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Thermodynamic Parameters for the Association of Fluorinated Benzenesulfonamides with Bovine Carbonic Anhydrase II

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Abstract: This paper describes a calorimetric study of the association of a series of seven fluorinated benzenesulfonamide ligands ($C_6H_nF_{5-n}SO_2NH_2$) with bovine carbonic anhydrase II (BCA). Quantitative structure–activity relationships between the free energy, enthalpy, and entropy of binding and pK_a and $\log P$ of the ligands allowed the evaluation of the thermodynamic parameters in terms of the two independent effects of fluorination on the ligand: its electrostatic potential and its hydrophobicity. The parameters were partitioned to the three different structural interactions between the ligand and BCA: the Zn^{II} cofactor–sulfonamide bond (≈65% of the free energy of binding), the hydrogen bonds between the ligand and BCA (≈10%), and the contacts between the phenyl ring of the ligand and BCA (≈25%). Calorimetry revealed that all of the ligands studied bind in a 1:1 stoichiometry with BCA; this result was confirmed by ^{19}F NMR spectroscopy and X-ray crystallography (for complexes with human carbonic anhydrase II).

Keywords: calorimetry · fluorinated ligands · ligand design · rational drug design · structure–activity relationships

Introduction

The primary motivation of this paper was to understand the interaction of arylsulfonamides with carbonic anhydrase II (CA; E.C. 4.2.1.1). Arylsulfonamides have the highest affinity and are the most widely used inhibitors for CA.[1–4] The structure of CA bound to arylsulfonamides has been defined in detail with X-ray crystallography.[1,2,5] CA binds most arylsulfonamides with the same geometry: the nitrogen atom of the ionized sulfonamide, $ArSO_2NH^-$, binds to the Zn^{II} cofactor (free energy $\Delta G^i_{Zn^{2+}-N}$),[6] one sulfonamide oxygen atom and the sulfonamide NH group engage in hydrogen bonds with residues of the active site of CA (free energy $\Delta G^i_{H-bonds}$), and the aryl ring interacts directly with a hydrophobic pocket of the enzyme (free energy ΔG^i_{ring}) (Scheme 1). Although computational approaches have provided rough estimates of the free energies of these interactions,[7] experimental estimates of the free energies of these interactions are still not available.[1] Furthermore, previous experimental and computational studies have not partitioned the thermodynamic parameters of enthalpy and entropy to the structural interactions between arylsulfonamide and CA.[1,4]

The present study attempts to address these deficiencies in the literature by examining the thermodynamics of binding (free energy, enthalpy, and entropy) of fluorinated benzenesulfonamides (compounds of the form $C_6H_nF_{5-n}SO_2NH_2$) to bovine carbonic anhydrase II (BCA). These results can be used to partition the thermodynamics of binding to the structural interactions between ligand and CA because fluorination of the phenyl ring involves only small changes in size and shape of the ligands. This study thus presents a useful perturbational approach to understanding the binding of benzenesulfonamide itself. The disadvantage of such an approach is that the variations in both structures and thermodynamic parameters are small, the data are noisy, and the conclusions are neither highly accu-

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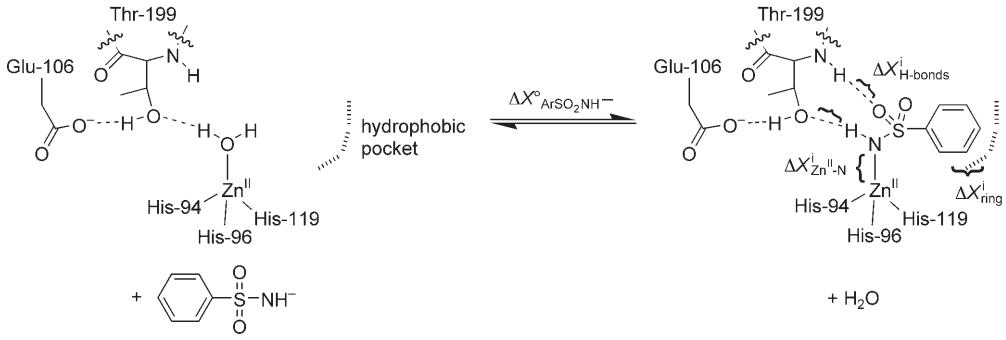


Scheme 1. Association of benzenesulfonamide ligands with carbonic anhydrase (CA). The diagram shows the equilibrium between the water-bound form of the enzyme (CA-Zn^{II}-OH_2^+) and the arylsulfonamide-bound form. We express the thermodynamic parameters (free energy: $X=G$, enthalpy: $X=H$, entropy: $X=S$) for the association of the arylsulfonamide anion with CA-Zn^{II}-OH_2^+ in terms of five contributions as follows: $\Delta X°_{ArSO_2NH^-} = \Delta X^i_{Zn^{2+}-N} + \Delta X^i_{H-bonds} + \Delta X^i_{ring} + \Delta X°_{trans+rot,ArSO_2NH^-} - \Delta X°_{trans+rot,H_2O}$. The terms with superscripts i indicate the intrinsic energies for structural interactions between ligand and CA (defined in the text) relative to the reference state of CA-Zn^{II}-OH_2^+; they are energies for the different interactions in the absence of losses in translational and rotational entropy.[6] $\Delta X°_{trans+rot,ArSO_2NH^-}$ and $\Delta X°_{trans+rot,H_2O}$ are the translational and rotational costs (primarily in entropy)[8,9] of complexation with CA of the arylsulfonamide anion and of water. We make the assumption that $\Delta X°_{trans+rot,ArSO_2NH^-} \approx \Delta X°_{trans+rot,H_2O}$ because of the weak (logarithmic) dependence of this primarily entropic term on molecular weight.[8] This assumption gives $\Delta X°_{ArSO_2NH^-} = \Delta X^i_{Zn^{2+}-N} + \Delta X^i_{H-bonds} + \Delta X^i_{ring}$.

rate nor amenable to reliable extrapolation to ligands with significantly different core structures.

A second motivation of this paper was to understand the use of fluorine in drug design. Fluorine has been used extensively in ligands that bind tightly to proteins or that limit metabolism in useful ways.[10,11] The physical properties of fluorine that have contributed to its use in medicinal chemistry include its slightly larger size compared to hydrogen (≈23% greater van der Waals radius of Bondi, ≈27% greater C–X covalent bond length, and ≈50% greater surface area for CX_3 groups), its high electronegativity (which significantly alters the inductive and electronic properties of fluorinated ligands relative to their nonfluorinated analogues), and its low polarizability.[11,12] Furthermore, fluorocarbons adopt significantly different conformations to hydrocarbons and are generally believed to be less polarizable and "more hydrophobic" than the analogous hydrocarbons.[11]

As an aryl subsitituent, fluorine significantly perturbs the electronic properties of the ring. For instance, the quadrupole moment of hexafluorobenzene is equal in magnitude but opposite in sign to that of benzene, and thus the interactions involving the face of hexafluorobenzene, which is partially positively charged, are different to those of benzene, which is partially negatively charged.[11,13] These myriad effects of fluorine make a clear understanding of the affinity of fluorinated ligands for proteins difficult, even more difficult than understanding the affinity of their nonfluorinated analogues (the affinity of nonfluorinated ligands for proteins is, to begin with, not well-understood).[14,15] Not being able to rationalize why fluorination increases the affinity of ligands makes the rational *design* of fluorinated ligands for target proteins particularly challenging.

A number of previous studies have examined the binding of fluorinated arylsulfonamide ligands to CA.[12,16–20] Gao et al. studied the binding of *para*-substituted benzenesulfonamides with linear hydrocarbon and fluorocarbon tails (p-$H_2NSO_2C_6H_4CONH(CX_2)_{n-1}CX_3$, where X = H or F and n = 1–6) to BCA.[12] As the length of the tails was increased, the values of the dissociation constant K_d^{obs} decreased by a factor of five for the hydrocarbon series and 15 for the fluorocarbon series. The contribution of hydrophobicity to affinity for BCA in the two series was the same when normalized to the molecular surface area (calculated by taking into account the larger size of fluorine compared to hydrogen) of the ligands. The affinities of the two series for BCA were not the same, however: the fluorocarbon ligands bound tighter (by ≈0.7 kcal mol^{-1}) than the analogous hydrocarbon ligands because of a hydrogen bond between the carboxamide of the ligand and residues of the active site of CA. This hydrogen bond should be stronger for the fluorocarbon than for the hydrocarbon ligands.

Kim et al. examined the binding of ligands of structure p-$H_2NSO_2C_6H_4CONHCH_2C_6H_nF_{5-n}$ (n = 0–5) to wild-type HCA II (HCA = human carbonic anhydrase) and to a

mutant of HCA II in which Phe-131 was mutated to Val.[16] Though the range in values of K_d^{obs} of these ligands for the two proteins was small (factor of <20), the investigators were able to construct a linear free-energy relationship ($R^2=0.83$) between affinity and the distinct electrostatic interactions between CA and the secondary (fluorinated) phenyl ring of the ligands. They determined that no single interaction dominated affinity for all of the ligands in the series, and suggested that multipole–multipole interactions contributed differently to affinity for different ligands. Their study emphasized that the effect of fluorination of the ligand on its affinity for CA is quite complicated, and that a number of effects compensate.

Supuran and co-workers studied the activity of arylsulfonamide ligands appended with fluoroalkyl tails or fluoroaryl rings.[17] Several of these ligands had high affinity (order of nM) towards HCA II and bovine carbonic anhydrase IV (a membrane-bound, medically relevant isozyme of CA),[1,2,4] moderate water solubility, and high activity in a rabbit model of glaucoma. These results suggest that fluorinated sulfonamides could be effective as therapeutic inhibitors of CA, but do not reveal the theoretical basis for this activity.

Gerig and co-workers studied the solution-phase binding of fluorinated benzenesulfonamides to HCA I and II by using ^{19}F NMR spectroscopy.[18–20] Their results suggested a binding stoichiometry of 2:1 for 2-fluorobenzenesulfonamide (2-FBS), 3-fluorobenzenesulfonamide (3-FBS), and 4-fluorobenzenesulfonamide (4-FBS) to wild-type HCA I and II,[19,20] and were in stark contrast to the 1:1 stoichiometry generally observed for the binding of all other arylsulfonamides to wild-type CA. Reconciling this conflict was a third motivation for our work. Accordingly, we observed 1:1 binding for all the fluorinated benzenesulfonamides (including 4-FBS) in this study by using biophysical and X-ray crystallographic techniques (Figure 1).

Experimental Design

The system of BCA and substituted benzenesulfonamides is the simplest one that we know for studying protein–ligand interactions; this system serves as a model to study the binding of structurally related ligands to a structurally well-defined protein.[1,4,5,21] BCA binds most substituted benzenesulfonamides with the same orientation. This conserved mode of binding has allowed the attribution of binding energies to particular interactions between the ligand and protein and, thus, to structural components of the ligand (e.g., a *para* substituent on the phenyl ring),[12,23,24] and has enabled the physical organic study of the affinity of arylsulfonamide ligands for CA.[1]

In the work described herein, fluorinated benzenesulfonamides constitute a series of simple ligands in which the contribution of fluorine to the electrostatic potential and to the hydrophobicity of the ligand, and the manifestations of these effects on affinity for BCA, can be easily separated. The conserved orientation of substituted benzenesulfonamides complexed with HCA II allows the attribution of differences in the thermodynamics of association of the various fluorinated benzenesulfonamides with BCA to differences in the strengths of conserved interactions between BCA and ligand (Scheme 1), and not to new, structurally different interactions in each case.

We used isothermal titration calorimetry (ITC) to follow the binding of the ligands to BCA because it allows the direct measurement of K_d^{obs}, the enthalpy of binding ($\Delta H°$), and the stoichiometry of binding from a single experiment, and the entropy of binding ($\Delta S°$) through the thermodynamic relation $\Delta G° = \Delta H° - T\Delta S°$.[24,25] Our results demonstrate that about 65% (≈ -8 kcal mol^{-1}) of the free energy of binding is contributed by the Zn^{II}–N bond, about 10% (≈ -1 kcal mol^{-1}) by the hydrogen-bond network, and about 25% (≈ -3.5 kcal mol^{-1}) by hydrophobic contacts between the phenyl ring and CA.

Given the small range in the thermodynamics of association (values of K_d vary by less than a factor of 10 across the series, and $\Delta H°$ and $T\Delta S°$ by 2.5–3 kcal mol^{-1}), we cannot generalize the quantitative aspects of our conclusions to the binding of arylsulfonamides more structurally complex than fluorinated benzenesulfonamides to CA. We believe, however, that our conclusions are generally qualitatively applicable.

Thermodynamic Framework and Background

The variation of K_d^{obs} with pH for all CA–sulfonamide complexes for which these data are reported gives a bell-shaped curve bound by two values of pK_a: the pK_a value for the acidic limb (≈ 6.9) is associated with an ionizable group of the enzyme (most likely Zn^{II}-bound water: $Zn^{II}–OH_2^+ \rightleftharpoons Zn^{II}–OH + H^+$), and the p$K_a$ value for the basic limb is equal to that of the sulfonamide.[1,26,27]

Although an exceptionally simple process in principle, the mechanism of interaction of CA with sulfonamides is still a matter of debate: both displacement of the Zn^{II}-bound water by the sulfonamide anion [Eq. (1)] and displacement of the Zn^{II}-bound hydroxide by the neutral sulfonamide [Eq. (2)] are consistent with the experimental data.

$$ArSO_2NH^- + CA\text{-}Zn^{II}\text{-}OH_2^+ \rightleftharpoons ArSO_2NH\text{-}Zn^{II}\text{-}CA + H_2O \quad (1)$$

$$ArSO_2NH_2 + CA\text{-}Zn^{II}\text{-}OH \rightleftharpoons ArSO_2NH\text{-}Zn^{II}\text{-}CA + H_2O \quad (2)$$

Although King and co-workers suggested that the mechanism involves three states (with an intermediate state that is weakly populated and has only hydrophobic contacts between CA and the ligand),[27,28] the details of the mechanism do not concern us here because we are only interested in the equilibrium between the separated reactants and the end-product complex, and because no intermediate in the pathway has been detected experimentally.[28] We only want to remove the dependence of the equilibrium thermodynam-

ic parameters on the fractions of arylsulfonamide and CA that are present in their active, charged forms (see next section).

We adopted the interaction of the sulfonamide anion ($ArSO_2NH^-$) with the Zn^{II}-bound-water form of CA (CA-Zn^{II}-OH_2^+) ([Eq. (1)], Scheme 1) as the standard reaction for the interpretation of thermodynamics because we believe that this is the most likely mechanism.[1]

Results and Discussion

X-ray Crystal Structures of Complexes of Fluorinated Benzenesulfonamides with HCA II

The X-ray crystal structures of HCA II complexed with 4-FBS, 2,6-difluorobenzenesulfonamide (2,6-FBS), and 3,5-difluorobenzenesulfonamide (3,5-FBS) demonstrate an invariant orientation of the sulfonamide group and phenyl ring for these ligands (Figure 1a). Table S1 shows the data collection and refinement statistics for these structures. Electron-density maps of each CA–ligand complex conclusively demonstrate a ligand to CA stoichiometry of 1:1 (Figure 1b–d). The ionized sulfonamide NH group of each ligand coordinates to the active-site Zn^{II} cofactor and donates a hydrogen bond to Thr-199, as in the structures of other substituted benzenesulfonamides complexed with HCA II.[1,2,5] Given that the active-site architectures of HCA II and BCA are identical, we can interpret the differences in the thermodynamics of association of the fluorinated benzenesulfonamides with BCA in terms of differences in the strengths of the same structural interactions between CA and ligand (Scheme 1).

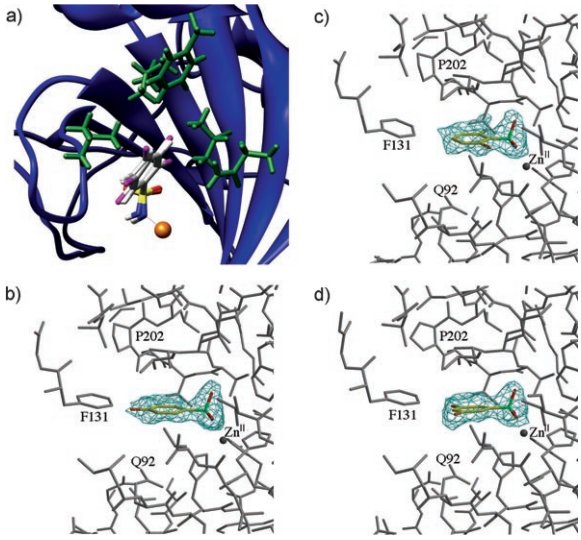
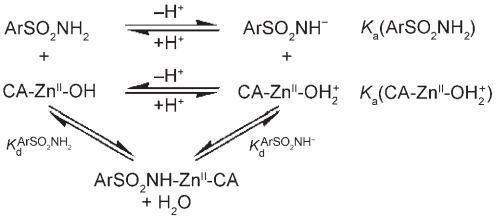


Figure 1. a) Overlay of binding of 4-FBS, 2,6-FBS, and 3,5-FBS to HCA II. The ligands are rendered as ball-and-stick models with the fluorine atoms in magenta; the phenyl rings of the ligands bind with an invariant orientation (they are coplanar). HCA II is depicted as a blue ribbon with the hydrophobic residues that are within van der Waals contact of the ligands displayed in green (Gln-92, Val-121, Phe-131, Leu-141, and Leu-198). The protein backbone and Zn^{II} cofactor (orange sphere) from only one structure are shown to facilitate visualization (the heavy-atom root-mean-square deviation of the side chains and backbone atoms of the protein in the different structures was <0.61 Å). b)–d) Difference electron-density maps (contoured at 2.2σ) of HCA II complexed with b) 4-FBS, c) 2,6-FBS, and d) 3,5-FBS, calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from each final model minus the ligand and active-site solvent molecules. This figure was prepared with Bobscript and Raster3D.[22]

Calculation of Thermodynamic Parameters for the Standard Reaction of the Binding of Sulfonamide Anions to CA-Zn^{II}-OH_2^+

We used Scheme 2 to calculate the thermodynamic parameters for the reaction in Equation (1). Equation (3) shows the



Scheme 2. Equilibria for the association of arylsulfonamide ligands ($ArSO_2NH_2$/$ArSO_2NH^-$) with carbonic anhydrase (CA-Zn^{II}-OH_2^+/CA-Zn^{II}-OH).

fractions (θ) of $ArSO_2NH^-$ and CA-Zn^{II}-OH_2^+:

$$\theta_{ArSO_2NH^-} = \left(1 + 10^{pK_a(ArSO_2NH_2)-pH}\right)^{-1} \quad (3a)$$

$$\theta_{CA-Zn^{II}-OH_2^+} = \left(1 + 10^{pH-pK_a(CA-Zn^{II}-OH_2^+)}\right)^{-1} \quad (3b)$$

where $pK_a(ArSO_2NH_2)$ is the acid dissociation constant for the neutral sulfonamide (hereon referred to as pK_a), pK_a(CA-Zn^{II}-OH_2^+) is the acid dissociation constant of the Zn^{II}-bound water, and pH is the pH of the solution.

Equations (4) and (5), derived in the Supporting Information, give the dissociation constant ($K_d^{ArSO_2NH^-}$) and enthalpy ($\Delta H^\circ_{ArSO_2NH^-}$) for the association of $ArSO_2NH^-$ with CA-Zn^{II}-OH_2^+ (Schemes 1 and 2):

$$K_d^{ArSO_2NH^-} = K_d^{obs}\theta_{ArSO_2NH^-}\theta_{CA-Zn^{II}-OH_2^+} \quad (4)$$

$$\begin{aligned}\Delta H^\circ_{ArSO_2NH^-} &= \Delta H^\circ_{obs} + (1 - \theta_{CA-Zn^{II}-OH_2^+}) \\ &\times(\Delta H^\circ_{ion,CA-Zn^{II}-OH_2^+} - \Delta H^\circ_{ion,buffer}) \\ &+(1 - \theta_{ArSO_2NH^-})(\Delta H^\circ_{ion,buffer} - \Delta H^\circ_{ion,ArSO_2NH_2})\end{aligned} \quad (5)$$

where K_d^{obs} is the experimentally observed dissociation constant of the CA–sulfonamide complex, ΔH°_{obs} is the experimentally observed enthalpy of binding of sulfonamide to CA, $\Delta H^\circ_{ion,ArSO_2NH_2}$ is the enthalpy of ionization of neutral sulfonamide in solution, $\Delta H^\circ_{ion,CA-Zn^{II}-OH_2^+}$ is the enthalpy of ionization of CA-Zn^{II}-OH_2^+ in solution, and $\Delta H^\circ_{ion,buffer}$ is the enthalpy of ionization in solution of the buffer ($H_2PO_4^-$;

$\Delta H^{\circ}_{ion,buffer} = 0.86$ kcal mol^{-1}).[29] Khalifah et al. undertook a similar analysis in their examination of the association of nitrogen heterocycles with HCA I.[30]

By titrating the benzenesulfonamides with sodium hydroxide, we measured values of pK_a by following the pH, and values of $\Delta H^{\circ}_{ion,ArSO_2NH_2}$ by measuring the heat released with ITC (Table 1; see Experimental Section). These procedures generated values in good agreement with those in the literature. Table 1 also lists values of pK_a and enthalpy of ionization for CA-Zn^{II}-OH$_2^+$ that were reported in the literature.[1,31,32]

Table 1. Physical properties of fluorinated benzenesulfonamides and the Zn^{II}-bound-water form of BCA (CA-Zn^{II}-OH$_2^+$).

Titrated molecule	pK_a	ΔH°_{ion} [kcal mol^{-1}]	P[a]	Log P[a]
BS	10.1[b,c]	9.10 ± 0.01[d,e]	1.94 ± 0.03[f]	0.29 ± 0.01
2-FBS	9.6[b]	7.89 ± 0.03[d]	1.82 ± 0.04	0.26 ± 0.01
3-FBS	9.7[b]	8.47 ± 0.06[d]	4.06 ± 0.12	0.61 ± 0.01
4-FBS	10.0[b]	8.58 ± 0.03[d]	3.42 ± 0.14	0.53 ± 0.02
2,6-FBS	9.1[b]	7.81 ± 0.14[d]	1.45 ± 0.04	0.16 ± 0.01
3,5-FBS	9.4[b]	8.58 ± 0.04[d]	9.0 ± 0.3	0.95 ± 0.02
pentaFBS	8.2[b,g]	7.60 ± 0.03[d]	11.1 ± 0.2	1.05 ± 0.01
CA-Zn^{II}-OH$_2^+$	6.9[h]	6.90[i]	–	–

[a] Partition coefficient between octanol and sodium phosphate buffer (pH 7.5) determined spectrophotometrically.[12] Uncertainties are the maximum deviation of a single measurement from the average of three independent measurements. [b] Determined potentiometrically (ionic strength $I = 0.052$ M with sodium chloride). Uncertainties from this procedure are estimated to be 0.2 pH units.[12] [c] Literature value of 10.1.[28] [d] Estimated from ITC by titration of sulfonamide with sodium hydroxide at pH 9.6 (near the pK_a), $I = 0.052$ M with sodium chloride. Uncertainties represent the standard deviation of the mean from seven to nine injections. [e] Literature value of 8.3 kcal mol^{-1}.[33] [f] Literature value of 1.63.[28] [g] Literature value of 8.05.[34] [h] Estimated from the pH dependence of BCA-catalyzed hydration of carbon dioxide.[31] We estimated an uncertainty of 0.05 pH units from other reported values for this parameter.[1] [i] Estimated from the temperature dependence of the pK_a of BCA-catalyzed hydrolysis of *p*-nitrophenylacetate.[32]

Table 2 lists the thermodynamic parameters that we observed experimentally (K_d^{obs}, ΔH°_{obs}, and $-T\Delta S^{\circ}_{obs}$), as well as those that we calculated for the binding of $ArSO_2NH^-$ to CA-Zn^{II}-OH$_2^+$ ($K_d^{ArSO_2NH^-}$, $\Delta H^{\circ}_{ArSO_2NH^-}$, and $-T\Delta S^{\circ}_{ArSO_2NH^-}$). In our analysis, we focused on the calculated thermodynamic parameters (for $ArSO_2NH^-$) because they are independent of the fractions of arylsulfonamide and CA in the active forms [Eq. (1)], and thus allow us to ascribe differences in the thermodynamics of binding of the ligands to structural interactions between the ligands and CA.

Quantitative Structure–Activity Relationships to Determine the Importance of Lewis Basicity and Hydrophobicity of the Fluorinated Benzenesulfonamide Anion

Although the ranges in values of the thermodynamic parameters are small ($\Delta G^{\circ}_{ArSO_2NH^-}$ varies by 1.3 kcal mol^{-1}, $\Delta H^{\circ}_{ArSO_2NH^-}$ by 2.8 kcal mol^{-1}, and $-T\Delta S^{\circ}_{ArSO_2NH^-}$ by 2.3 kcal mol^{-1}; Table 2), we believe that the application of a quantitative structure–activity relationship (QSAR) to the data clarifies the importance of the different structural interactions between these ligands and BCA. The narrow ranges do not, however, allow us to generalize our results to the association of structurally complex arylsulfonamides with CA. Similar QSAR analyses were reported by other investigators, but the thermodynamic parameters for the association of the arylsulfonamide anion, $ArSO_2NH^-$, with CA-Zn^{II}-OH$_2^+$ were not examined.[4,35]

As discussed in the Introduction, the important interactions between $ArSO_2NH^-$ and CA are 1) the bond between the arylsulfonamide nitrogen atom and the Zn^{II} cofactor, 2) the hydrogen bonds between the SO_2NH group and residues of the active site, and 3) the contacts between the aryl ring of the arylsulfonamide and the hydrophobic pocket of CA (Scheme 1 and Figure 1). The first two of these interactions should be influenced by the Lewis basicity of $ArSO_2NH^-$, and thus by the pK_a of $ArSO_2NH_2$ (Brønsted relationship assumed). The third interaction should be influenced by the hydrophobicity of the ligand, estimated by the logarithm of the partition coefficient (log P) of the ligand between octanol and sodium phosphate buffer (pH 7.5). We estimated values of log P by UV/Vis spectroscopy (Table 1) as previously described.[12] We determined that pK_a and log P do not correlate with one another ($R^2 = 0.28$).

We constructed QSARs between $\Delta G^{\circ}_{ArSO_2NH^-}$ ($K_d^{ArSO_2NH^-}$), $\Delta H^{\circ}_{ArSO_2NH^-}$, or $-T\Delta S^{\circ}_{ArSO_2NH^-}$ and pK_a and log P of the benzenesulfonamide ligands to determine the relative importance of electrostatic (pK_a-dependent) and hydrophobic (log P-dependent) interactions to the free energy, enthalpy, and entropy of binding (Figure S1). The fits that we obtained were only modest for all three thermodynamic parameters ($R^2 = 0.46$–0.63). To account for the possibility that the set

Table 2. Thermodynamic parameters for the observed binding of fluorinated benzenesulfonamides to BCA, and for the binding of $ArSO_2NH^-$ to CA-Zn^{II}-OH$_2^+$ (calculated with Equations (3) and (4)).

Ligand	K_d^{obs}[a] [nM]	ΔH°_{obs}[a] [kcal mol^{-1}]	$-T\Delta S^{\circ}_{obs}$[b] [kcal mol^{-1}]	$K_d^{ArSO_2NH^-}$[c] [nM]	$\Delta H^{\circ}_{ArSO_2NH^-}$[d] [kcal mol^{-1}]	$-T\Delta S^{\circ}_{ArSO_2NH^-}$[e] [kcal mol^{-1}]
BS	730 ± 60	−9.0 ± 0.5	+0.7 ± 0.6	0.34 ± 0.18	−12.3 ± 0.6	−0.6 ± 0.6
2-FBS	230 ± 20	−10.2 ± 0.2	+1.1 ± 0.2	0.39 ± 0.18	−12.2 ± 0.2	−0.6 ± 0.4
3-FBS	75 ± 8	−9.0 ± 0.2	−0.7 ± 0.2	0.11 ± 0.05	−11.6 ± 0.2	−2.0 ± 0.4
4-FBS	590 ± 40	−7.8 ± 0.5	−0.7 ± 0.5	0.4 ± 0.2	−10.6 ± 0.5	−2.2 ± 0.6
2,6-FBS	190 ± 14	−9.4 ± 0.4	+0.2 ± 0.4	1.0 ± 0.5	−11.2 ± 0.5	−1.1 ± 0.6
3,5-FBS	57 ± 12	−9.6 ± 0.3	−0.3 ± 0.3	0.16 ± 0.08	−12.3 ± 0.3	−1.1 ± 0.5
pentaFBS	25 ± 4	−8.9 ± 0.1	−1.4 ± 0.1	0.8 ± 0.4	−9.5 ± 0.5	−2.9 ± 0.5

[a] Uncertainties are the maximum deviation of one measurement from the average of three independent measurements. [b] Uncertainties were estimated by propagating errors in K_d^{obs} and ΔH°_{obs}. [c] Calculated with [Eq. (3)]. Uncertainties were estimated by propagating the errors in all of the parameters in [Eq. (3)] assuming that they were independent. [d] Calculated with [Eq. (4)]. Uncertainties were estimated by propagating the errors in all of the parameters in [Eq. (4)] (except for $\Delta H^{\circ}_{ion,buffer}$) assuming that they were independent. [e] Uncertainties were estimated by propagating errors in $K_d^{ArSO_2NH^-}$ and $\Delta H^{\circ}_{ArSO_2NH^-}$.

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of ligands did not interact in a constant way with CA, and that this inhomogeneity resulted in the poor fits, we also constructed QSARs in which we omitted one of the ligands from the analysis (considered as an outlier). The best fits that we obtained for $\Delta G^{\circ}_{ArSO_2NH^-}$ ($K_d^{ArSO_2NH^-}$) and $\Delta H^{\circ}_{ArSO_2NH^-}$ involved the omission of 4-FBS ($R^2=0.83$ for both; Figure 2a and b). The best fit for $-T\Delta S^{\circ}_{ArSO_2NH^-}$ involved the omission of 3,5-FBS ($R^2=0.83$; data not shown), but omitting 4-FBS also gave a reasonable QSAR to the $-T\Delta S^{\circ}_{ArSO_2NH^-}$ data ($R^2=0.70$; Figure 2c). As a simplifying approximation, we assumed that 4-FBS interacts with CA in a way that is different from that of the other ligands, and consider it in a separate section below.

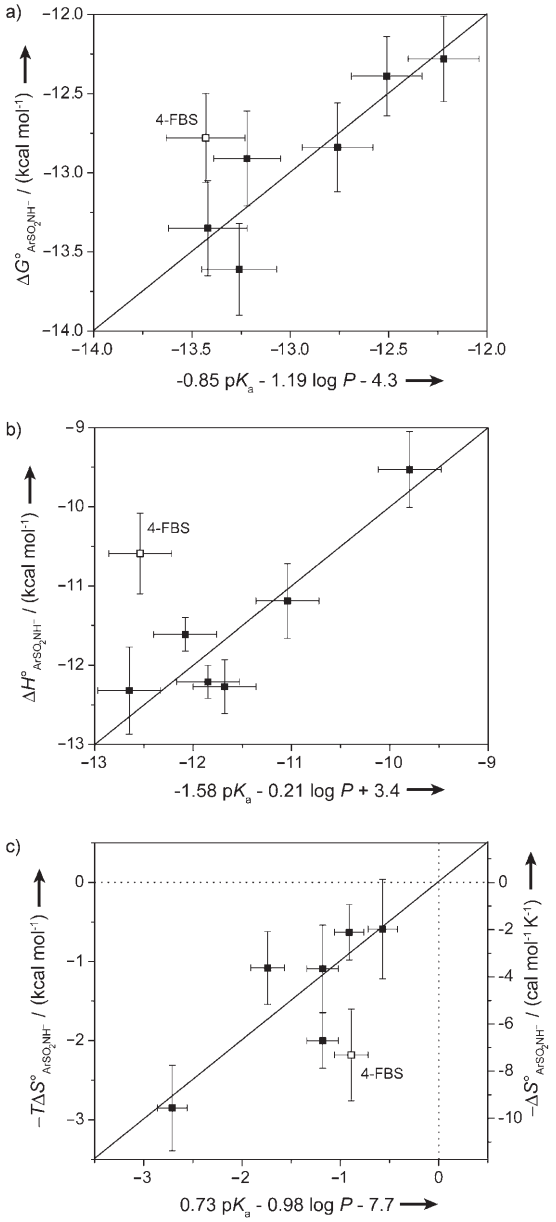


Figure 2. QSARs between a) $\Delta G^{\circ}_{ArSO_2NH^-}$, b) $\Delta H^{\circ}_{ArSO_2NH^-}$, and c) $-T\Delta S^{\circ}_{ArSO_2NH^-}$ and pK_a and log$P$ for fluorinated benzenesulfonamides. QSARs are shown in which the data for 4-FBS (open squares) was omitted; QSARs to the data for all the ligands are shown in Figure S1. The y error bars are uncertainties described in Table 2, and the x error bars were obtained by propagating uncertainties in pK_a and log$P$. The horizontal and vertical dotted lines in c) separate favorable ($-T\Delta S^{\circ}<0$) from unfavorable ($-T\Delta S^{\circ}>0$) entropy of binding.

Equations (6)–(8) give the QSARs obtained when 4-FBS was omitted from the optimization. We discuss each of the equations and their implications on the nature of the interactions between the benzenesulfonamide ligands and CA in turn in the following sections.

$$\Delta G^{\circ}_{ArSO_2NH^-} = -0.85(\pm0.23)pK_a - 1.19(\pm0.40)\log P - 4.3(\pm2.3) \quad (6)$$

$$\Delta H^{\circ}_{ArSO_2NH^-} = -1.58(\pm0.47)pK_a - 0.21(\pm0.82)\log P + 3.4(\pm4.7) \quad (7)$$

$$-T\Delta S^{\circ}_{ArSO_2NH^-} = 0.73(\pm0.53)pK_a - 0.98(\pm0.92)\log P - 7.7(\pm5.3) \quad (8)$$

Although the uncertainties in the parameters are relatively large, we believe that we can cautiously use the QSARs to partition the free energy, enthalpy, and entropy to the structural interactions between CA and ligand in a semi-quantitative way.

Electrostatic Effects of the Benzenesulfonamide Ligands (Zn^{II}–N Bond and Hydrogen-Bond Network) Primarily Influence the Enthalpy of Binding

Equation (7) shows that $\Delta H^{\circ}_{ArSO_2NH^-}$ is more sensitive to changes in pK_a (electrostatic effects) than to the same changes in log$P$ (hydrophobic effects). The weak influence of log$P$ is consistent with the widely held belief that hydrophobic interactions in protein–ligand binding are manifested as the "hydrophobic effect" and appear primarily in the entropy of binding with an enthalpy of binding near zero at 298 K.[36]

The equilibrium shown in Scheme 1 allows the partitioning of $\Delta H^{\circ}_{ArSO_2NH^-}$ into the structural, component interactions between $ArSO_2NH^-$ and CA [Eq. (9)].

$$\Delta H^{\circ}_{ArSO_2NH^-} = \Delta H^{i}_{Zn^{II}-N} + \Delta H^{i}_{H-bonds} + \Delta H^{i}_{ring} \quad (9)$$

The first two terms ($\Delta H^{i}_{Zn^{II}-N}$ and $\Delta H^{i}_{H-bonds}$) are expected to depend only on pK_a, and the third term (ΔH^{i}_{ring}), which represents van der Waals contacts (induced dipole–induced dipole forces) between the phenyl ring and active-site residues of CA, on log$P$ as in Equation (10).

$$\Delta H^{i}_{Zn^{II}-N} + \Delta H^{i}_{H-bonds} = ApK_a + c_1 \quad (10a)$$

$$\Delta H^{i}_{ring} = B\log P + c_2 \quad (10b)$$

We disregarded possible interactions between polar and charged residues of the active site of CA and the multipoles

(quadrupole and dipole) of the fluorinated rings of the ligands because X-ray crystal structures revealed that there are no such amino acid residues of CA in the vicinity of the ring (Figure 1).[37] There is, of course, the possibility of contacts between nonpolar residues of the active site of CA and the multipoles of the rings of the ligands. We cannot take such contacts into account without overparameterizing the QSAR because of the small number of ligands studied. Moreover, Kim et al. previously estimated that contacts between the dipole of the fluorinated phenyl rings of sulfonamide ligands and nonpolar residues of CA contributed $<0.4\,\mathrm{kcal\,mol^{-1}}$ to the free energy of binding.[16] Thus, our approach of neglecting these multipole–induced dipole contacts, though certainly not ideal, is defensible as a first-order approximation.

To solve for the constant c_2 in Equation (10b), we assumed that the strength of van der Waals contacts between the phenyl ring and CA disappears when the ring is not present (e.g., for the molecule HSO_2NH_2, for which $c\log P = -2.21$, $\Delta H^{i}_{ring} = 0 = -2.21B + c_2$). We solved for c_1 by subtracting this value for c_2 from the constant in Equation (7).

Equation (11) gives the contributions from electrostatic interactions (Zn^{II}–N bond and hydrogen-bond network [Eq. (11a)]) and from hydrophobic interactions [Eq. (11b)] to the enthalpy of association (by combining Equations (7) and (10)).

$$\Delta H^{i}_{Zn^{II}-N} + \Delta H^{i}_{H-bonds} = -1.58(\pm 0.47)pK_a + 3.9(\pm 5.0) \quad (11a)$$

$$\Delta H^{i}_{ring} = -0.21(\pm 0.82)\log P - 0.5(\pm 1.8) \quad (11b)$$

Table 3 lists the values, calculated by using Equation (11), of these contributions. The Zn^{II}–N bond and hydrogen-bond network contribute very favorably ($\approx -11\,\mathrm{kcal\,mol^{-1}}$) to the enthalpy of association, whereas the phenyl ring makes a very small ($\approx -1\,\mathrm{kcal\,mol^{-1}}$) favorable contribution (presumably due to van der Waals contacts of the phenyl ring).

Electrostatic and Hydrophobic Interactions Both Influence the Entropy of Binding

Increasing the Brønsted basicity of $ArSO_2NH^-$ (i.e., pK_a of $ArSO_2NH_2$) increases $-T\Delta S^{\circ}_{ArSO_2NH^-}$ [Eq. (8)] and decreases $\Delta H^{\circ}_{ArSO_2NH^-}$ [Eq. (7)]. The observation that increasing the enthalpy of the pK_a-dependent structural, component interactions (Zn^{II}–N bond and hydrogen-bond network; Scheme 1) lowers the entropy of these interactions is compatible with the phenomenon of enthalpy/entropy compensation.[38,39] In the physical model for enthalpy/entropy compensation, more-exothermic binding occurs with a less-favorable entropy than less-exothermic binding because of the lower mobility at the protein–ligand interface for the more-exothermic binding event.[38]

Equation (8) shows that increasing the hydrophobicity (i.e., $\log P$) of the benzenesulfonamide ligand decreases $-T\Delta S^{\circ}_{ArSO_2NH^-}$. This observation is compatible with the widely held belief that the hydrophobic effect is primarily entropic when $T \approx 298$ K.[36]

We solved for the component (intrinsic) entropies of interaction [Eq. (12)] as we did above for the component (intrinsic) enthalpies [Eq. (11)] and Scheme 1).

$$-T\Delta S^{i}_{Zn^{II}-N} - T\Delta S^{i}_{H-bonds} = 0.73(\pm 0.53)pK_a - 5.5(\pm 5.7) \quad (12a)$$

$$-T\Delta S^{i}_{ring} = -0.98(\pm 0.92)\log P - 2.2(\pm 2.0) \quad (12b)$$

Table 3 shows the calculated results for the component entropies of association. Electrostatic contacts (Zn^{II}–N bond and hydrogen-bond network) make small, unfavorable contributions to $-T\Delta S^{\circ}_{ArSO_2NH^-}$ ($\approx$1–2 $\mathrm{kcal\,mol^{-1}}$). That these interactions are entropically unfavorable is compatible with the phenomenon of enthalpy/entropy compensation (see above). In line with our intuition, the phenyl ring makes a favorable (≈ -3 to $-2\,\mathrm{kcal\,mol^{-1}}$) contribution to $-T\Delta S^{\circ}_{ArSO_2NH^-}$, presumably due to solvent release as the basis of the hydrophobic effect.[36]

Table 3. Free energies, enthalpies, and entropies ($\mathrm{kcal\,mol^{-1}}$) for the different structural interactions between the fluorinated benzenesulfonamide anion and CA-Zn^{II}-OH_2^+ (Scheme 1).

Ligand	Calculated $\Delta G^{\circ}_{ArSO_2NH^-}$ [a]	ΔG^{i} Zn^{II}-N bond[a,b]	ΔG^{i} H-bond network[a,c]	ΔG^{i} Ring contacts[a,d]	Calculated $\Delta H^{\circ}_{ArSO_2NH^-}$ [a]	ΔH^{i} ES[a,e]	ΔH^{i} Ring contacts[a,f]	Calculated $-T\Delta S^{\circ}_{ArSO_2NH^-}$ [a]	$-T\Delta S^{i}$ ES[a,g]	$-T\Delta S^{i}$ Ring contacts[a,h]
BS	-13 ± 3	-9 ± 3	-2 ± 3	-2.9 ± 0.9	-12 ± 7	-12 ± 7	-1 ± 2	-1 ± 8	2 ± 8	-2 ± 2
2-FBS	-13 ± 3	-8 ± 3	-1 ± 3	-2.9 ± 0.9	-12 ± 7	-11 ± 7	-1 ± 2	-1 ± 7	1 ± 8	-2 ± 2
3-FBS	-13 ± 3	-8 ± 3	-1 ± 3	-3.3 ± 0.9	-12 ± 7	-11 ± 7	-1 ± 2	-1 ± 7	2 ± 8	-3 ± 2
4-FBS	-13 ± 3	-8 ± 3	-1 ± 3	-3.2 ± 0.9	-12 ± 7	-12 ± 7	-1 ± 2	-1 ± 7	2 ± 8	-3 ± 2
2,6-FBS	-12 ± 3	-8 ± 3	-1 ± 3	-2.8 ± 0.9	-11 ± 6	-10 ± 7	0 ± 2	-1 ± 7	1 ± 7	-2 ± 2
3,5-FBS	-13 ± 3	-8 ± 3	-1 ± 3	-3.7 ± 1.0	-12 ± 6	-11 ± 7	-1 ± 2	-2 ± 7	1 ± 8	-3 ± 2
pentaFBS	-12 ± 3	-8 ± 3	-1 ± 3	-3.9 ± 1.0	-10 ± 6	-9 ± 6	-1 ± 2	-3 ± 7	0 ± 7	-3 ± 2

[a] Uncertainties were estimated by propagating uncertainties in all of the parameters in the appropriate equation (assuming that these uncertainties were independent). [b] From [Eq. (14a)]. [c] From [Eq. (14b)]. [d] From [Eq. (13b)]. [e] Electrostatic contacts: Zn^{II}–N bond and hydrogen-bond network from [Eq. (11a)]. [f] From [Eq. (11b)]. [g] Electrostatic contacts: Zn^{II}–N bond and hydrogen-bond network from [Eq. (12a)]. [h] From [Eq. (12b)].

Partitioning the Free Energy of Binding Into the Different Structural Interactions between Fluorinated Benzenesulfonamide Anions and CA-Zn^{II}-OH_2^+

Equation (13) shows the partitioning of $\Delta G^{\circ}_{ArSO_2NH^-}$ into the structural, component interactions between $ArSO_2NH^-$ and CA using an analysis similar to that used to partition $\Delta H^{\circ}_{ArSO_2NH^-}$ and $-T\Delta S^{\circ}_{ArSO_2NH^-}$ above.

$$\Delta G^{i}_{Zn^{II}-N} + \Delta G^{i}_{H-bonds} = -0.84(\pm 0.23)pK_a - 1.7(\pm 2.1) \quad (13a)$$

$$\Delta G^{i}_{ring} = -1.2(\pm 0.4)\log P - 2.6(\pm 0.9) \quad (13b)$$

Koike et al. reported a small-molecule model of the active site of CA that consists of a macrocyclic triamine chelated to Zn^{II}.[40] The triamine provides a good model because 1) it has a distorted tetrahedral geometry about Zn^{II} with the fourth site occupied by H_2O with a pK_a of 7.3 (close to the value for CA-Zn^{II}-OH_2^+; Table 1),[1,31,32] 2) it catalyzes the hydrolysis of *p*-nitrophenylacetate (a model substrate for CA),[1,32] and 3) it binds arylsulfonamides as anions. The binding of arylsulfonamides to the triamine only probes the Zn^{II}–N bond ($\Delta G^{i}_{Zn^{II}-N}$), with no effects of the hydrogen-bond network or of hydrophobic contacts of the ring ($\Delta G^{i}_{ring} = \Delta G^{i}_{H-bonds} = 0$; Scheme 1). The value of the slope (related to β) of the plot of $\Delta G^{\circ}_{ArSO_2NH^-}$ for the binding of arylsulfonamides (and monoanions) to the triamine model versus the pK_a of these ligands was -0.40 (the uncertainty in the value of β from the standard error for linear least-squares fitting was 0.07). We took the value of -0.40 to be the pK_a dependence of the Zn^{II}–N bond ($\Delta G^{i}_{Zn^{II}-N}$) for the binding of fluorinated benzenesulfonamides to BCA. This assumption gives a value for the pK_a dependence of the hydrogen-bond network ($\Delta G^{i}_{H-bonds}$) of -0.44. Interestingly, this analysis suggests that the Zn^{II}–N bond and hydrogen-bond network have the same sensitivity to the pK_a of the sulfonamide.

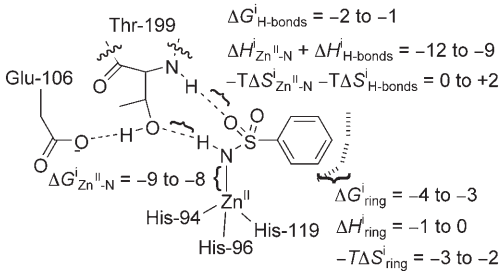
Liang et al. showed that mutating Thr-199 to Ala in HCA II decreased the affinity of HCA for dansylamide by about 0.8 kcal mol^{-1}, presumably due to the removal of the hydrogen bond between the side chain hydroxy group of Thr-199 and the NH group of the sulfonamide (Scheme 1).[41] No experimental value is available for the strength of the other hydrogen bond (between the backbone amide of Thr-199 and one of the sulfonamide oxygen atoms). Krebs et al. examined the catalytic activity of, but not the binding of sulfonamides to, an HCA mutant in which Thr-199 was replaced by Pro, a mutation that would be expected to abolish both hydrogen bonds.[42] We assumed that the two hydrogen bonds are equal in energy, thus giving $\Delta G^{i}_{H-bonds} = -1.5$ kcal mol^{-1} for dansylamide ($pK_a = 9.8$).[43]

These assumptions allowed us to divide the effect of electrostatics (pK_a-dependent terms; [Eq. (13a)]) on $\Delta G^{\circ}_{ArSO_2NH^-}$ into contributions from the Zn^{II}–N bond and the hydrogen-bond network [Eq. (14)].

$$\Delta G^{i}_{Zn^{II}-N} = -0.40(\pm 0.06)pK_a - 4.5(\pm 3.2) \quad (14a)$$

$$\Delta G^{i}_{H-bonds} = -0.44(\pm 0.24)pK_a + 2.8(\pm 2.4) \quad (14b)$$

Table 3 shows the calculated intrinsic free energies. Most of the binding energy (≈75%) for the fluorinated benzenesulfonamides is contributed by electrostatic contacts (with the Zn^{II}–N bond making up about 65% and the hydrogen-bond network about 10%), with the remainder (≈25%) from hydrophobic contacts (mainly in the entropy of association) of the phenyl ring (Scheme 3).



Scheme 3. Estimated free energies, enthalpies, and entropies (kcal mol^{-1}) for the different structural interactions between a fluorinated benzenesulfonamide anion and CA-Zn^{II}-OH_2^+ (data from Table 3).

Our results suggest the dominant role of the Zn^{II}–N bond in affinity and compare extremely well to a computational study of Menziani et al., which reported a value of about 60% for this interaction.[7] Our results differ from those of Menziani et al., however, in that they ascribed the variation of $\Delta G^{\circ}_{ArSO_2NH^-}$ (for the binding of a series of substituted benzenesulfonamide anions to CA-Zn^{II}-OH_2^+) to the variation in the strengths of van der Waals contacts between the ligands and CA, with the strength of the Zn^{II}–N bond being relatively constant across the series. Our results suggest that variations in the strengths of the Zn^{II}–N bonds ($\Delta G^{i}_{Zn^{II}-N}$) and the ring contacts (ΔG^{i}_{ring}) are of comparable magnitude, with neither alone explaining the variation in $\Delta G^{\circ}_{ArSO_2NH^-}$ (compare BS and pentaFBS in Table 3).

Entropy of Binding Partially Compensates for Enthalpy

Figure 3 shows a plot of $\Delta H^{\circ}_{ArSO_2NH^-}$ versus $-T\Delta S^{\circ}_{ArSO_2NH^-}$; the slope gives the compensation (1.1 ± 0.2) between the two. A stronger Zn^{II}–N bond has a proportionally greater entropic cost of association. The compensation value is slightly greater than unity; this result demonstrates that increasing the exothermicity of binding correlates with increasing the affinity of $ArSO_2NH^-$ for CA-Zn^{II}-OH_2^+.

We did not make too much of the linear relationship between enthalpy and entropy, however. The range in $\Delta G^{\circ}_{ArSO_2NH^-}$ for the series of fluorinated benzenesulfonamides is much smaller than that in $\Delta H^{\circ}_{ArSO_2NH^-}$ or $-T\Delta S^{\circ}_{ArSO_2NH^-}$, and thus such a linear relationship is required.[44]

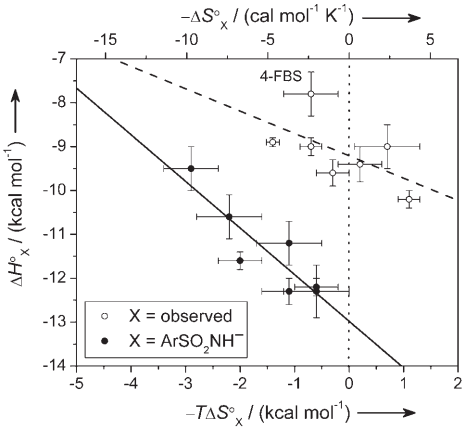


Figure 3. An enthalpy/entropy compensation plot for the binding of fluorinated benzenesulfonamides to BCA. Error bars are uncertainties described in Table 2. The solid and dashed lines are linear fits to the data. For X = $ArSO_2NH^-$ (closed circles), the best-fit line gave a value for compensation (negative of the slope) of 1.1 ± 0.2, and a value of R^2 of 0.88. For X = observed (the experimentally observed data; open circles), the best-fit line gave a value for compensation of 0.5 ± 0.3, and a value of R^2 of 0.36; the fit improved only marginally when 4-FBS was excluded from the analysis ($R^2 = 0.45$). The poorer fit to the observed data than to the data calculated for the idealized reaction (X = $ArSO_2NH^-$; Equations (1) and (5)) illustrates the difficulty of rationalizing the thermodynamics for processes that have a number of steps (e.g., ionization of arylsulfonamide and CA, and binding) without first disentangling the thermodynamics of the individual steps. Uncertainties were given by the linear least-squares fitting procedure. The dotted vertical line separates favorable ($-T\Delta S^\circ < 0$) from unfavorable ($-T\Delta S^\circ > 0$) entropy of binding.

The Outlier: 4-Fluorobenzenesulfonamide

The ligand 4-FBS interacts with a less-favorable $\Delta H^\circ_{ArSO_2NH^-}$ (Figure 2b) and a more-favorable $-T\Delta S^\circ_{ArSO_2NH^-}$ (Figure 2c) than that expected based on the QSARs for the other ligands. This deviation suggests either 1) greater mobility of the CA/4-FBS complex (either of the protein or the ligand in the complex) than expected based on the other ligands of the series, or 2) greater contribution of hydrophobic contacts with CA of 4-FBS than expected based on the QSAR for the other ligands. Possibility 2) seems unlikely because the hydrophobicity ($\log P$) of 4-FBS does not differ appreciably from the other ligands (Table 1), and all the ligands bind in a similar way to CA (Figure 1). We are currently exploring possibility 1) by conducting molecular dynamics simulations of CA/ligand complexes. We will report the results of these studies in due course.

4-Fluorobenzenesulfonamide Binds to BCA, HCA I, and HCA II with a Stoichiometry of 1:1

ITC provides the stoichiometry of binding of ligand to protein as one of the fitting parameters. The stoichiometries for all CA–FBS complexes examined in this study were unity (1.02 ± 0.05). This observation is in contrast to the results reported by Gerig and co-workers.[19,20] On the basis of binding titrations followed by ^{19}F NMR spectroscopy, these investigators reported that 2-FBS, 3-FBS, and 4-FBS bound with ligand-to-protein stoichiometries of 2:1 in complexes with HCA I and II, and that the bound ligands were in fast exchange on the NMR timescale (and thus were likely to be in the active site).[19,20] These results are very surprising given the 1:1 stoichiometry that has been demonstrated for almost all other sulfonamide–wild-type CA complexes by a number of biophysical techniques.[1,45,46]

In search of a reconciliation between these conclusions, we speculated that Gerig and co-workers might have been observing a second, weak binding site ($K_d^{obs} \approx 5$ μM) that we would not observe under the conditions we used for ITC, because of the greater (50–100-fold) concentration of CA used in NMR spectroscopy (0.5–1 mM) than in ITC (10 μM).[47] We repeated the ITC measurements with 4-FBS using about 70 μM of BCA so as to observe a weak secondary binding site ($K_d^{obs} \leq 7$ μM). Again, our data fitted well to a single-site binding model with a stoichiometry of 1.06 (Figure S2). The possibility remained that the second ligand bound with an enthalpy below our limit of detection ($|\Delta H^\circ_{obs}| < 0.2$ kcal mol^{-1}).

To address this issue, we attempted to replicate the ^{19}F NMR titration experiments of Gerig and co-workers.[18–20] Our results demonstrate that 4-FBS binds to BCA with a 1:1 stoichiometry (Figure 4). Although BCA has very high sequence identity (81%) and homology (88%) and is completely identical in its active site with HCA II, there are subtle differences outside the active site (e.g., I91V, C206S) between the two that could contribute to differences in the binding of ligands.[1,48]

We repeated the ^{19}F NMR titration with HCA I and 4-FBS, and again observed a binding stoichiometry of 1:1 (Figure S3). We ensured high activity of the NMR samples of BCA and HCA I by following fluorometrically the binding of ethoxzolamide (an inhibitor that has been shown to bind in a 1:1 stoichiometry to CA and to quench the fluores-

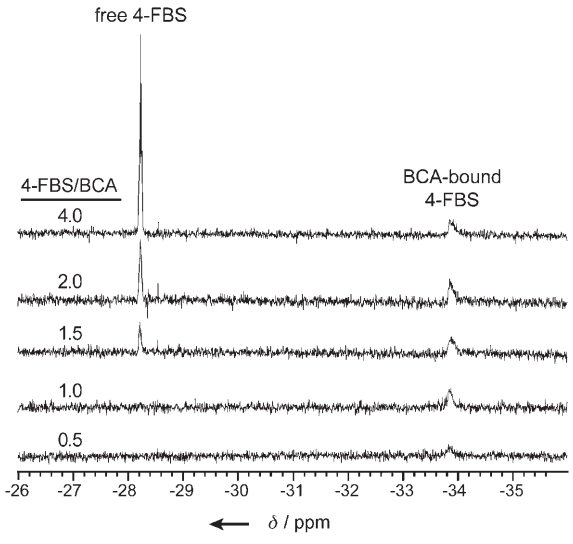


Figure 4. ^{19}F NMR spectra of 4-FBS in the presence of BCA (0.5 mM). The number of equivalents of 4-FBS to BCA is indicated. Chemical shifts (δ in ppm) are reported relative to trifluoroacetic acid as external standard (in a sealed capillary). All samples were in 20 mM $Na_2D_2PO_4$ ("pH" 7.5) at 298 K.

cence of tryptophan residues of the protein).[43,49] Our results indicate high activity of both enzymes: about 90% for BCA and about 82% for HCA I (Figure S4).

Our solution-phase results are consistent with the crystal structure of 4-FBS complexed with HCA II (Figure 1a); this structure conclusively demonstrates a binding stoichiometry of 1:1. There is no evidence in the electron-density map for a second or alternative binding conformation of 4-FBS (Figure 1b). The Zn^{II} coordination polyhedron remains tetrahedral in the 4-FBS complex and does not adopt the pentavalent geometry proposed by Gerig and Dugad.[19]

We have no explanation for the difference reported by Gerig and co-workers.[19,20] Based on our current understanding of the system, we deduce that their conclusions are incorrect and based on some unrecognized source of error in the data.

Conclusions

We have reported the enthalpy and entropy for the association of fluorinated benzenesulfonamides with BCA. We have used the experimentally observed data to calculate thermodynamic parameters for the idealized reaction of the binding of the sulfonamide anion ($ArSO_2NH^-$) to the Zn^{II}–water form of BCA (CA-Zn^{II}-OH_2^+) (Scheme 1). We constructed QSARs for $\Delta G^\circ_{ArSO_2NH^-}$, $\Delta H^\circ_{ArSO_2NH^-}$, and $-T\Delta S^\circ_{ArSO_2NH^-}$ of the idealized reaction with pK_a and $\log P$ of the fluorinated benzenesulfonamides (Figure 2). With our results and those of others, we semiquantitatively partitioned these thermodynamic parameters to the different structural interactions (component intrinsic interactions) between $ArSO_2NH^-$ and CA-Zn^{II}-OH_2^+ (Schemes 1 and 3).

The QSARs demonstrate that increasing fluorination on the benzenesulfonamide has three effects on the dissociation constant ($K_d^{ArSO_2NH^-}$) of $ArSO_2NH^-$ for CA-Zn^{II}-OH_2^+: 1) it increases the hydrophobicity ($\log P$) of the ligand, an effect that lowers $K_d^{ArSO_2NH^-}$, 2) it decreases the Lewis basicity of the anion for the Zn^{II} cofactor of CA (by decreasing the pK_a of the neutral sulfonamide), an effect that increases $K_d^{ArSO_2NH^-}$, and 3) it decreases the strength of the hydrogen-bond network between the SO_2NH group and residues of the active site of CA, an effect that increases $K_d^{ArSO_2NH^-}$. These three effects minimize the variation in $K_d^{ArSO_2NH^-}$ (the affinity varies by less than a factor of ten) across the series. This small range in $K_d^{ArSO_2NH^-}$ (and in $\Delta H^\circ_{ArSO_2NH^-}$ and $-T\Delta S^\circ_{ArSO_2NH^-}$) does not allow us to generalize these results to the binding of structurally complex arylsulfonamides to CA.

The partitioning of the thermodynamic parameters into the different structural interactions between the benzenesulfonamide ligands and CA have clarified the relative importance of the different interactions: electrostatic contributions are dominant with about 65% (≈ -8 kcal mol^{-1}) of the free energy being contributed by the Zn^{II}–N bond and about 10% (≈ -1 kcal mol^{-1}) by the hydrogen-bond network. Hydrophobic interactions between the aryl ring and CA contribute the remaining roughly 25% (≈ -3.5 kcal mol^{-1}) (Scheme 3); this result is consistent with the roughly 10^3 times (≈ -4 kcal mol^{-1}) higher affinity of HCA for benzenesulfonamide than for methanesulfonamide.[1,50]

One of the ligands, 4-FBS, interacts differently with CA from the others. It binds with a less-favorable enthalpy and a more-favorable entropy than anticipated from QSARs to the other ligands (Figure 2). We believe that the complex of 4-FBS and CA has greater mobility than the complexes of the other ligands and CA. We are currently pursuing computational studies to test this hypothesis.

Our results highlight the importance of examining the thermodynamic parameters for the association of $ArSO_2NH^-$ to CA-Zn^{II}-OH_2^+ (both ligand and protein in their active forms). Most studies (even QSARs in the literature) have only discussed the experimentally observed thermodynamic parameters. Such analyses confound too many variables: for instance, a lower pK_a for arylsulfonamide increases the fraction present as the anion (active form), but also decreases the Lewis basicity of the anion.

The "best" value for the pK_a of the ligand to give the lowest value for K_d^{obs} should be near the pH of the solution (≈ 7.4) because of these two competing effects of pK_a.[1,51] Pentafluorobenzenesulfonamide has a pK_a of only 8.2; a larger ring system (e.g., naphthalene) would allow the addition of more electron-withdrawing substituents to decrease the pK_a to about 7.4. Fluorine is the best choice for these substituents because it decreases the value of pK_a and also increases the hydrophobicity of the ligand.

Our results show that the different binding interactions between protein and ligand can be separated experimentally. An examination of the magnitude of these separate interactions for different ligands in a series could allow for the optimization of affinity by tuning the strengths of the different interactions independently. Finally, our results reveal that, even for a well-characterized protein such as CA, there are still outliers that show that our understanding of noncovalent interactions remains painfully incomplete.[14]

Experimental Section

General Methods

Fluorinated benzensulfonamides and proteins (BCA, pI 5.9, HCA I, and soybean trypsin inhibitor) were purchased from Sigma–Aldrich (St. Louis, MO). D_2O (99.9% D), hexadeuterated dimethyl sulfoxide ([D_6]DMSO; 99.9% D), and NaOD (99.5% D) were purchased from Cambridge Isotopes (Andover, MA). The benzenesulfonamides were recrystallized from water before use, and the other reagents were used as received. Enzymes were quantified by UV/Vis spectroscopy: BCA $\varepsilon_{280} = 55\,300\ M^{-1}cm^{-1}$, HCA I $\varepsilon_{280} = 47\,000\ M^{-1}cm^{-1}$ (molecular weights 29.09 and 28.85 kDa, respectively).[1,52] $^{19}F\{^1H\}$ NMR spectroscopy was carried out on a Varian Inova spectrometer operating at 376 MHz (^{19}F). ITC was performed with a VP-ITC microcalorimeter from MicroCal (Northampton, MA). UV/Vis spectroscopy was conducted on a Hewlett Packard 8453 spectrophotometer (Palo Alto, CA) and fluorescence spectroscopy on a Perkin–Elmer LS50B fluorometer (Boston, MA) with temperature controlled by a circulating water bath.

Quantification of Stock Solutions of Fluorinated Benzenesulfonamides

The fluorinated benzenesulfonamides (except pentafluorobenzenesulfonamide) were prepared gravimetrically to 10–20 mM in D_2O or

[D_6]DMSO. Stock solutions were diluted 1:10 with maleic acid (2.00 mM) in D_2O (prepared gravimetrically to 1 M and then diluted), and an excess of NaOD was added to facilitate deprotonation of the sulfonamide. Proton resonances due to the benzenesulfonamide were normalized relative to that of maleic acid (allowing a 10-s delay between pulses) to determine accurately the concentration of the stock solutions. Pentafluorobenzenesulfonamide, which was rigorously dried, was prepared to 10.0 mM gravimetrically.

Measurement of pK_a of Fluorinated Benzenesulfonamides

The benzenesulfonamides (5 mM in 52 mM NaCl) were titrated with NaOH, and the pH was monitored with a glass pH electrode. The values of pK_a were obtained by fitting the data (treating pH as the independent variable and volume of NaOH added as the dependent variable) to the full solution for the titration by using nonlinear least-squares optimization and the activity coefficients for proton and hydroxide at an ionic strength of 0.05 M.[53]

ITC to Determine Enthalpies of Ionization of Fluorinated Benzenesulfonamides

Samples of the benzenesulfonamides ($\approx$5 mM in 52 mM NaCl with pH adjusted to 9.6, near their values of pK_a) were titrated with NaOH (10.0 mM) in NaCl (52 mM) at 298 K. Ten 6.0-μL injections were preceded by one 2.0-μL injection, which was omitted for data analysis. After the injections, it was verified that the pH of the samples had not changed during the titration. The peaks of the thermogram were integrated, subtracted by the background enthalpies, and normalized to the amount of NaOH added. The average and standard deviation, which was taken to be the uncertainty, of these values are reported. To determine appropriate background enthalpies (of dilution and mechanical effects), buffers with values of p$K_a \approx 9.6$ and with well-characterized enthalpies of ionization (values of $\Delta H°_{ion,buffer}$ in kcal mol^{-1}: cyclohexylaminoethanesulfonic acid = 9.453, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid = 11.15, ethanolamine = 12.05)[29] were titrated as described. The difference between these experimental values and those from the literature gave the background enthalpies, which were very small (<0.3 kcal mol^{-1}) in all cases.

ITC to Examine the Binding of Fluorinated Benzenesulfonamide Ligands to BCA

For most experiments, BCA ($\approx$10 μM) in sodium phosphate buffer (pH 7.5, 20 mM, with 0.6% D_2O) was titrated with benzenesulfonamide ligand ($\approx$110 μM) in the same buffer at 298 K. Twenty-five 12.0-μL injections were preceded by one 2.0-μL injection, which was omitted for data analysis. After subtraction of background enthalpies, the data were analyzed by a single-site binding model using the Origin software (provided by Microcal) with the values of binding stoichiometry, $\Delta H°$, and K_d allowed to vary to optimize the fit. Measurements were conducted 3–4 times with a new stock solution of ligand prepared and quantified for each measurement. To verify the binding stoichiometry of 4-FBS to BCA, BCA (68.2 μM) in sodium phosphate buffer (20 mM, with 5% [D_6]DMSO) was titrated with 4-FBS (1.00 mM) in the same buffer at 298 K. Twenty-eight 10.0-μL injections were preceded by one 2.0-μL injection, which was omitted for data analysis. The data were analyzed as described.

^{19}F NMR Binding Titrations to Verify Stoichiometry of 4-FBS to CA

BCA (0.5 mM) or HCA I (0.75 mM) was solubilized in sodium phosphate buffer (pH 7.5, 20 mM). The sample was lyophilized and then redissolved in an equal volume of D_2O. Aliquots of 4-FBS ($\approx$20 mM in D_2O) were added to the sample, and $^{19}F\{^1H\}$ NMR spectra were acquired with a time between pulses of 2.0 s when the ligand-to-protein stoichiometry was less than or equal to unity and 20.0 s otherwise, to take into account the reported values of T_1 by Dugad and Gerig.[19] The resonances are reported relative to trifluoroacetic acid in a sealed capillary as external standard. It was verified that the "pH" of the samples did not change appreciably (<0.2 "pH" units) during the titration.

X-ray Crystal Structures of HCA II–Ligand Complexes

Recombinant HCA II was prepared as described[54] and crystallized by the hanging-drop vapor-diffusion method. Typically, 5 μL of protein solution (8–12 mg mL^{-1} protein, 1 mM methyl mercuric acetate, 20 mM tris sulfate (pH 8)) and 5 mL of precipitant buffer (2.75 M ammonium sulfate, 50 mM tris sulfate (pH 8)) were combined in a single drop suspended over a 1-mL reservoir of precipitant buffer at 277 K. Crystals appeared within two weeks and belonged to the space group $P2_1$ with average unit-cell parameters $a = 43.4$, $b = 42.2$, $c = 73.5$ Å, $\beta = 104.2°$.

Prior to ligand-soaking experiments, crystals of HCA II were cross-linked by adding 5 mL of glutaraldehyde solution (0.8% glutaraldehyde (*v*/*v*), 4.0 M ammonium sulfate, 50 mM tris sulfate (pH 8.0)) to the hanging drop and allowing it to equilibrate at 277 K for 48 h. Crystals were then transferred to a precipitant buffer containing 1–5 mM of the fluorinated benzenesulfonamide and soaked for 1 week at 4 °C.

X-ray diffraction data were collected at room temperature with an R-AXIS IIc image plate detector (Molecular Structure Corporation) mounted on a Rigaku RU-200HB rotating anode X-ray generator (operating at 50 kV and 100 mA) supplying $Cu_{K\alpha}$ radiation focused with Yale double mirrors. Raw diffraction data were processed with the HKL suite of programs.[55] The 1.54-Å resolution structure of native HCA II retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB accession code 2CBA)[56] was used as the starting coordinate set for the crystallographic refinement of the structure of each enzyme–ligand complex. Electron-density maps calculated with Fourier coefficients $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ and phases derived from the in-progress atomic model were generated with X-PLOR and viewed with O;[57] these maps consistently revealed the binding of only one ligand molecule per enzyme molecule throughout refinement. Refinement converged smoothly to final crystallographic *R* factors in the range 0.211–0.221. Data collection and refinement statistics are recorded in Table S1. The atomic coordinates of the complexes of HCA with 4-FBS, 2,6-FBS, and 3,5-FBS were deposited at the RCSB with the accession codes 1IF4, 1IF5, and IF6, respectively.

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[47] According to the data of Gerig and co-workers, there is no peak apparent at the chemical shift of the free ligand until the ligand-to-protein stoichiometry is greater than 2:1. Assuming that free ligand was present at about 7% (but below the limit of detection by ^{19}F NMR spectroscopy), and given the total concentration of CA of 1 mM, we calculated a value for K_d^{obs} for this presumptive secondary site of about 5 μM.

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