in its renumbering as His–198. The high resolution structure of the B enzyme shows the 1'–N nitrogen of this residue to be located 5.6 Å from the zinc and 8.5 Å from the sulfur atom of the sulfonamide group in the Diamox complex (59). A histidyl residue at this site is apparently not essential for activity, because it is replaced by Thr–198 in the sequence of the C enzyme (see Figure 3). On the other hand His-63 mentioned previously is conserved in both the B and C structures, and is located 8.6 Å from the Zn(II) in the B enzyme (1.5 Å closer in the C enzyme) (59). It can be modified with bromopyruvate in the C isozyme with partial loss of activity, but cannot be modified in the B isozyme. Evidence previously reviewed (57, 58, 60) clearly suggests that bromo- or iodoacetate first bind to the anion binding site of the B isozyme by coordinating the metal ion via the carboxylate group (equation 7*a*). The carboxymethylation of the 3'–N of His–198 is then facilitated by this noncovalent intermediate (equation 7*b*). At neutral pH values, the activity (both CO_2 hydration and esterase activity) of the carboxymethylated enzyme is reduced to between 10 and 20% of the normal value; even so it is still a very active enzyme. As the pH is raised, the activity rises and the modified enzyme shows an apparent sigmoid pH-rate profile with a pK_a shifted to ~8.8 instead of ~7 as in the native enzyme (58). The pK_a describing the transition between the two spectral forms of the Co(II) enzyme is ~9.5 in the carboxymethylated enzyme rather than ~7 as observed in the normal Co(II) enzyme (50). Both the activity and spectral titrations are compatible with a competition between OH^- ions and an intramolecular carboxylate anion for the Zn(II) or Co(II) coordination site. (equation 7*c*). Generation of the active metal-hydroxide form of the enzyme thus requires a higher pH and the result is an apparent shift in the pK_a of the enzyme ionization. It has been stated that the spectra of the Co(II) enzyme show no evidence for substantial interaction between the carboxymethyl group and the metal (50). This is in fact not true, because the spectra of the acid form of the Co(II) enzyme, the acetate complex, and the bromoacetate or iodoacetate complexes (both noncovalent and covalent) are very similar, indicating similar coordination geometries (2, 60).



7.

Changes in the spectrum of the carboxymethylated derivative on removal of the excess reagent by dialysis reflect primarily the pH at which the dialysis is carried out which determines how much alkaline enzyme forms. Above pH 7, the spectra are a combination of the acetate-coordinated species and the normal alkaline form.

The interest of the carboxymethylated derivative in the present context concerns the effect of this modification on sulfonamide and anion binding. Complex formation with both sulfonamides and anions is not prevented by carboxymethylation. The visible absorption spectra of p-nitrobenzenesulfonamide complexes of the unmodified Co(II) enzyme B and the carboxymethylated Co(II) enzyme B are almost identical (50). Addition of CN^- to the carboxymethylated Co(II) enzyme also generates the same visible absorption spectrum as observed for the CN^- adduct of the unmodified enzyme except that full formation of the complex requires a higher concentration of CN^- (2, 60). Thus, the reactions indicated by equations 7*d* and 7*e* can take place, although the dissociation constants of the resulting complexes are significantly increased.

Affinities of both the anion and sulfonamides are reduced from 1 to 4 orders of magnitude by carboxymethylation (50, 51). There are large increases in the dissociation rate constants and a decrease of 2 orders of magnitude in k_a for a sulfonamide carrying a carboxylate substitution (51). More significantly the pH-dependency of binding for both the anion and the sulfonamides is shifted about 1.5 pH units to alkaline pH (50, 51). The lower apparent association rate and the shift of the binding curve to alkaline pH have been attributed to an alteration in the relative populations of the sulfonamide-combining and noncombining forms of the enzyme, i.e. protonated and nonprotonated enzyme, as reflected by the shift in the enzyme $pK_{a_{enz}}$ from ~7 to ~9.5. All these apparent shifts including the apparent shift in the enzyme pK_a can be explained by the ligand competition for the metal between incoming anion (sulfonamide), the intramolecular carboxylate anion, and solvent OH^- according to equation 7. While a shift in the intrinsic pK_a of the group on the enzyme involved in activity would suffice as a formal description of the alteration in binding, consideration of carboxymethylation as resulting in a set of competing ligand exchange reactions has different mechanistic implications.

CONCLUSIONS

Studies of the binding of sulfonamides to carbonic anhydrase provide a uniquely detailed prototype for the interactions of small molecules (drugs) with an enzymatically active macromolecule. The extensive studies of binding in solution as monitored by equilibrium and rapid-flow spectroscopic techniques coupled with the three-dimensional structures at high resolution for several crystalline carbonic anhydrase-sulfonamide complexes as determined by X-ray diffraction provide a particularly complete comparison between data obtained in solution and in a protein crystal. The bifunctional nature of the carbonic anhydrase-sulfonamide interaction involving both coordination to a metal ion at the active site and hydrophobic, hydrogen bonding, and van der Waals interactions with the protein indicate how the macromolecular environment can alter both kinetic and thermodynamic aspects of binding compared to binding reactions between small molecules. Particularly striking are: 1. changes in association rates rather than dissociation rates make the major contribution to changes in stability of the complex as a function of chemical structure of the sulfonamide; 2. the complex is stabilized by a large favorable enthalpy change associated with the binding reaction; and 3. an ordinarily weak metal-coordination bond is enormously stabilized by the cooperative interactions of the organic side chain with the macromolecule.

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