Fluoroalkyl and Alkyl Chains Have Similar Hydrophobicities in Binding to the "Hydrophobic Wall" of Carbonic Anhydrase

Supporting Information

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Aliphatic Fluorocarbons and Hydrocarbons. Alkyl and fluoroalkyl groups have considerably different properties, including: i) refractive indexes (1.2515 for hexane and 1.3751 for perfluorohexane);[1] ii) dipole moments (1.85 D for CH_3F, 1.64 D for CHF_3); iii) C-X bond lengths (1.09 Å for X = H, and 1.35 Å for X = F);[2] iv) van der Waals radii (1.20 Å for H, 1.47 Å for F);[2] v) molecular surface areas (46 $Å^2$ for $–CH_3$, 59 $Å^2$ for $–CF_3$); vi) conformations of X with respect to the C-C bond (all-antiperiplanar vs. helical);[1] and vii) solubility in water.

The Classical View of Hydrophobic Interactions. The term "hydrophobic interaction", which implies the tendency of a nonpolar surface to minimize contact with water, refers to the favorable free energy of formation of aggregates of hydrated hydrocarbons (or fluorocarbons) in water.[3-5] Early thermodynamic analyses by Frank and Evans indicated that the unfavorable free energy of dissolution of nonpolar gases in water was determined by an unfavorable entropic term at room temperature, where the enthalpy of dissolution is nearly zero.[6] As temperature increased, however, the magnitude of the enthalpy of dissolution of nonpolar gases also increased. This observation indicates that the change in heat capacity of dissolving nonpolar solutes in water is positive. Both the increase in heat capacity and the unfavorable entropy at room temperature led Frank and Evans to rationalize the poor solubility of nonpolar compounds in water to be the result of an increase in the order of the water that solvates these compounds, and they introduced the term "iceberg" to describe the structure of water molecules that hydrate nonpolar solutes.[6]

Kauzmann—based on the analyses of Frank and Evans—hypothesized that the folding of proteins was due to an unfavorable entropy of hydrating the nonpolar side chains of Val, Leu, Ile, and Phe.[7] Kauzmann inferred that ordering of water molecules near hydrophobic amino acids in solution might explain the entropic driving force for the folding of proteins. At the same time, structural studies of methane hydrates showed that the ordering of water around methane in the

solid state produced a network of hydrogen bonds that is almost indistinguishable from the structure of hexagonal ice.[8] Tanford coined the term "hydrophobic effect" for the unfavorable free energy of hydration of nonpolar molecules in water.[9]

Modern Views of Hydrophobic Interactions. The community of biophysical chemists, in the decades since, has embraced the concept that water is more ordered near hydrophobic solutes than it is in the bulk. Most of the support for structured water, however, derives from spectroscopic studies of water near macroscopic interfaces with nonpolar phases,[10-13] while a few studies (by neutron diffraction spectroscopy) of solutions of nonpolar solutes in water seem to provide contradicting results.[14,15] Theoretical studies in the decades since KT, too, seem to provide conflicting results: although some theoretical treatments of hydrophobic effects—primarily those presented by Stillinger, Pratt, Chandler, Hammer, and others—predict structured water near extended (> 1 nm^2) surfaces, and a lack of structured water near smaller solutes,[16-19] molecular dynamics simulations that have explicitly studied the behavior of water near small nonpolar solutes have, in many cases, supported the original KT speculation.[20-22] Little consensus exists in the literature to support the notion of structured water near small solutes—those having areas less than ~1 nm^2—in aqueous solution.[3,23] Moreover, little work has focused on the behavior of water near fluoroalkyl groups in aqueous medium.[24]

Hydrophobic Interactions in Protein-Ligand Association. Molecular dynamics simulations of water in the binding pockets of proteins portray the complicated nature of the structure and energy of water near protrusions and cavities on the surfaces of proteins.[25-32] Simulations predict that i) water in a hydrophobic cavity is less favorable in enthalpy than water in bulk solution because, near hydrophobic groups in these cavities, water forms fewer hydrogen bonds than does

water in bulk solution, and that ii) water near polar groups, by contrast, forms hydrogen bonds with the entropic cost of being more ordered than water in bulk solution.[30]

This complicated picture of water in protein binding pockets coincides with thermodynamic signatures of hydrophobic interactions that are somewhat different from those of the hydrophobic effects described by KT. Numerous experimental studies of protein-ligand association have demonstrated that negative values for the change in constant pressure heat capacity of binding ($\Delta Cp°_b$), rather than unfavorable values of $-T\Delta S°_b$, correlate with hydrophobic interactions in protein binding.[33-40] Nonetheless, the repeated observation that binding of hydrophobic molecules to proteins has negative values of $\Delta Cp°_b$ is consistent with the hypothesis that the structure of water in the binding pockets of proteins determines the thermodynamics of hydrophobic effects.

Purification of HCA.

Wild-type HCA II was expressed in E. Coli BL21 cells, and purified from the crude cell lysate in two steps. First, the lysate was added to agarose resin that was functionalized with aminomethyl-benzenesulfonamide, washed with Triton-X (0.1%) and with Triton-X (0.1%) and sodium sulfate (500 mM), and eluted with sodium azide (400 – 600 mM). Second, this eluate was purified further by size-exclusion chromatography on Superdex 75 using 10 mM sodium phosphate, pH 7. 6 as running buffer. The concentration of the pooled fractions of HCA II (100-500 μM) was measured by UV-Vis spectroscopy. The purified protein was diluted to 50 μM in buffer (10 mM sodium phosphate, pH 7.6) and used for ITC experiments.

Isothermal Titration Calorimetry.

Sulfonamides were dissolved in DMSO at a concentration of ~20 mM. These solutions were diluted to ~2.0 μM in sodium phosphate (10 mM, pH = 7.6), mixed, and used for ITC experiments.

Titration experiments were conducted at 298.15 K, using an Auto VP-ITC instrument (MicroCal). The titration of an HCA II sample (20 μM) into a solution of sulfonamide ligand (~ 2.0 μM) comprised 10 injections (one initial injection of 1 μL followed by nine injections of 29 μL per injection, with a 400-second interval between injections). The initial data point from the 1 μL injection was deleted from the integrated data to minimize the effect of diffusive mixing that occurs during equilibration of the instrument.

Crystal Growth and Ligand Soaking Experiments.

Aliquots of HCA II (purified as described in the text) were concentrated to 200 – 800 μM in 50 mM Tris-SO_4 buffer (pH = 7.6). These solutions were mixed in equal volume with crystallization buffer (1.14 – 1.25 M sodium citrate, pH = 7.8) and suspended on glass slides over 1.0 mL of crystallization buffer in the well of a 24-well plate at 4°C. Crystals typically grew within one week to typical sizes of 250 x 200 x 100 μm, and were stored at 4°C until needed. Soaking experiments were performed as described in the main text.

X-ray Diffraction Experiments.

Crystals of HCA II soaked with ligand were harvested with cryoloops, flash frozen in liquid nitrogen, and shipped to the National Synchrotron Light Source. Data were collected on an

ADSC Quantum Q315 CCD detector at beamlines X25 and X29 in collaboration with the Mail-In-Program. The dataset were indexed and scaled using HKL2000.[41]

Solution of Crystal Structures.

Diffraction data were analyzed using the CCP4i suite of crystallography software.[42] To obtain phase information for each data set, we used Phaser[43] for molecular replacement with a previously reported structure of native HCA II (PDBID: 2ILI), from which we removed the atoms of water and zinc. After one round of restrained refinement using REFMAC,[44] we used OMIT to generate a weighted difference electron density map (OMIT map). For every data set, visual inspection showed contiguous electron density (at ~1σ on the OMIT map) for residues 3 – 259, and well-defined electron density for the ligands in the active site. We added the atoms of the ligand and refined them locally. We added molecules of water in 4 – 6 cycles (with 5 – 10 intervening rounds of restrained refinement) to the peaks in the difference map that were >5σ.

Figure S1. Dependence of $\Delta G°_b$ for binding of benzenesulfonamide ligands ($H_2NSO_2C_6H_4$-$CONHCH_2(CX_2)_nCX_3$, X = H, F) on surface area (top) and volume of ligands (bottom).

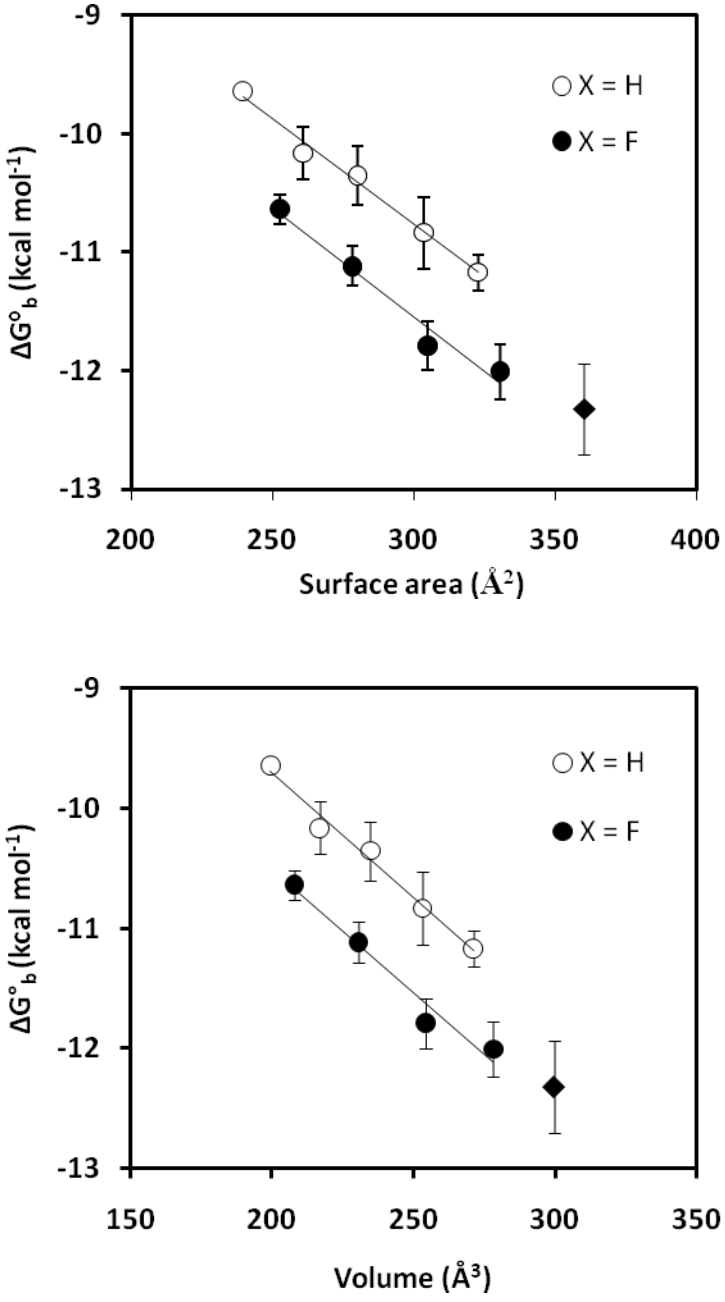


Figure S2. Dependence of $\Delta H°_b$ for binding of benzenesulfonamide ligands ($H_2NSO_2C_6H_4$-$CONHCH_2(CX_2)_nCX_3$, X = H, F) on surface area (top) and volume of ligands (bottom).

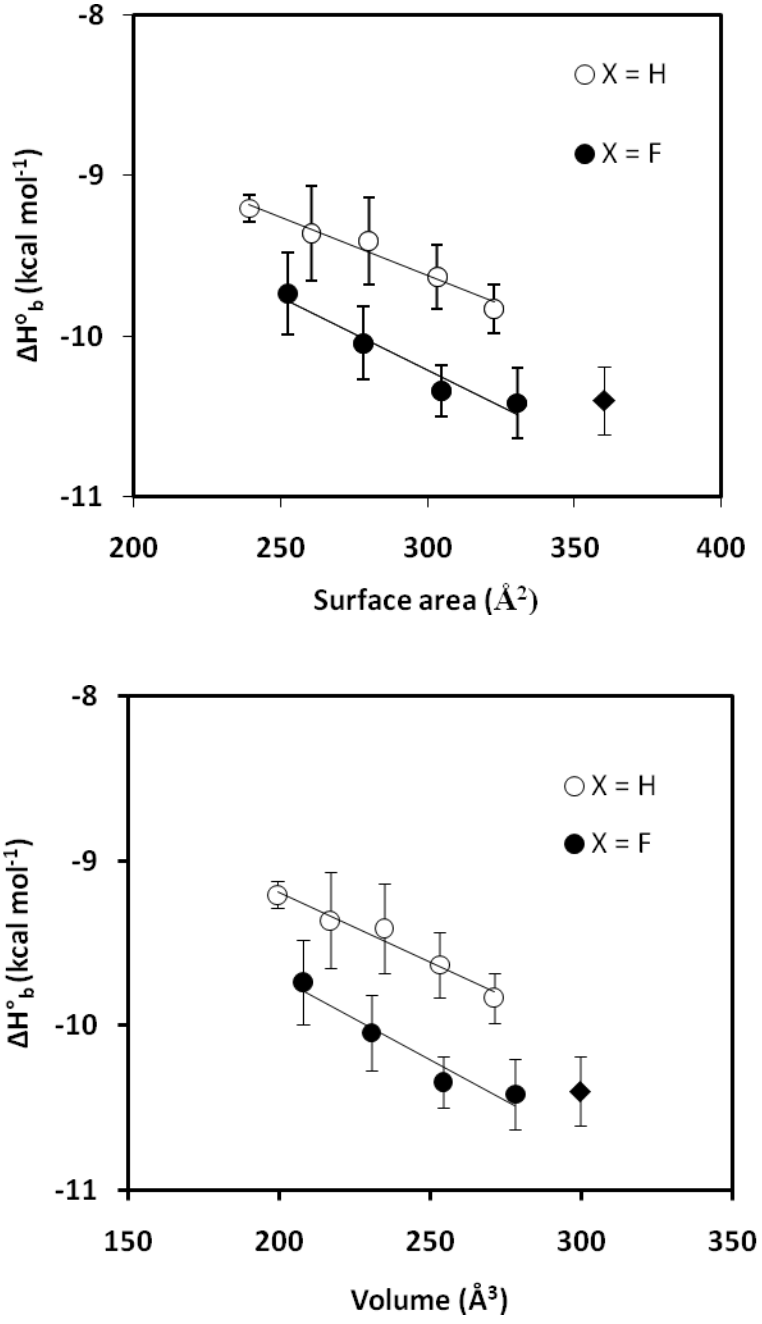
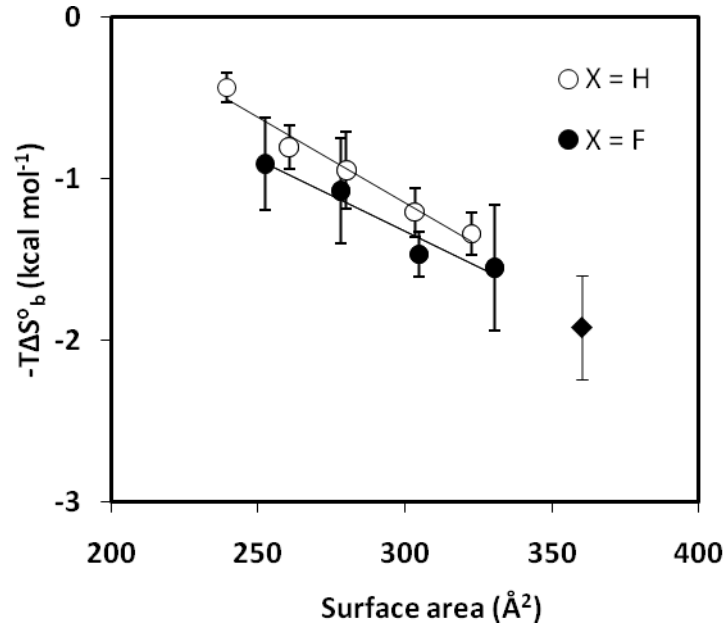


Figure S3. Dependence of $-T\Delta S°_b$ for binding of benzenesulfonamide ligands ($H_2NSO_2C_6H_4$-$CONHCH_2(CX_2)_nCX_3$, X = H, F) on surface area (top) and volume of ligands (bottom).



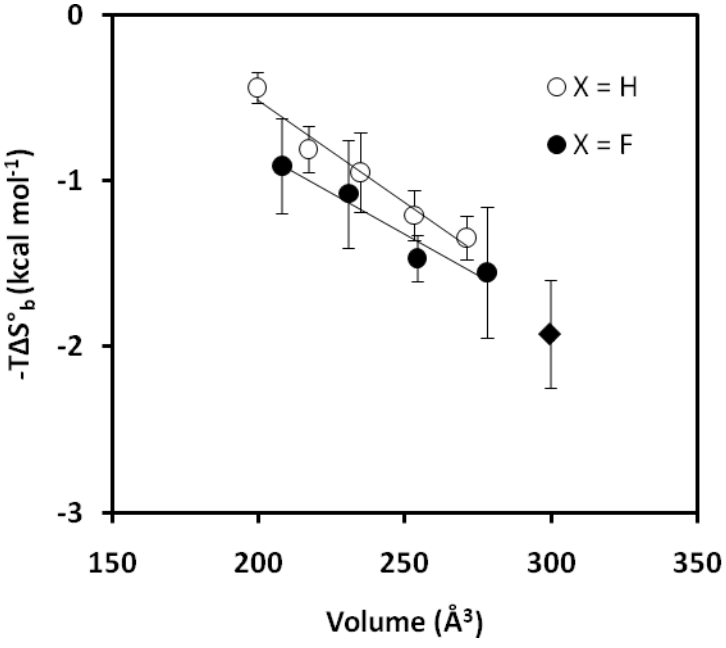


Figure S4. Electron density map shows ligand in the active site. The atoms of HCA and those of the ligands (R_H; n = 4) appear as stick representations, the Zn^{2+} appears as a silver sphere, and one water molecule appears as a red sphere. The mesh represents the electron density ($F_o - F_c$) derived from molecular replacement using the coordinates for a reported structure of the native protein (PDB: 2ILI). Electron density for the ligand appears dark blue and that for the protein light blue.

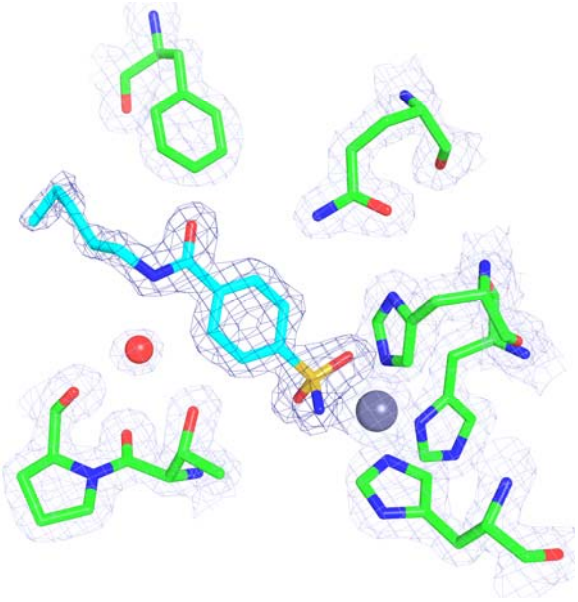


Figure S5. Comparing the ^{1}H NMR chemical shift of the amide hydrogen for R_H and R_F. Ligands exhibit a significant difference in the chemical shift between the two series ($\Delta\Delta H°_b$ = ~0.7 ppm), but the change in chemical shift across each series is negligible.

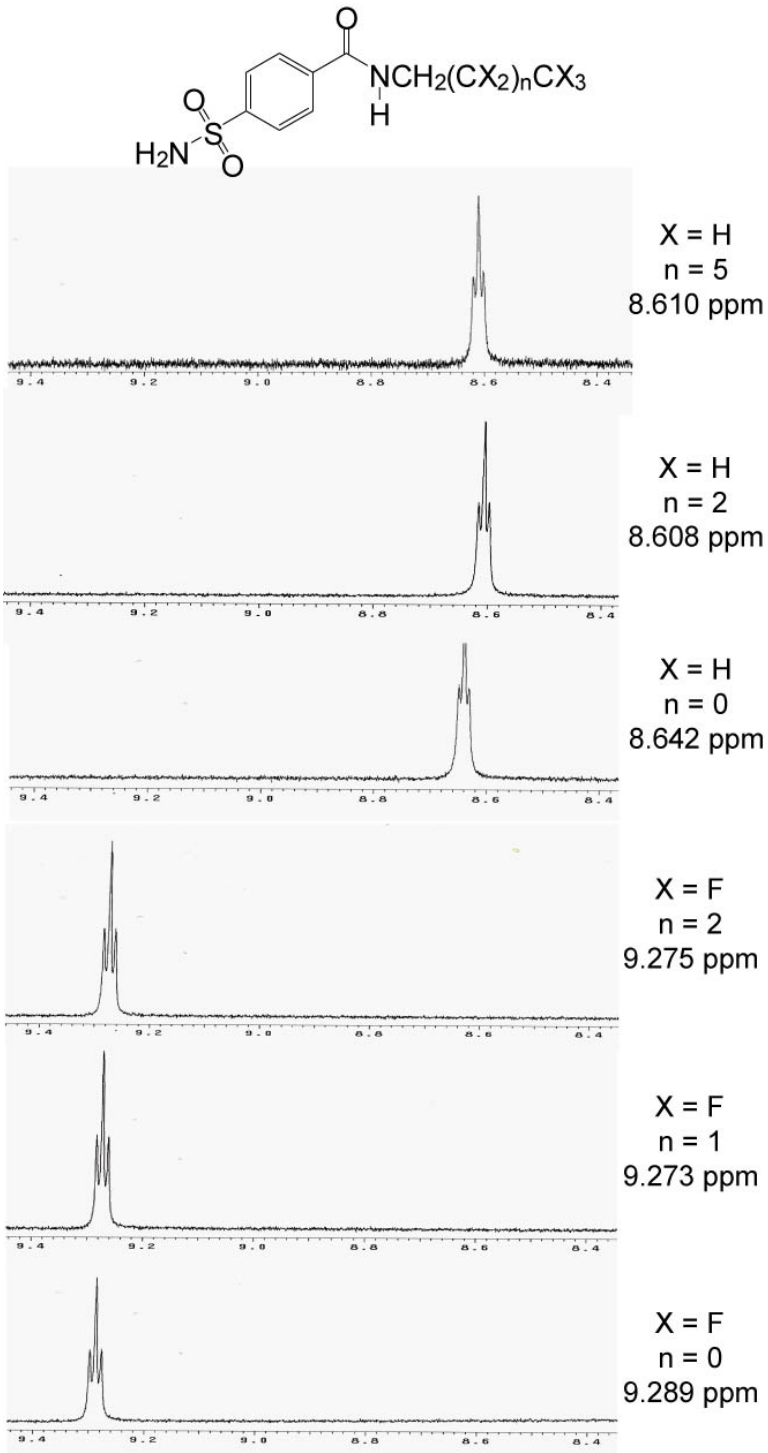


Figure S6. Individual images of the each of the ligands coordinated to the active site zinc ion. Structures for both R_H and R_F are presented from two different viewpoints.

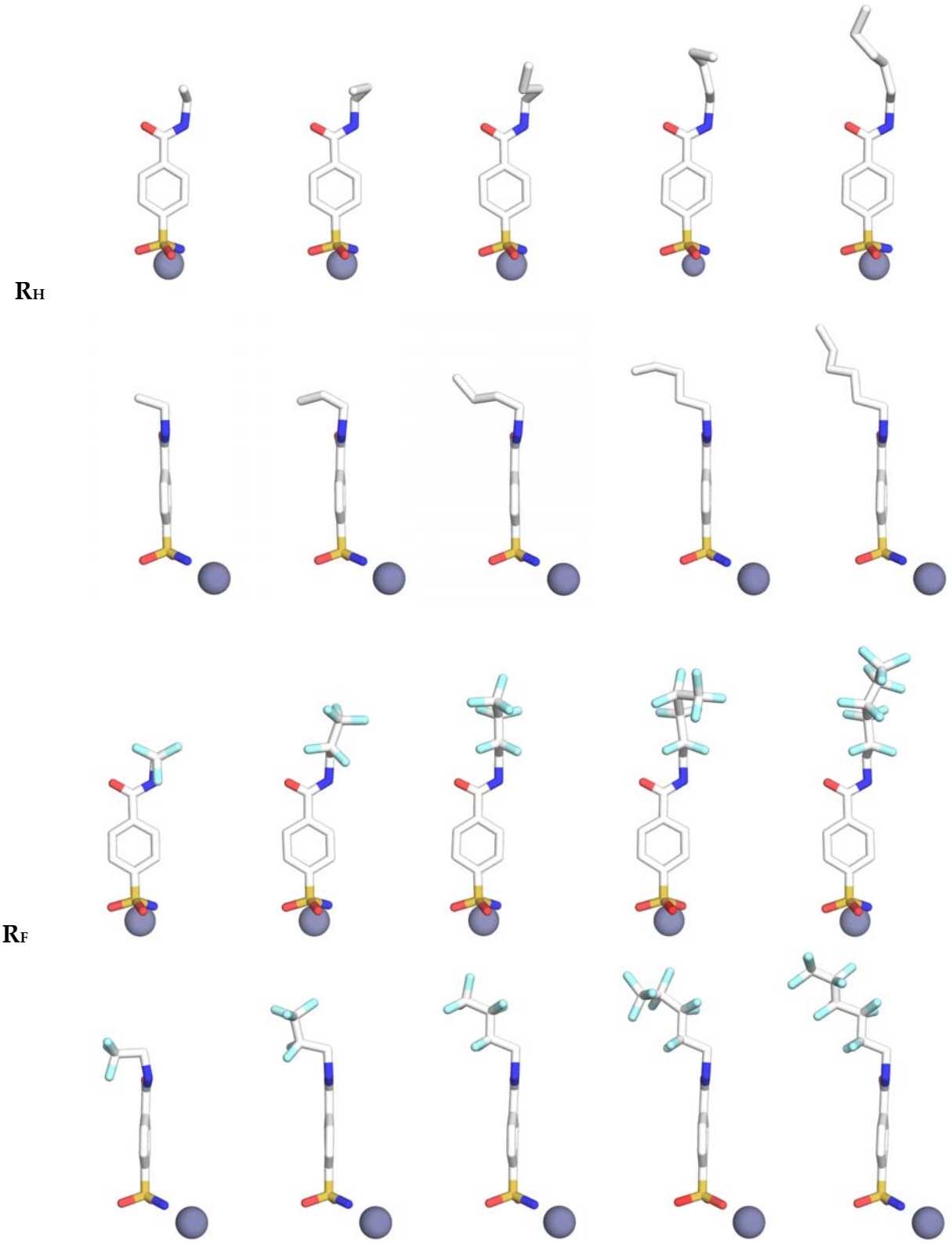
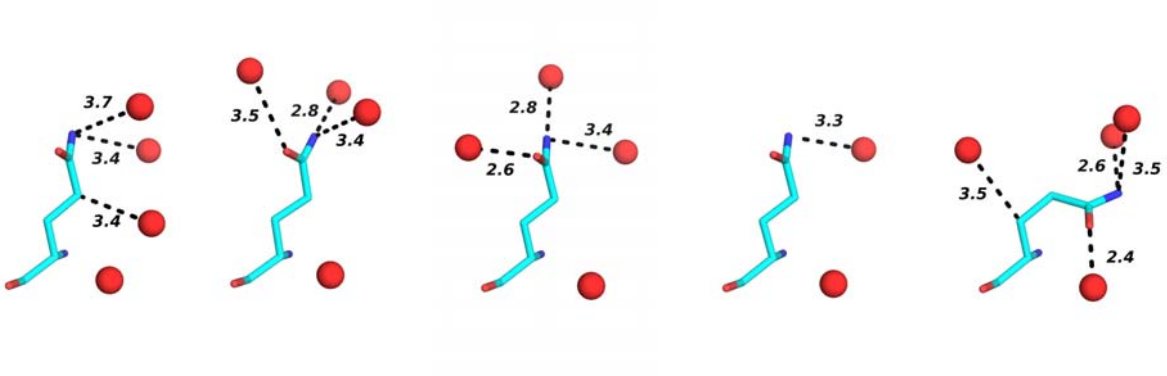


Figure S7. Crystallographically defined water molecules contacting Gln136 in structures with fluorinated ligands.



n = 0 n = 1 n = 2 n = 3 n = 4

Table S1. Crystallographic data.

X, n	**H, 0**	**H, 1**	**H, 2**	**H, 3**	**H, 4**	**F, 0**	**F, 1**	**F, 2**	**F, 3**	**F, 4**
Unit cell[a] (Å)	42.37, 41.45, 72.09	42.00, 41.18, 71.45	42.61, 41.87, 72.35	42.30, 41.63, 72.30	42.22, 41.54, 72.18	42.55, 41.72, 72.34	42.58, 41.78, 72.35	42.41, 41.70, 72.36	42.28, 41.68, 72.33	42.37, 41.88, 72.56
(°)	90.00, 104.54, 90.00	90.00, 104.51, 90.00	90.00, 103.37, 90.00	90.00, 104.52, 90.00	90.00, 104.56, 90.00	90.00, 104.20, 90.00	90.00, 103.58, 90.00	90.00, 104.81, 90.00	90.00, 104.66, 90.00	90.00, 104.65, 90.00
Resolution Range[b] (Å)	35.62-1.20 (1.22-1.19)	20.98-1.15 (1.19-1.16)	21.26-1.80 (1.85-1.80)	39.98-1.65 (1.69-1.65)	30.63-1.70 (1.75-1.70)	23.38-1.40 (1.43-1.39)	20.02-1.60 (1.64-1.60)	30.78-1.37 (1.41-1.37)	35.81-1.51 (1.55-1.51)	35.10-1.80 (1.84-1.80)
Reflections (#)	72206 (5133)	79221 (5376)	20971 (1502)	25854 (1287)	25463 (1865)	41793 (1369)	27856 (1642)	51504 (2950)	36181 (2588)	21145 (1484)
Completeness (%)	100 (100)	95.00 (91.33)	100 (100)	92.13 (61.56)	99.81 (99.19)	100 (100)	100 (100)	92.30 (83.30)	98.92 (96.50)	96.00 (91.52)
R_{factor}	0.133 (0.215)	0.124 (0.184)	0.138 (0.124)	0.148 (0.34)	0.130 (0.206)	0.131 (0.361)	0.165 (0.352)	0.138 (0.244)	0.127 (0.177)	0.128 (0.183)
R_{free}	0.168 (0.227)	0.152 (0.221)	0.206 (0.225)	0.202 (0.487)	0.186 (0.326)	0.170 (0.393)	0.240 (0.400)	0.183 (0.307)	0.177 (0.265)	0.203 (0.353)
Bond Length	0.026	0.028	0.023	0.024	0.024	0.025	0.023	0.025	0.025	0.023
Angles	2.275	2.312	1.984	1.807	1.94	2.063	2.005	2.138	2.089	1.956
PDBID	3RYV	3RYY	3RZ0	3RZ5	3RZ8	3RYJ	3RYX	3RYZ	3RZ1	3RZ7

[a] All crystals belonged to the $P2_1$ space group. [b]Values in parentheses represent those for the highest resolution shell.

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