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Antihydrophobic Solvent Effects: An Experimental Probe for the Hydrophobic Contribution to Enzyme−Inhibitor Binding

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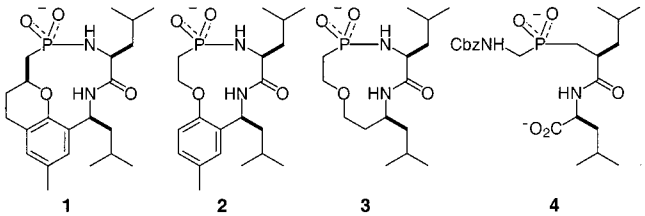
Abstract: The hydrophobic component to the binding affinities of one acyclic phosphinate (**4**) and three macrocyclic phosphonamidate inhibitors (**1**−**3**) to the zinc peptidase thermolysin was probed by varying the solvent composition. Increasing the percentage of ethanol in the buffer solution over the range 0−9% increases the inhibition constants, K_i, by up to an order of magnitude. This approach represents an experimental method for distinguishing solvation from conformational or other effects on protein−ligand binding. The size of the "antihydrophobic effect" is correlated with the amount of hydrophobic surface area sequestered from solvent on association of the inhibitor and enzyme, although it is attenuated from that calculated from the surface tension of ethanol−water mixtures. The results are consistent with the Lum−Chandler−Weeks explanation for the size dependence of the hydrophobic effect.

Introduction

The hydrophobic effect is an important component of the binding affinity in enzyme−inhibitor association, and indeed in virtually all solvent-mediated, noncovalent interactions between molecules of biological interest.[1,2] Common understanding ascribes the magnitude of the effect in protein−ligand association to the amount of hydrophobic surface area—on both components—that is desolvated on complexation.[3,4] Although easily conveyed by the "oil and water do not mix" analogy, the hydrophobic effect is remarkably complex at the molecular level, comprising both entropic and enthalpic terms that vary in importance with scale and configuration.[5−7] Notwithstanding our conceptual understanding of the phenomenon, evaluating the effect quantitatively is quite difficult, for the simple reason that it is not readily probed in isolation from other influences. Structural modifications to change contact areas also alter conformational preferences and steric interactions and thus result in perturbations in binding affinity that encompass more than solvation effects alone. However, Breslow and co-workers have shown that the hydrophobic effect can be assessed experimentally, in the context of bimolecular organic reactions, by modifying the solvent itself.[8,9] For example, the dimerization of cyclopentadiene is greatly accelerated in water, relative to organic solvents, because the hydrocarbon surface that is exposed decreases as the two substrate molecules come together in the transition state. Breslow et al. found that the rate of this reaction is attenuated progressively as the concentration of an organic cosolvent is increased. We reasoned that a similar strategy could be used to probe the hydrophobic contribution in an enzyme−inhibitor association process.

We chose an experimental system with a closely related series of inhibitors whose binding interactions with the target enzyme have been well characterized structurally. The three macrocyclic phosponamidates **1**−**3**, along with the acyclic phosphinate **4**, are potent inhibitors of the zinc peptidase thermolysin.[10,11] The structures of the complexes with all four compounds have been determined by X-ray crystallography, and the conformations adopted by the macrocycles in solution have been determined with a combination of NMR and molecular modeling.[10] Thermolysin itself is a robust enzyme that is resistant to denaturation both thermally and in the presence of organic cosolvents.[12]



1 **2** **3** **4**

Results and Discussion

The inhibition constants for **1**−**4** were determined in a conventional fashion using 2-furanacryloyl-Gly-Leu-NH_2 as sub-

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Table 1. Effect of Ethanol Concentration on Inhibitor Affinity (K_i) and Substrate Turnover (V/K) for Thermolysin and on Solubility (S) of Naphthalene[a]

% ethanol	inhibitor K_i values (nM) 1	2	3	4	V/K (%)	naphthalene solubility, S (mM)
0	7.7[b]	150[b]	1500[b]	290[b]	100	0.207
3	35[c]	640	5300	740	25	0.248
6	63	1100	8100	1300	9	0.301
9	84	1700	12000	1700[d]	4	0.366

[a] Buffer: 0.11 M MOPS at pH 7.0, containing 0.57 M NaBr, 11 mM $CaCl_2$, 2.5% DMF (v/v), 0.1% BSA (w/v), and the indicated amount of ethanol (0−12%, v/v); the substrate was 2-furanacryloyl-Gly-Leu-NH_2.[b] K_i^0. [c] K_i = 21 nM in 1.5% ethanol. [d] K_i = 2000 nM in 11% ethanol.

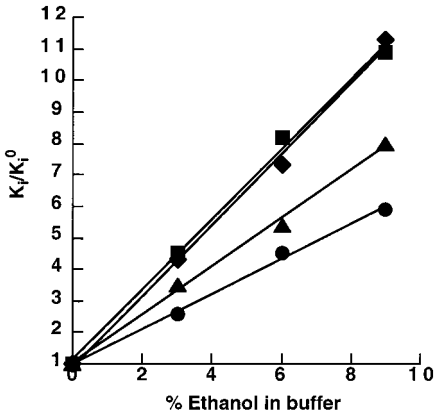

Figure 1. Increase in inhibition constant K_i as a function of solvent composition (K_i^0 = inhibition constant at 0% ethanol: ■, **1**; ◆, **2**; ▲, **3**; and ●, **4**).

strate (Table 1),[13] and in the presence of increasing amounts of ethanol as cosolvent. The high salt concentration typically used for thermolysin assays (e.g., [NaBr] = 2.5 M)[14] could not be attained in the presence of ethanol, so NaBr concentration was reduced to 0.57 M. Enzyme activity itself is attenuated significantly by increasing ethanol concentration because substrate binding, which is already weak in aqueous buffer at low salt concentrations (K_m > 10 mM),[13] becomes even weaker (Table 1).[15] The structure of the enzyme did not appear to be perturbed by the cosolvent: there was essentially no change in the CD spectrum of the enzyme over the 0−12% ethanol range.[12]

Two effects on inhibitor binding are immediately apparent from the data: the affinity decreases significantly as the concentration of ethanol increases, and the effect is greater for the larger inhibitors **1** and **2** than it is for **3** and **4**. A simple plot of the normalized inhibition constants (K_i/K_i^0, K_i^0 = inhibition constant at 0% ethanol) as a function of ethanol concentration illustrates these effects quite clearly (Figure 1).

From the X-ray structures of the inhibitor−thermolysin complexes, the total decrease in exposed surface area on binding can be calculated (Table 2). For the three complexes with the cyclic inhibitors **1**, **2**, and **3**, the differences in solvent-exposed surface area between the dissociated and associated forms are 510, 513, and 472 Å^2, respectively. Despite the differences in size of the tricyclic and bicyclic inhibitors **1** and **2**, the same

Table 2. Calculation of Surface Areas (Å^2) Sequestered from Solvent on Enzyme−Inhibitor Binding[a]

inhibitor	enzyme	inhibitor − total[b]	E·I complex	ΔA total (Å^2)
1	11 598	364	11 452	−510
2	11 598	349	11 434	−513
3	11 598	292	11 418	−472

[a] Surface areas were calculated using the Connolly algorithm implemented in InsightII and using the atomic radii of the original publication (no hydrogens in the molecules).[16,17] [b] Inhibitor surface areas were calculated from conformations determined in solution, with side chains fixed in the bound conformation.[10] This approach may overestimate the surface area of the free inhibitor in cases of "hydrophobic collapse".[18]

amount of surface area is buried when they bind because the additional ethylene unit in **1** does not contact the enzyme in the complex (Figure 2a). However, the aromatic rings of these inhibitors are stacked face-to-face with the His-231 imidazole,[11] an interaction that is missing from the complex with the monocyclic analogue **3** (Figure 2b); as a result, there is less hydrophobic surface sequestered from solvent when the latter binds.

The calculations of Table 2 are qualitatively consistent with the observation that solvent composition affects the affinities of **1** and **2** identically, and to a greater extent than the affinity of **3**. To quantitate these differences is more challenging. If the interactions of enzyme and inhibitors were purely hydrophobic, the effect of solvent on binding should be proportional to the effect on the solubility of a hydrocarbon such as naphthalene, with the magnitudes of the effect proportional to surface area buried.[9] We determined the solubility of naphthalene in the same buffer solutions; however, the effect of ethanol is quite different than that for the inhibitors (see Figure 3). The assumption that the enzyme−inhibitor interactions are purely hydrophobic is, of course, false: there are important ionic and polar interactions, such as that between the phosphonate anion and the active site zinc cation, which are enhanced by a reduction in solvent polarity. Thus, it is not surprising that the inhibition constants and naphthalene solubility are affected differently, and it appears that the linear correlations of inhibitor K_i/K_i^0 with solvent composition are purely coincidental.

The influence of solvent on the free energy of binding can be understood by considering how the various components of ΔG° are affected by solvent and by the structures of the inhibitor and the active site (eq 1). For the present discussion, ΔG° is

$$\Delta G^\circ_{bind} = \Delta G^\circ_{phobe} + \Delta G^\circ_{polar} + \Delta G^\circ_{nonsolv} \qquad (1)$$

dissected into the free energies of solvation of the nonpolar (hydrophobic) elements (ΔG°_{phobe}) and of the polar (ionic and hydrogen bonding) moieties (ΔG°_{polar}), and the contributions from intra- and intermolecular interactions (e.g., steric and conformational effects, $\Delta G^\circ_{nonsolv}$) that are independent of solvent but which can differ significantly among inhibitors.[19]

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(19) Williams has pointed out that most attempts to partition binding free energy between specific interactions fail to take into account the cooperativity among them that arises in any multipoint association process.[20,21] As a result of this cooperativity, the strength of one interaction is affected by the magnitude of another. Because perturbation of the solvent does not affect the intermolecular protein−ligand interactions that give rise to this effect, any influence of cooperativity on the free energy of binding is subsumed in the solvent-independent term $\Delta G^\circ_{nonsolv}$.

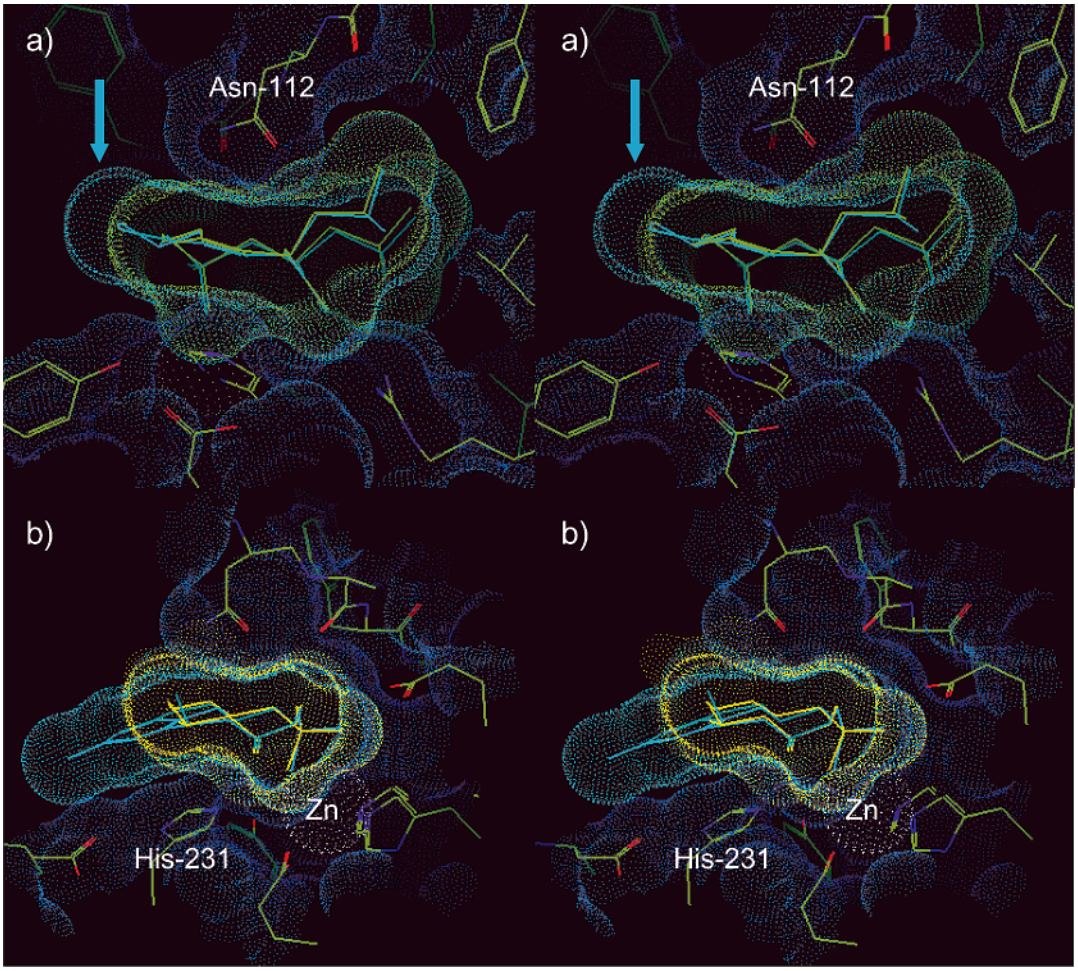

Figure 2. Views of the thermolysin active site with bound forms of macrocyclic inhibitors **1** (cyan), **2** (green), and **3** (yellow) superimposed (dots represent solvent-accessible surfaces of the individual components).[10] (a) Comparison of **1** and **2**: arrow indicates the bridging ethylene moiety of **1** that is absent in **2** but does not contact the protein (the aromatic rings of the inhibitors are truncated by the front clipping plane in this image). (b) Comparison of **1** and **3**, showing additional hydrophobic contact between the aryl moiety of **1** and the His-231 imidazole.

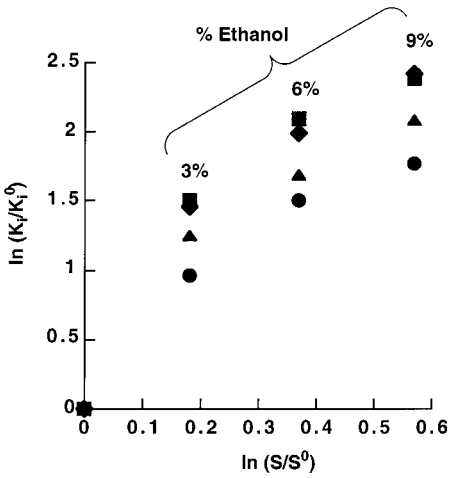

Figure 3. Logarithmic comparison of the effect of ethanol on inhibition constants (K_i/K_i^0) against naphthalene solubility ($S/S°$): ■, **1**; ◆, **2**; ▲, **3**; and ●, **4**.

Because the effects of solvent polarity are quite different for hydrophobic and polar surfaces, their relative importance cannot be determined quantitatively for any given inhibitor−enzyme pair.

However, comparisons within and between inhibitor series allow the individual contributions to $\Delta G°_{bind}$ to be isolated. From the relationship between $\Delta G°_{bind}$ and K_i, eq 1 can be transformed into eq 2 as a function of solvent composition,

$$-RT\ln(K_i/K_i^0) = \Delta\Delta G°_{phobe} + \Delta\Delta G°_{polar} \qquad (2)$$

whereby the solvent-independent term $\Delta G°_{nonsolv}$ drops out. Both $\Delta\Delta G°_{phobe}$ and $\Delta\Delta G°_{polar}$ are dependent on solvent composition as well as the respective surfaces that are buried on association (eq 3), although as noted above, these functions are

$$-RT\ln(K_i/K_i^0) = f(\text{solvent})\cdot\Delta\text{Å}^2{}_{phobe} + f'(\text{solvent})\cdot\Delta\text{Å}^2{}_{polar} \qquad (3)$$

different. Nevertheless, since the polar solvation effects are likely to be the same for the three cyclic inhibitors, this term can be negated for comparisons *between* inhibitor series (eq 4).

$$-RT\ln\frac{(K_i/K_i^0)^a}{(K_i/K_i^0)^b} = f(\text{solvent})\cdot\Delta\Delta\text{Å}^2{}_{phobe} \qquad (4)$$

Because the differences in structure among the cyclic inhibitors are almost entirely hydrophobic in nature, the differences in surface area buried on binding should closely approximate $\Delta\Delta$Å$^2{}_{phobe}$. But what is the appropriate parameter for estimating

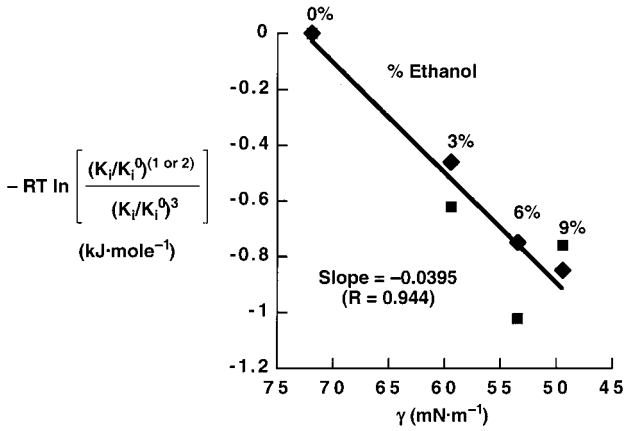

Figure 4. Comparison of tricyclic (■, **1**) and bicyclic (◆, **2**) inhibitors vs the monocyclic inhibitor (**3**), plotted against the surface tension, γ, according to eq 4.

f(solvent)? The surface tension, γ, as a measure of the energy per unit area required to create an interface with a nonmiscible fluid, has served as the experimental starting point for computational estimations of the hydrophobic effect.[6] Using the K_i/K_i^0 values for monocyclic inhibitor **3** in the denominator, the ratio-of-ratios of eq 4 is plotted in Figure 4 for the tri- and bicyclic inhibitors **1** and **2** against the surface tension of ethanol−water mixtures.[22] A good linear correlation is observed for bicycle **2**, and although that for tricycle **1** is inferior, similar slopes are found for each (the linear fit of Figure 4 is to the aggregated data for **1** and **2** vs **3**, since similar differences in $\Delta\Delta$Å^2 are involved).[23]

Several caveats must be acknowledged before any conclusion is drawn from this relationship. First, the ratio-of-ratios in eq 4 compounds any experimental errors in the K_i determinations. Second, the values for the surface tension are those of ethanol−water alone; the surface tension of the assay solution is altered by the presence of salts and 2.5% DMF. Third, and perhaps most importantly, the effect observed is substantially less than that calculated directly for the ca. 40 Å^2 differences in hydrophobic surface area buried on binding the tri- and bicyclic inhibitors **1** and **2** compared to the monocycle **3** (see Table 2). The units of surface tension are equivalent to energy per unit area: 1 mN·m^{-1} = 6.023 J·mol^{-1}·Å^{-2}. Thus, a reduction in surface tension of ca. 20 mN·m^{-1} should produce a change in $-RT \ln [(K_i/K_i^0)^a/(K_i/K_i^0)^b]$ of ca. $-6 \times 20 \times 40 = -4.8$ k J·mol^{-1} for a surface area difference of 40 Å^2·molecule^{-1}. A change of only 0.8 k J·mol^{-1} is observed for the correlation of Figure 4. Some of this difference arises from stabilizing van der Waals interactions between solvent and solute; for example, the water−hydrocarbon surface tension is 25% less than that of the air−water interface, where such interactions are absent.[6] However, most of the difference is explained by the Lum−Chandler−Weeks (LCW) treatment of hydrophobicity.[7] The magnitude of the hydrophobic effect is attenuated at molecular dimensions by the fact that concave surfaces with radii <10 Å disrupt the hydrogen bonding network of surrounding water much less than do extended surfaces. For example, for a solute with radius 2.5 Å (surface area of 79 Å^2), the hydrophobic effect *per Å^2 surface area* is only 25% that for a solute of radius 10 Å. Observation of a hydrophobic effect over 40 Å^2 that is only 1/6 of that predicted from macroscopic values of solvent−air surface tensions is thus consistent with the LCW analysis. Since the hydrophobic effect varies nonlinearly with surface area at the molecular level and also depends strongly on the configuration of the interface (e.g., concave, flat, convex), it is an oversimplification to use the macroscopic parameter of surface tension as *f*(solvent) in eq 4. Thus, even though we are able to probe the hydrophobic effect in isolation from other influences on binding, ready quantitation of its magnitude remains elusive at the molecular level.[6]

Conclusions

Our results suggest that antihydrophobic solvent effects can be used to isolate experimentally the hydrophobic component of binding from the multitude of other influences on protein ligand affinity. In the case of the thermolysin inhibitors, the results are consistent with the structural evidence, in that the antihydrophobic effect is the same for the inhibitors that bury comparable amounts of hydrophobic surface on binding, and smaller for the inhibitors that bury less. Since the creation of surface in a hydroxylic solvent and the creation of hydrophobic contact are both resisted by hydrogen bonding, the correlation of $-RT \ln [(K_i/K_i^0)^a/(K_i/K_i^0)^b]$ with surface tension (eq 4) is consistent with long-established understanding of the hydrophobic effect. However, while a qualitative relationship is found as a function of inhibitor structure and surface tension (Figure 4), and the magnitude of the effect observed is consistent with the Lum−Chandler−Weeks theory, the quantitative correlation remains elusive.

Experimental Section

Synthesis of Inhibitors. The synthesis of tricyclic inhibitor **1** and acyclic inhibitor **4** and details of the structures of their thermolysin complexes have been described previously.[11,14] The syntheses and binding characteristics of the bi- and monocyclic inhibitors **2** and **3** will be described elsewhere.[10]

Assay Procedures. The standard aqueous buffer for all assays was 0.114 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.570 M NaBr, and 11.4 mM $CaCl_2$ and 2.5% v/v DMF, adjusted to pH 7.00 at room temperature. The assay buffer included 0.1% w/v of bovine serum albumin (BSA) and the indicated amount of absolute ethanol. Thermolysin was obtained from Calbiochem (3× recrystallized) and used without further purification; the concentration of enzyme stock solutions was determined by UV absorbance (ϵ_{280} (1%) 17.65 cm^{-1}).[24] The concentrations of stock solutions of enzyme, furanacryloyl-glycyl-leucinamide (FaGLa, ϵ_{345} 766 M^{-1} cm^{-1}), and inhibitors **1**, **2**, and **4** were determined by UV absorbance; that of inhibitor **3** was determined from an accurately weighed sample. Assay samples included thermolysin to a concentration of 1.1 to 8.5% of K_i for inhibitors **3** and **4** and between 15 and 64% of K_i for inhibitors **1** and **2**, inhibitor to a concentration of 0.4−10 times K_i in a total volume of 100 μL. The assay was initiated by the addition of FaGLa (1.2 mM) and differences in absorbance at 345 nm were measured. All velocities were determined for ≤10% of total reaction and were reproducible within ±8%. The K_i

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(23) The acyclic analogue **4** fits quite poorly in this surface tension correlation, if referenced to one of the macrocycles. The carbamate and carboxylate moieties of this inhibitor, which are not present in the macrocycles, do not allow the effect of solvent on the binding of polar groups (*f′*(solvent), see eq 4) to be factored out.
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values were determined from Dixon plots[25] for inhibitors **3** and **4** and from Henderson plots[26] for the more potent inhibitors **1** and **2**. The $[S]/K_m$ term was neglected for all analyses because the substrate concentration (1.2 mM) was much smaller than K_m (>10 mM).

Determination of the Solubility of Naphthalene. Excess solid naphthalene was equilibrated with buffers identical with those used to determine the inhibition constants by shaking the mixture at room temperature. Concentrations of naphthalene in the supernatant were determined spectrophotometrically (log ϵ_{266} 3.7) after 7 days and again after 9 days to ensure that the solutions were saturated.

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Supporting Information Available: Dixon and Henderson plots for determination of inhibition constants as a function of ethanol concentration; CD spectra of thermolysin in ethanol/buffer mixtures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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