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The hydrophobic effect—a rationalization of the insolubility of nonpolar molecules in water—is centrally important to biomolecular recognition. Despite extensive research devoted to the hydrophobic effect, its molecular mechanisms remain controversial, and there are still no reliably predictive models for its role in protein-ligand binding. Here we describe a particularly well-defined system of protein and ligands—carbonic anhydrase and a series of structurally homologous heterocyclic aromatic sulfonamides—that we use to characterize hydrophobic interactions thermodynamically and structurally. In binding to this structurally rigid protein, a set of ligands (also defined to be structurally rigid) shows the expected gain in binding free energy as hydrophobic surface area is added. Isothermal titration calorimetry demonstrates that enthalpy determines these increases in binding affinity, and that changes in the heat capacity of binding are negative. X-ray crystallography and molecular dynamics simulations are compatible with the proposal that the differences in binding between the homologous ligands stem from changes in the number and organization of water molecules localized in the active site in the bound complexes, rather than (or perhaps in addition to) release of structured water from the apposed hydrophobic surfaces. These results support the hypothesis that structured water molecules—including both the molecules of water displaced by the ligands and those reorganized upon ligand binding—determine the thermodynamics of binding of these ligands at the active site of the protein. Hydrophobic effects in various contexts have different structural and thermodynamic origins, although all may be manifestations of the differences in characteristics of bulk water and water close to hydrophobic surfaces.

The hydrophobic effect—the energetically favorable association of nonpolar surfaces in an aqueous solution—often dominates the free energy of binding of proteins and ligands (1–5). Frequently, increasing the nonpolar surface area of a ligand decreases its dissociation constant (K_d ; i.e., increases the strength of binding) (6), and simultaneously decreases its equilibrium constant for partitioning from a hydrophobic phase to aqueous solution (K_p) (7). Modern, structure-guided, ligand design has relied upon the “lock-and-key” notion of conformational association between the atoms of the ligand and the binding pocket of a protein; the detailed molecular basis for the hydrophobic effect, however, continues to be poorly understood (1–5). This lack of understanding of the hydrophobic effect prevents accurate prediction of the free energy of binding of proteins and ligands.

The first, and currently most pervasive, rationale for the hydrophobic effect was based on studies of the thermodynamics of partitioning of nonpolar solutes from hydrophobic phases (i.e., the gas phase or a hydrophobic liquid phase) into water. The thermodynamics of partitioning of solute molecules is characterized by a dominant, unfavorable entropy and an increase in heat capacity of the aqueous system (8). The classical mechanism for

Alternative theoretical approaches for modeling the hydrophobic effect—including those based on scaled-particle theory and its intellectual progeny (17–20)—also rationalize the unfavorable entropic component of transferring nonpolar molecules from a hydrophobic phase to an aqueous phase. These theories propose that the accumulation of “void volume” sufficient to accommodate a nonpolar solute in water is entropically unfavorable. Although these void volume theories have been criticized because they do not rationalize the heat capacities of partitioning (1), they do predict a size-dependence of the thermodynamics of water near nonpolar solutes: Small solutes (less than approximately 1 nm in diameter, similar in size to the nonpolar gases studied by Frank) fit into the hydrogen bonded network of liquid water without breaking hydrogen bonds, but the solvation of larger solutes requires water to sacrifice hydrogen bonds to maintain van der Waals contact with the solute. Water near solutes with diameters greater than approximately 1 nm, in the void volume models, have a structure that is enthalpically less favorable than that of bulk water (2). A body of spectroscopy studies support the prediction that molecules of water near extended hydrophobic surfaces participate in fewer hydrogen bonds than do molecules of bulk water (21, 22).

The prediction that the thermodynamics of water near solutes depends on the size—and implicitly on the shape—of the solute has influenced modern models of the hydrophobic effect. In particular, the inhomogeneous solvation theory of Lazaridis predicts that water in chemically heterogeneous cavities (like those that characterize the binding pockets of many proteins) possess structures that have free energies less favorable than the structure

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Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3S75, 3S71, 3S78, 3S74, 3S77, 3S73, 3S76, and 3S72).

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of bulk water (23, 24). This theory is consistent with speculations derived from many experimental studies of molecular recognition in aqueous solution (25–31). Experimentalists have rationalized enthalpically favorable association by invoking either (i) enthalpically favorable interactions between hosts and guests in the bound complex (so-called “nonclassical” hydrophobic effects) (4, 32, 33), or (ii) the “release” of water molecules upon association that, because of the structure of the binding pocket, adopt configurations that are enthalpically less favorable than bulk water (25, 34, 35). Many of these experimental studies, which have relied heavily on modern isothermal titration calorimeters (ITC), have also shown negative values of the heat capacity of association (ΔC_p°) (26, 27, 36–40), a term that has since become the sign-post of hydrophobic interactions—even though changes in heat capacity result, in principle, from myriad structural changes that occur with association in aqueous solution (41–43).

A series of recent computational studies of explicitly modeled water in the binding pockets of proteins is compatible with the rationale that water in binding pockets is less favorable in free energy than bulk water (44–50). The hydrophobic effect that determines the free energy of displacing these waters from the binding pocket appears to be quite different from the hydrophobic effects that determine the free energy of water near small, nonpolar solutes, and that of water near large nonpolar surfaces. Although an entropy-dominated picture of hydrophobic interactions continues to pervade thinking in contemporary biochemistry (51)—primarily because of Kauzmann’s plausible and understandable proposal that protein folding is stabilized by the burial of hydrophobic amino acids (9)—the evidence from both theoretical and experimental studies over the last few decades paints a far more complicated picture, and one that is considerably more challenging to interpret: There is no single model that is consistent with all of the thermodynamic and structural characterizations of hydrophobic interactions, per se. Hydrophobic effects in alternative contexts may have different structural and thermodynamic origins, although all may be manifestations of the differences in characteristics of bulk water and water close to surfaces.

The motivation for our work was (i) to define the thermodynamics of the hydrophobic effect experimentally in interactions between a protein and a ligand in the simplest and structurally best-defined system that we could design, (ii) to obtain biostructural data from X-ray crystallography that would define the character of the interacting nonpolar interfaces, (iii) to interpret the thermodynamics of association obtained by ITC in terms of the biostructural data, and (iv) to compare the experimental results with estimates of thermodynamic parameters from molecular dynamics simulations. We wished, in particular, to define the hydrophobic effect in a system uncomplicated by protein plasticity, or by conformational mobility of the ligand.

We selected human carbonic anhydrase II (HCA, E.C. 4.2.1.1) as our model protein. HCA is conformationally rigid: An extensive literature—and results we describe here—establish that it does not undergo an observable (> 1 Å) conformational change upon binding of most arylsulfonamide ligands (52). A metal coordination bond and several hydrogen bonds fix the geometry of the sulfonamide-Zn^{II} structure (Fig. 1A). The chemical environment of the binding pocket of HCA is heterogeneous: One side of the pocket presents a nonpolar surface (Phe 131, Pro 201, Pro 202, and Leu 198), and the other side presents a polar surface (Asn 62, His 64, and Asn 67).

Our analysis is based on the comparison of the binding of five-membered heterocyclic sulfonamides, and of benzo derivatives of these compounds, in the active site of HCA. We reasoned that, although data from a series of different structures might be difficult to interpret, a common perturbation to these structures would be interpretable. The perturbation we chose is what we call here “benzo-extension” (Fig. 1B), which extends the nonpolar

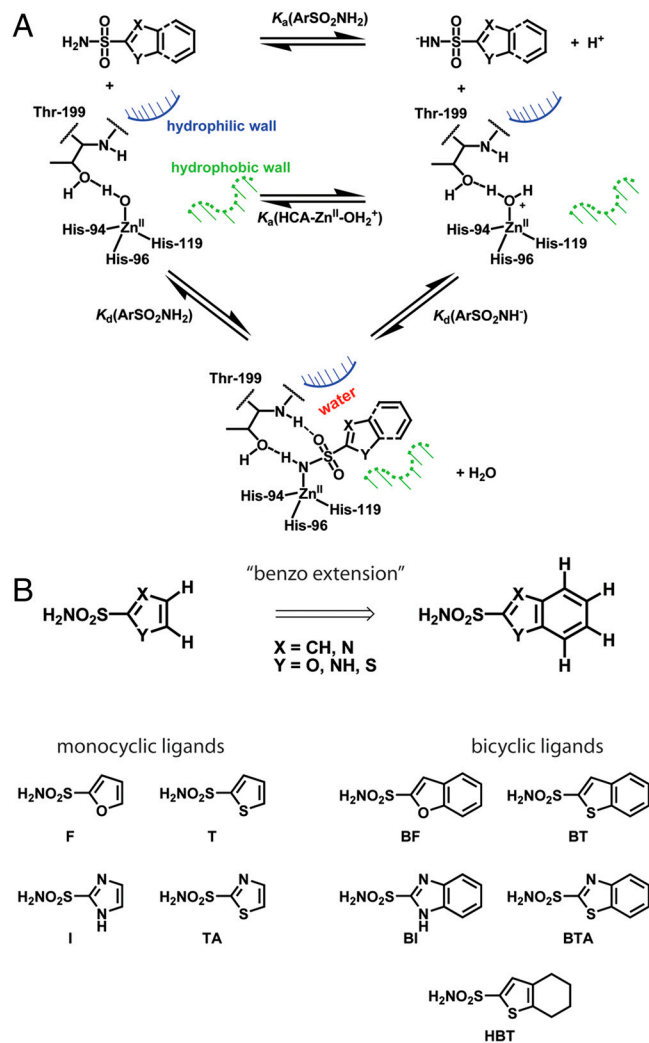
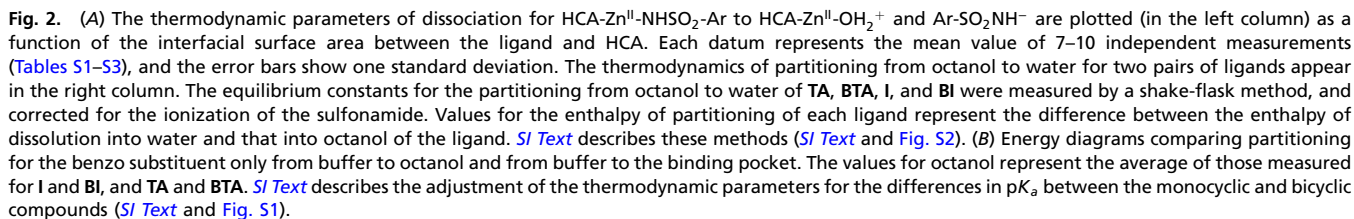


Fig. 1. Schematic representations of the model system. (A) The covalent binding of sulfonamides to HCA involves the deprotonation of the sulfonamide. We converted the observed thermodynamic binding parameters of each ligand to the binding parameters of each sulfonamide anion ($\text{Ar-SO}_2\text{NH}^-$) to the Zn^{II}-bound water form of HCA ($\text{HCA-Zn}^{\text{II}}\text{-OH}_2^+$; Fig. 1A). This conversion allowed us to compare the thermodynamic binding parameters of different ligands in a scheme that is independent of the pK_a values of the ligands. The derivation of the equations used to calculate the binding parameters of each sulfonamide anion ($\text{Ar-SO}_2\text{NH}^-$) appear in *SI Text*. (B) Available biostructural evidence suggests that benzo-extension would extend the nonpolar surface of the heteroaromatic ligand over the nonpolar “hydrophobic wall” of the active site of HCA, increase the area involved in hydrophobic interactions, and make the free energy of binding more favorable. The structures of each of the ligands used in this study appear with their abbreviations.

surface of the heteroaromatic ligand over the nonpolar face of the active site of HCA.

Results and Discussion

Fig. 2A plots the thermodynamic parameters describing the dissociation of the ligand from the protein as a function of the difference in solvent-accessible surface area ($\Delta\text{SASA}_{\text{unbound}} = \text{SASA}_{\text{ligand}} + \text{SASA}_{\text{protein}} - \text{SASA}_{\text{complex}}$) between the bound and unbound states. Values of $\Delta\text{SASA}_{\text{unbound}}$ between protein and ligand established that the average decrease in ΔG° of benzo-extension was $-20 \text{ cal mol}^{-1} \text{ \AA}^{-2}$; this value is consistent with the widely reported range of values of -20 to $-33 \text{ cal mol}^{-1} \text{ \AA}^{-2}$ for hydrophobic interactions (10). ITC demonstrates, however, that the contributions of enthalpy and entropy to $\Delta\Delta G^\circ_{\text{unbound,benzo}}$ are the opposite of those predicted by either the iceberg or void



Although hydrophobic effects dominated by enthalpy have been observed previously (4), an important additional piece of information in this system is that the addition of a fused cyclohexyl ring in **HB**T produces the same thermodynamic signature as the addition of a fused benzo ring in **BT**. This result suggests that the hydrophobic effect in this system is indistinguishable for the surfaces of aryl and alkyl groups: There is, thus, no “nonclassical hydrophobic effect” resulting from interactions between the aromatic group of the ligand and the protein (4).

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We chose the buffer phase as a reference state and calculated the free energy, enthalpy, and entropy of the benzo group in octanol and in the binding pocket of HCA (Fig. 2*B*). This calculation indicates that: (i) solvation of the benzo group in aqueous buffer is roughly +2 kcal mol⁻¹ higher (less favorable) in free energy than it is either in solution, in octanol, or in association with the binding pocket of HCA; (ii) association of the benzo group with the binding pocket is roughly -3 kcal mol⁻¹ more favorable in enthalpy than solvation of that group in octanol; and (iii) association of the benzo group with the binding pocket is +3 kcal mol⁻¹ less favorable in entropy than solvation of that group in octanol. Entropy and enthalpy, thus, compensate in the transfer of the benzo substituent from nonpolar solvent to the binding pocket, with binding of the hydrophobic group being characterized by a favorable enthalpic term.

To rationalize these results, we used X-ray crystallography to characterize each of the eight HCA-ligand complexes; the resolution of these structures was in the range of 1.25–1.97 Å (Table S4). The average root-mean-squared deviation of the alignment of the heavy atoms of the proteins was 0.15 ± 0.04 Å, and that of all residues having at least one atom within 5 Å of atoms of the ligand was 0.10 ± 0.02 Å (Fig. 3*A*). The crystal structures also indicated that each of the ligands bind to HCA with the same geometry (Fig. 3*B*). We inferred from the structural data that the thermodynamics of binding are not due to changes in the crystallographically determined structure of the protein on binding the ligands, or due to differences between the geometry of the Zn^{II}-N bond of the monocyclic and bicyclic ligands.

The structural data also indicate that the atoms of the fused benzo ring make few contacts with residues in the hydrophobic shelf (Fig. 3*C*): The fused rings are in contact with three nonpolar residues from the hydrophobic shelf (Phe 131, Pro 202, and Leu 198), and with two polar groups (the hydroxyl of Thr 200 and the carbonyl oxygen of Pro 201). Remarkably, the hydropho-

bic effect, in this system, thus does not involve extensive contact of the apposed hydrophobic surfaces of protein and ligand. Of the three nonpolar contacts, only two (Phe 131 and Pro 202) differentiate the benzo ring from the five-membered ring. There is, therefore, a net increase of only two additional hydrophobic contacts (approximately 90 Å²) upon the addition of the fused benzo ring. One face of the benzo ring, thus, forms a cavity, which has a volume of 50 Å³, with residues Phe 131 and Pro 202 of the hydrophobic wall: The value of $\Delta\Delta G^{\circ}_{\text{unbind,benzo}}$, in this case, is not the result of conformational association of the “lock” and the “key.”

Values of $\Delta C^{\circ}_{p,\text{bind}}$ for two ligands (**T** and **BT**) over the temperature range of 283–303 K (Fig. 4*A*) are negative ($\Delta C^{\circ}_{p,\text{bind}} = -38 \pm 7$ cal mol⁻¹ K⁻¹ for **T**, and -96 ± 6 cal mol⁻¹ K⁻¹ for **BT**); these values indicate the involvement of solvent in determining the thermodynamics of binding (3, 5, 36). Calorimetric studies that correlate values of ΔC°_p of unfolding to the difference in surface area between the folded and unfolded states of numerous proteins predict a contribution of $-0.28 - 0.51$ cal mol⁻¹ K⁻¹ Å⁻² of buried nonpolar surface area, and a contribution of $+0.14 - +0.26$ cal mol⁻¹ K⁻¹ Å⁻² of buried polar surface area (43). Our estimate of $\Delta\Delta C^{\circ}_{p,\text{bind}} = -58 \pm 9$ cal mol⁻¹ K⁻¹ is, thus, approximately twice that predicted from the crystal structures for the burial of surface area alone ($\Delta\Delta C^{\circ}_{p,\text{predicted}} = -30 \pm 5$ cal mol⁻¹ K⁻¹).

Changes in heat capacity, however, also occur with the ordering of water molecules in protein-ligand complexes (29, 39). Connolly has estimated a maximum value of -9 cal mol⁻¹ K⁻¹ for the ordering of one water molecule in a protein-ligand complex (42). Our thermochemical data, thus, are consistent with the ordering of two to four additional water molecules in the HCA-bicyclic ligand complexes than are ordered in the structures of HCA-monocyclic ligand complexes.

Crystallography is compatible with the hypothesis that water in the binding pocket of HCA determines the hydrophobic effect in this system: In the structures of HCA with the bicyclic ligands, between three and five molecules of water appear ordered between the benzo group and the polar wall. Crystallography does not, however, allow us to examine molecules of water that are not localized. Also, we cannot correlate (based solely on the positions of the crystallographically observable molecules of water) the influence of the change in the structure of the network of water molecules and the thermodynamics of binding of the ligands.

To estimate the contribution from water to the free energy of binding, we used the WaterMap method (Schrödinger Inc.) to predict the positions, enthalpies, and entropies of the water molecules in the binding pockets of the complexes of HCA-**T**, HCA-**BT**, HCA-**F**, and HCA-**BF** (Fig. 4 and Table S5). These calculations use an explicit-solvent molecular dynamics simulation, followed by clustering of the water molecules into “hydration sites,” which are nonoverlapping spheres of 1.0 Å radius (46). WaterMap uses inhomogeneous solvation theory to estimate the enthalpy and entropy of each hydration site (23, 24). To estimate the enthalpy and entropy of hydration of each complex, we calculated the sum of the 30 hydration sites closest to the ligand; this group of sites occupied a volume extending over a distance of roughly 10 Å from the surface of the ligand. Beyond this distance, the water-water correlation diminishes to zero and the radial distribution function of the hydration sites approaches one (i.e., that of bulk water). Because the relevant thermodynamic parameters in our case were those of benzo-extension, we compared the hydration of each monocyclic-HCA complex to that of the bicyclic-HCA complex (e.g., $\Delta\Delta G^{\circ}_{\text{WM,benzo}} = \Delta G^{\circ}_{\text{BT}} - \Delta G^{\circ}_{\text{T}}$). Our rationale was that—independent of the absolute accuracies of any calculations—the positions and relative energies of the hydration sites would reflect changes to the local structure of water induced by benzo-extension.

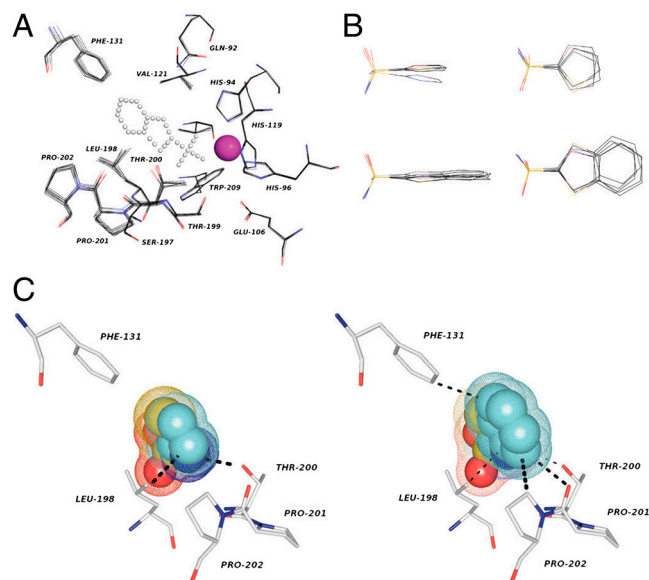


Fig. 3. Structural alignment of the eight available HCA-ligand complex structures **F**, **BF**, **T**, **BT**, **I**, **BI**, **TA**, and **BTA**. (*A*) Residues of the protein with atoms within 5 Å of the ligand in each structure appear as line diagrams. The magenta sphere indicates the position of the Zn^{II} ion. The white dashed lines indicate the position of the ligand. (*B*) Detailed view of all the heavy atoms of the sulfonamide ligands from the aligned HCA-ligand complex structures. (*C*) A representation of the contacts between the atoms of protein and the atoms of **TA** (*Left*) and **BTA** (*Right*). The atoms of the ligands appear as a space-filling representation; the green mesh represents the van der Waals contact surface. Dashed black lines indicate contacts between the ligands and the protein, and the radii of the lines are proportional to the interfacial contact area between the residue and the benzo substituent.

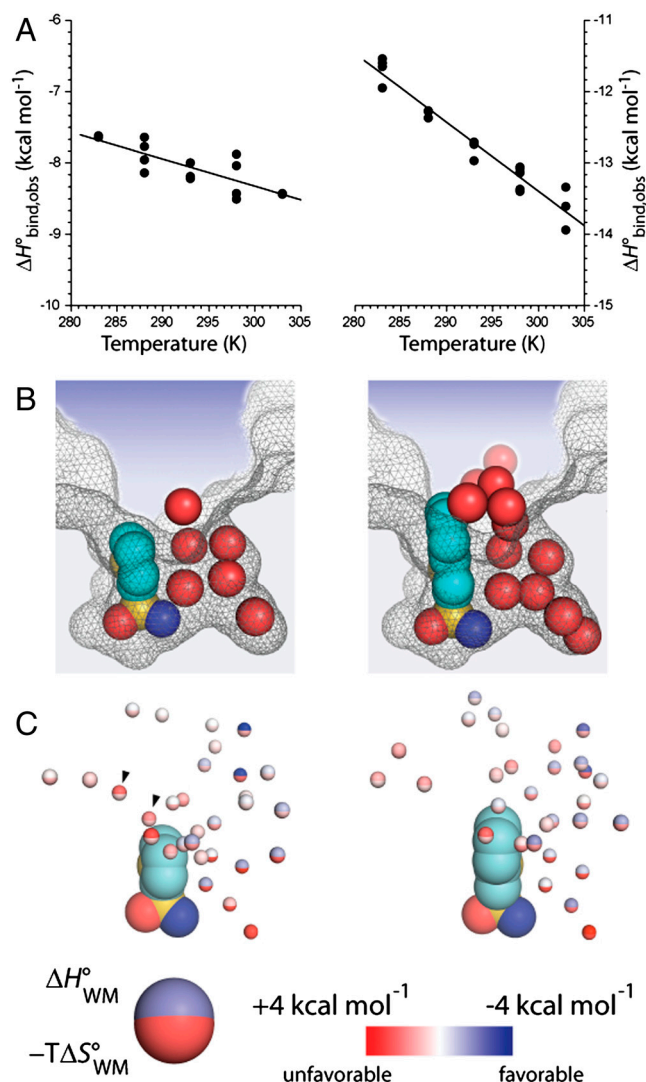


Fig. 4. (A) Observed enthalpies of binding as a function of temperature between 283 and 303 K. Enthalpy of binding was measured by ITC with independent titrations conducted at each temperature. Each datum represents a single ITC experiment. The black line indicates a linear fit of the data. The slope of the regression line was $-38 \pm 7 \text{ cal mol}^{-1} \text{ K}^{-1}$ for **T**, and was $-96 \pm 6 \text{ cal mol}^{-1} \text{ K}^{-1}$ for **BT**. (B) Positions of the molecules of water observed in the crystal structures of **T** and **BT**. The binding pocket of HCA appears as a mesh surface representation. Atoms of the ligands and crystallographically determined molecules of water appear as sphere representations. The corresponding images of six additional HCA–ligand complexes appear in *SI Text* (Fig. S3). (C) Results of the WaterMap calculations. The heavy atoms of the ligand appear as sphere representations (Left, **T** and Right, **BT**). The hydration sites from WaterMap appear as spheres that are color-coded to represent the predicted values of enthalpy and entropy: The top hemisphere represents enthalpy and the bottom hemisphere represents entropy. The color scale is set such that red represents hydration sites that are less favorable than bulk water, and blue represents those more favorable than bulk water.

Thermodynamic parameters calculated by WaterMap for benzo-extension were consistent with those determined experimentally by ITC (i.e., WaterMap also indicates that the gain in binding free energy comes from enthalpic stabilization). Average values for converting **F** to **BF** and **T** to **BT** were $\Delta\Delta G^{\circ}_{\text{WM,benzo}} = -2.8 \text{ kcal mol}^{-1}$, $\Delta\Delta H^{\circ}_{\text{WM,benzo}} = -3.0 \text{ kcal mol}^{-1}$, and $-T\Delta\Delta S^{\circ}_{\text{WM,benzo}} = 0.2 \text{ kcal mol}^{-1}$ (*SI Text* and Table S5 detail the results of each calculation). The calculated values of entropy and enthalpy for each hydration site, moreover, provide some structural rationale for the enthalpically favorable hydrophobic effect. In particular, water molecules in two enthalpically unfa-

vorable hydration sites (indicated by arrows in Fig. 4C) in the binding pocket near the monocyclic ligand are displaced by benzo-extension. Additional changes to the network of water molecules in the binding pocket—including the ordering of water between the benzo group and the hydrophilic wall—result in entropy-enthalpy compensation, with the increased water ordering producing a loss in entropy. The cumulative effect of benzo-extension results in computed thermodynamic parameters that are indistinguishable from those determined experimentally by ITC. Although this agreement does not prove that changes in the water network are the origin of the hydrophobic effect in this system, it is compatible with that hypothesis.

An important conclusion from this work is that the phrase “hydrophobic effect” can have different molecular-level interpretations when it refers to partitioning of a ligand from a hydrophobic liquid phase to an aqueous phase, and from a hydrophobic binding pocket to the same aqueous phase. Thus, hydrophobic effects (plural) in biomolecular recognition and in partitioning between water and a nonpolar phase may have different structural and thermodynamic origins, although both may be manifestations of the differences in characteristics of water in bulk, and close to, surfaces. It thus appears that even for a given set of ligands, it is necessary to discuss multiple hydrophobic effects (with very different thermodynamic signatures) rather than a single hydrophobic effect: The tendency of nonpolar surfaces to associate reflects quite different balances of enthalpic and entropic effects, depending on molecular context, even though all (or many) may be manifestations of the structure of water.

Theoretical discussions by Rossky, Berne, Friesner, Lazaridis, and others (44–50, 53) have repeatedly suggested that the structure and thermodynamics of water adjacent to a nonpolar surface would depend on the molecular-scale topography of the surface. In this view, the difference in the structure and thermodynamics between water in the active site, and water in bulk, may determine the hydrophobic effect in the declivities that make up most binding pockets.

Although negative values of ΔC_p are the sine qua non of hydrophobic interactions between proteins and ligands, the physical interpretation of this parameter remains as obscure as the experimental support for structured water near hydrophobic groups in dilute aqueous solution. We conclude that combined thermodynamic, biostructural, and computational studies of ΔC_p of binding in systems of proteins and ligands continue to be necessary to untangle our understanding of hydrophobic effects. In that regard, HCA and arylsulfonamides seem to be an especially appropriate model system.

The combination of thermodynamic analysis, X-ray crystallography, and simulations described in this work is compatible with the hypothesis that the hydrophobic effect in biomolecular recognition, in this system, reflects changes in the structure of water extending across (and beyond) the active site region. The hydrophobic effect, here, cannot be attributed solely to the waters that are in contact with the nonpolar surfaces of the ligand, and it is not due to conformal association of the protein and ligand. In this view, the shape of the water in the binding cavity may be as important as the shape of the cavity.

Methods

SI Text details the experimental procedures for the purification of protein, the measurement of the thermodynamics of binding and partitioning, the measurement of the pK_a of the ligands, the preparation and crystallography of the protein–ligand complexes, and the calculation of the energies of the hydration sites for the HCA–ligand complexes.

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