HIV-1 Protease Inhibitors: Enthalpic versus Entropic Optimization of the Binding Affinity†

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Received October 15, 1999; Revised Manuscript Received December 7, 1999

ABSTRACT: Existing experimental as well as computational screening methods select potential ligands or drug candidates on the basis of binding affinity. Since the binding affinity is a function of the enthalpy (ΔH) and entropy (ΔS) changes, it is apparent that improved binding can be achieved in different ways: by optimizing ΔH, ΔS, or a combination of both. However, the behavior of enthalpically or entropically optimized inhibitors is fundamentally different, including their response to mutations that may elicit drug resistance. In the design of HIV-1 protease inhibitors, high binding affinity has usually been achieved by preshaping lead compounds to the geometry of the binding site and by incorporating a high degree of hydrophobicity. The thermodynamic consequence of that approach is that the binding affinity of the resulting inhibitors becomes entropically favorable but enthalpically unfavorable. Specifically, the resulting high binding affinity is due to an increased solvation entropy (hydrophobic effect) combined with a reduced loss of conformational entropy of the inhibitor upon binding (structural rigidity). Here we report that tripeptide inhibitors derived from the transframe region of Gag-Pol (Glu-Asp-Leu and Glu-Asp-Phe) bind to the HIV-1 protease with a favorable enthalpy change. This behavior is qualitatively different from that of known inhibitors and points to new strategies for inhibitor design. Since the binding affinities of enthalpically favorable and enthalpically unfavorable inhibitors have opposite temperature dependence, it is possible to design fast screening protocols that simultaneously select inhibitors on the basis of affinity and enthalpy.

Ligand or inhibitor design involves the optimization of the binding affinity of a lead compound toward a selected target. Since the binding affinity is determined by the Gibbs energy [$K_a = \exp(-\Delta G/RT)$] and ΔG is given by $\Delta G = \Delta H - T\Delta S$, it is apparent that binding optimization can be accomplished either by making ΔH more negative, making ΔS more positive, or an appropriate combination of both. Because experimental screening methods as well as computer-based algorithms select for affinity, the reasons for binding improvements are often not immediately known; however, they can have important consequences from the point of view of side effects (e.g., drug resistance). All known inhibitors of the HIV-1 protease for which data are available, including those in clinical use, bind to the active site with an unfavorable or only slightly favorable enthalpy change, depending on the exact temperature, in a process driven by a large favorable entropy change. Most of the entropy gain arises from the desolvation of nonpolar groups upon binding (a consequence of the hydrophobicity of the inhibitors) and from a rather small loss in conformational entropy upon binding (a consequence of preshaping the inhibitor to the geometry of the binding site). The unfavorable enthalpy change, on the other hand, is due to the burial of hydrophobic groups and, in part, to the energy required to bring the flap regions into the bound conformation (*1*−*3*).

We have previously suggested that the reduced flexibility of inhibitors preshaped to the wild-type binding site accentuates their susceptibility to mutations that distort the protease binding site (*2*). The difficulty of rigid inhibitors to adapt to an altered binding site geometry lowers the strength of van der Waals, hydrogen bond, and other favorable interactions, resulting in a diminished binding affinity. It is apparent that flexible inhibitors will be more accommodating to protease mutations. However, adding flexibility to existing inhibitors will lower their binding affinity because a greater conformational entropy will be lost upon binding. Therefore, flexibility needs to be compensated by additional favorable interactions. These interactions cannot be hydrophobic because the existing inhibitors are already highly hydrophobic and because flexible hydrophobic ligands will lack specificity. The alternative is an enthalpic optimization that will provide the required additional binding affinity and the necessary target specificity. However, lead compounds that bind with a favorable enthalpy are difficult to find due to the high hydrophobic character of most chemical libraries. By using high-sensitivity calorimetry and structural analysis, we have characterized a new type of peptide inhibitor that binds to the HIV-1 protease with a negative enthalpy change. To our knowledge, these are the first HIV-1 protease inhibitors that

† Supported by grants from the National Institutes of Health (GM 51362 and GM 57144). A.V.-C. was partially supported by a postdoctoral fellowship from the Universidad de Granada, Spain (Plan Propio 1,999).

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10.1021/bi992399d CCC: $19.00
Published on Web 02/12/2000

exhibit a favorable binding enthalpy. These characteristics as well as the structural distribution of their interactions point to new strategies for inhibitor design.

EXPERIMENTAL PROCEDURES

Protease Purification. HIV-1 protease was prepared as described previously (*4*) using a procedure optimized for the high yield, activity, and stability required for calorimetric analysis. Plasmid-encoded mutant protease (Q7K/L33I/L63I; a gift from A. G. Tomasselli, designed to remove three hypersensitive autolytic sites) was expressed as inclusion bodies in *Escherichia coli* 1458. Cells were suspended in extraction buffer [20 mM Tris, 1 mM EDTA, and 10 mM 2-ME (pH 7.5)] and broken with two passes through a French pressure cell (≥15000 psi). Cell debris and protease-containing inclusion bodies were collected by centrifugation (18000*g* for 20 min at 4 °C). Inclusion bodies were resuspended in extraction buffer using a glass homogenizer, then diluted with an equal volume of 100% acetic acid to solubilize the protease, and centrifuged (30000*g* for 20 min at 4 °C) to remove nonsoluble constituents. Protease was purified from washed inclusion bodies using two ion exchange chromatographic steps. HOAc-solubilized inclusion bodies (pH ≈1.0) were applied directly to a cation exchange S-Sepharose column (SP-Sepharose HP, Pharmacia) equilibrated with S buffer [20 mM MES, 1 mM glycyl-glycine, 1 mM EDTA, 20 mM 2-ME, and 6 M urea (pH 6.0)]. The column was washed with S buffer until the pH reached 6.0, and then with 2.5 volumes of S buffer at pH 9.0. HIV-1 protease remained bound through both washes (i.e., pI = 9.2) and was eluted, after re-equilibrating with S buffer at pH 6.0, using a steep salt gradient to 0.5 M NaCl. Fractions containing protease were pooled, concentrated, and dialyzed overnight with Q buffer [10 mM Tris, 1 mM EDTA, 20 mM 2-ME, and 6 M urea (pH 8.5)] and washed through an anion exchange Q-Sepharose column (Q-Sepharose HP, Pharmacia). The protease passed through the column and was acidified by adding 25 mM formic acid immediately upon elution from the column. After being passed through the two columns, protease-containing fractions were pooled, concentrated, and stored at 4 °C at 5−10 mg/mL.

The HIV-1 protease was folded by 10-fold stepwise dilution into 10 mM formic acid at 0 °C. The pH was gradually increased to 3.8, and then the temperature was raised to 30 °C. Sodium acetate (pH 5.0) was added to a final concentration of 100 mM, and the protein was concentrated. Folded protease was desalted into 1 mM sodium acetate at pH 5.0 (PD-10, Pharmacia) and stored at either 4 or −20 °C (≥2.5 mg/mL). After folding, protease was estimated to be ≥99% pure.

Spectrophotometric Enzymatic Assays. The specific activity of the HIV-1 protease preparations was measured by following the hydrolysis of the chromogenic substrate Lys-Ala-Arg-Val-Nle-nPhe-Glu-Ala-Nle-NH_2 where Nle is norleucine and nPhe[1] is *p*-nitrophenylalanine (California Peptide Research Inc., Napa, CA). Protease was added to a 120 μL microcuvette containing substrate at 25 °C. Final concentrations in the standard assay were as follows: 100 nM protease, 100 μM substrate, 100 mM sodium acetate, and 1 M NaCl (pH 5.0). The absorbance was monitored at five wavelengths (296−304 nm) using a HP 8452 diode array spectrophotometer (Hewlett-Packard) and corrected for spectrophotometer drift by subtracting the average absorbance at 446−454 nm. An extinction coefficient for the difference in absorbance upon hydrolysis (1800 M^{-1} cm^{-1} at 300 nm) was used to convert absorbance change to reaction rates. Hydrolysis rates were obtained from the initial portion of the data, where at least 80% of the substrate remains free. Typical protease preparations hydrolyzed chromogenic substrate at a rate of 4 s^{-1} (per dimer) at 25 °C.

Differential Scanning Calorimetry. The heat capacity function of the HIV-1 protease was measured as a function of temperature with a high-precision differential scanning VP-DSC microcalorimeter (Microcal Inc., Northampton, MA). Protein samples and reference solutions were properly degassed and carefully loaded into the cells to avoid bubble formation. Exhaustive cleaning of the cells was undertaken before each experiment. Thermal denaturation scans were performed with freshly prepared protease, buffer-exchanged into 10 mM glycine (pH 3.6). HIV-1 protease (at 0.21 mg/mL), with different concentrations of Glu-Asp-Leu (California Peptide Reseach Inc.), was examined for thermal denaturation at a scanning rate of 1 °C/min, from 15 to 85 °C. Protease specific activity was determined in unheated samples and compared to those of samples that underwent thermal denaturation. Reversibility for a single cycle was at least 80%. Data were analyzed by software developed in this laboratory.

Isothermal Titration Calorimetry. Isothermal titration calorimetry experiments were performed using a high-precision VP-ITC titration calorimetric system (Microcal Inc.). The enzyme solution (62.8 μM dimer) in the calorimetric cell was titrated with Glu-Asp-Leu or Glu-Asp-Phe dissolved in the same buffer (at a concentration of 3 mM in the injection syringe). The heat evolved after each inhibitor injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction between the inhibitor and the enzyme was obtained as the difference between the heat of reaction and the corresponding heat of dilution. Analysis of the data was performed using software developed in this laboratory.

Temperature Dependence of Inhibition. The temperature dependence of the protease inhibition by Glu-Asp-Leu and acetyl pepstatin (Peptides International, Louisville, KY) was assessed as described for the spectrophotometric assays using a thermostated cuvette holder. These experiments were performed at substrate concentrations much lower than the K_m for the chromogenic substrate to evaluate the temperature dependence of the inhibition constant with negligible influence from the temperature dependence of K_m. It can be shown that at low substrate concentrations ([S] ≪ K_m), both competitive and noncompetitive inhibitors will exhibit the same apparent kinetic behavior. For competitive inhibition, the initial velocity v(I) in the presence of a concentration of inhibitor [I]

$$v(\mathrm{I}) = \frac{k_{cat}[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_I}\right)+[\mathrm{S}]} \approx \frac{k_{cat}[\mathrm{S}]}{K_m\left(1+\frac{[\mathrm{I}]}{K_I}\right)} \qquad (1)$$

and for noncompetitive inhibition

[1] Abbreviations: Sta, statine; Nle, norleucine; nPhe, *p*-nitrophenylalanine.

$$v(\mathrm{I}) = \frac{k_{cat}[\mathrm{S}]}{K_m\left(1 + \frac{[\mathrm{I}]}{K_I}\right) + [\mathrm{S}]\left(1 + \frac{[\mathrm{I}]}{K_I}\right)} \approx \frac{k_{cat}[\mathrm{S}]}{K_m\left(1 + \frac{[\mathrm{I}]}{K_I}\right)} \quad (2)$$

From the above equations, it follows that under conditions in which $[S] \ll K_m$, the fractional decrease in activity due to the presence of inhibitor $[v(I)/v(0)]$ is simply

$$\frac{v(\mathrm{I})}{v(0)} = \frac{K_I}{(K_I + [\mathrm{I}])} \quad (3)$$

indicating that under these conditions, the temperature dependence of K_I can be isolated from that of K_m.

RESULTS AND DISCUSSION

Binding Energetics. Figure 1A shows the results of a calorimetric titration of the tripeptide inhibitor Glu-Asp-Leu into the HIV-1 protease. Analysis of the data yields a binding affinity of $4.5 \times 10^4\ M^{-1}$ ($K_D = 22\ \mu M$) under the conditions of this experiment [10 mM glycine (pH 3.6)]. This value is very close to the inhibition constant ($K_I = 20\ \mu M$) published previously by Louis et al. (*5*) under similar conditions. The most notable feature of this experiment is the fact that the heat effect associated with binding is exothermic. In fact, the association of the inhibitor Glu-Asp-Leu with the HIV-1 protease is characterized by a favorable enthalpy change of −3.6 kcal/mol. Calorimetric experiments performed with a second tripeptide inhibitor derived from the transframe region of Gag-Pol (Glu-Asp-Phe) (Figure 1B) also yielded a favorable enthalpy change of −4.5 kcal/mol. Under the conditions of the experiments whose results are depicted in Figure 1 [10 mM glycine (pH 3.6)], at which a decrease in pH no longer influences the binding affinity significantly (*5*), the binding reaction is also favored by a positive entropy change of 9.2 and 6.0 cal K^{-1} mol^{-1}.

We have shown previously that structural stability and inhibitor binding are linked functions, and that the temperature stability of the protease is a function of the concentration and binding affinity of the inhibitor (*3*). Because of the negative binding enthalpy, the binding affinity of Glu-Asp-Leu is not temperature-independent but a decreasing function of temperature. Figure 2 shows the dependence of the denaturation temperature of the HIV-1 protease on the concentration of Glu-Asp-Leu. The solid line in the figure represents the expected dependence of the denaturation temperature on inhibitor concentration. The Gibbs energy for the structural stability of the protease was calculated according to the master equation derived experimentally in a previous publication (*4*):

$$\Delta G(T,\mathrm{pH},[\mathrm{I}]) = -9000 + 3200(T - 298.15) - T[-79.15 + 3200\ln(T/298.15)] - 4RT\ln\left(\frac{(1 + 10^{4.3-\mathrm{pH}})}{(1 + 10^{2.9-\mathrm{pH}})}\right) + RT\ln(1 + K_a[\mathrm{I}]) \quad (4)$$

The first part on the right-hand side of eq 4 represents the intrinsic and protonation contributions to the Gibbs energy (*4*) and the last term the effect of inhibitor concentration [I] on the stability of the protease. The inhibitor binding constant K was calculated with the parameters measured in this paper. The agreement between experimental and calculated values confirms the accuracy of the thermodynamic parameters obtained by isothermal titration calorimetry and the exothermic nature of Glu-Asp-Leu binding.

The experiments presented above demonstrate that the binding of Glu-Asp-Leu or Glu-Asp-Phe to the HIV-1 protease is qualitatively different from that of other protease inhibitors. For comparison, the inset in Figure 1A shows the results of a calorimetric titration obtained with the well-known protease inhibitor acetyl pepstatin (Ac-Val-Val-Sta-Ala-Sta) under identical conditions. It is clear that the heat effects have opposite signs. Additional calorimetric titrations with Indinavir, Saquinavir, and Nelfinavir, protease inhibitors currently in clinical use, also yield positive enthalpies on the order of 3−5 kcal/mol (M. J. Todd and E. Freire, in preparation). Hoog et al. (*6*) previously found that another synthetic inhibitor (SB203386) also exhibited a positive binding enthalpy of 6.4 kcal/mol with the homologous SIV protease. This behavior should be contrasted with that of Glu-Asp-Leu and Glu-Asp-Phe which exhibit exothermic binding.

In addition to the enthalpy change, another important thermodynamic quantity is the heat capacity change, ΔC_p, which is related to changes in solvation associated with binding (*7*−*10*). Experimentally, ΔC_p is equal to the temperature dependence of the enthalpy change and can be obtained from titrations performed at different temperatures (*9*). For Glu-Asp-Leu, experiments performed between 15 and 35 °C (not shown) yield a ΔC_p of − 60 cal K^{-1} mol^{-1}. This value is smaller than that obtained with more hydrophobic inhibitors. In particular, under identical conditions the heat capacity change associated with the binding of acetyl pepstatin is −452 cal K^{-1} mol^{-1} (*2*, *3*).

Temperature Dependence of Enzyme Inhibition. The most immediate difference between a binding process characterized by a positive or negative enthalpy change is in the temperature dependence of the binding affinity. An exothermic binding process will be characterized by a decrease in binding affinity upon a temperature increase, while an endothermic binding process will exhibit the opposite behavior. In enzyme inhibition assays, the temperature dependence of the inhibition constant must be dissected from the temperature dependence of K_m and V_{max}. Figure 3 shows the dependence of $v(I)/v(0)$ on inhibitor concentration at two different temperatures. The experiments were performed with Glu-Asp-Leu, an enthalpically favorable inhibitor (see above), and with acetyl pepstatin, an enthalpically unfavorable inhibitor (*2*, *3*). It is evident that the binding affinity of Glu-Asp-Leu is lower at 35 °C than at 15 °C, whereas acetyl pepstatin exhibits a higher affinity at 35 °C. Analysis of the data in terms of eq 3 (solid lines) yields inhibition constants of 9 and 16 μM for Glu-Asp-Leu and 1.0 and 0.3 μM for acetyl pepstatin at 15 and 35 °C, respectively. These values are consistent with those obtained by other methods. The van't Hoff enthalpies calculated from the data in Figure 3 are −5 and 10.5 kcal/mol for Glu-Asp-Leu and acetyl pepstatin, respectively, which are close to those measured directly by calorimetric titrations (−3.6 and 7.3 kcal/mol, respectively).

The results shown in Figure 3 indicate that fast screening methods need not be restricted to binding affinity and that by performing the experimental screening of potential

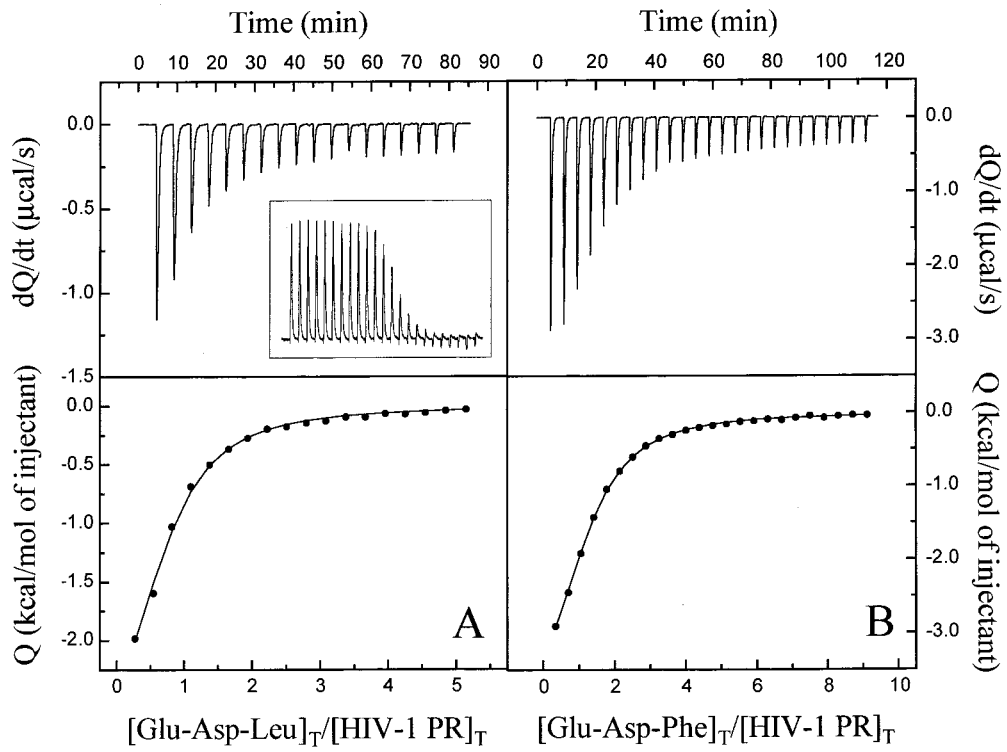

FIGURE 1: Calorimetric titration of HIV-1 protease with the inhibitor Glu-Asp-Leu (A) and Glu-Asp-Phe (B) in 10 mM glycine (pH 3.6). Top panels show the heat effects associated with the injection of Glu-Asp-Leu or Glu-Asp-Phe (10 μL per injection of a 3 mM solution) into the calorimetric cell (1.4 mL) containing HIV-1 protease at a concentration of 62.8 μM dimer. The experiments were performed at 25 °C. Bottom panels show the binding isotherm corresponding to the data in the top panels and the best fitted curves (see the text for details). The inset in panel A shows the result of a calorimetric titration of the HIV-1 protease with the inhibitor acetyl pepstatin (*3*). Note that the heat effects for Glu-Asp-Leu or Glu-Asp-Phe are exothermic whereas those for acetyl pepstatin are endothermic. In fact, contrary to other protease inhibitors, the binding of these tripeptide inhibitors is characterized by a favorable enthalpy change.

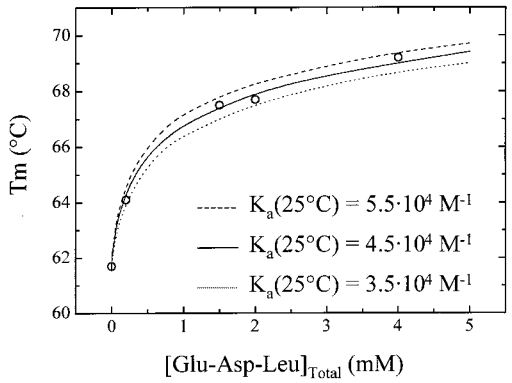

FIGURE 2: Dependence of the denaturation temperature of the HIV-1 protease on Glu-Asp-Leu concentration. The solid lines were calculated with the master equation for the Gibbs energy for the structural stability of the HIV-1 protease (*3*) and the thermodynamic parameters for the binding of Glu-Asp-Leu. The solid line shows the predicted values obtained with the association constant measured at 25 °C [K_a(25 °C)]. The dashed and dotted lines represent the upper and lower bounds for the association constant at 25 °C, respectively. In all cases, the binding enthalpy was −3.6 kcal/mol at 25 °C.

inhibitors at a minimum of two temperatures it is also possible to identify those compounds that bind to their targets with either positive or negative enthalpies.

Structure-Based Thermodynamic Analysis. The structure of the complex of Glu-Asp-Leu and the HIV-1 protease has been determined crystallographically at 2.0 Å resolution (PDB file 1A30) (*5*). Analysis of the complex using the structural parametrization of the energetics published previously (*7*, *10*−*15*) yields a binding enthalpy of −5.6 kcal/mol and a ΔC_p of −80 cal K^{-1} mol^{-1} in good agreement with the experimental data. Figure 4A−C shows the distribution of binding interactions within the inhibitor−protease complex. In contrast to those of other inhibitors that span the active site, the interactions of Glu-Asp-Leu are not symmetrically distributed within the two subunits of the protease molecule. One of the subunits contributes more than 70% of the binding contacts. Particularly strong interactions are seen between polar residues Glu and Asp with Gly 27, Asp 29, Asp 30, and Gly 48 from the first subunit and Arg 8 from the second subunit. The side chain of the terminal Leu is completely buried, making a significant hydrophobic contribution to the Gibbs energy and also establishing strong interactions with Asp 25 and Val 82 in the second subunit.

According to the structure-based thermodynamic analysis, most of the enthalpically favorable interactions are due to the polar interactions established by Glu and Asp, whereas the highly hydrophobic C-terminal Leu makes a slightly unfavorable contribution to the binding enthalpy (Figure 4B). The large contribution of the C-terminal Leu to the binding affinity is primarily due to the large increase in solvent entropy resulting from the burial of a significant hydrophobic surface area (∼115 Å^2) (Figure 4C). In total, 400 Å^2 of nonpolar and 380 Å^2 of polar surface area are buried upon binding of Glu-Asp-Leu (a nonpolar/polar ratio of close to 1). The large fraction of polar surface area buried upon binding gives rise to the favorable enthalpy change (*12*). These results should be contrasted with those obtained for

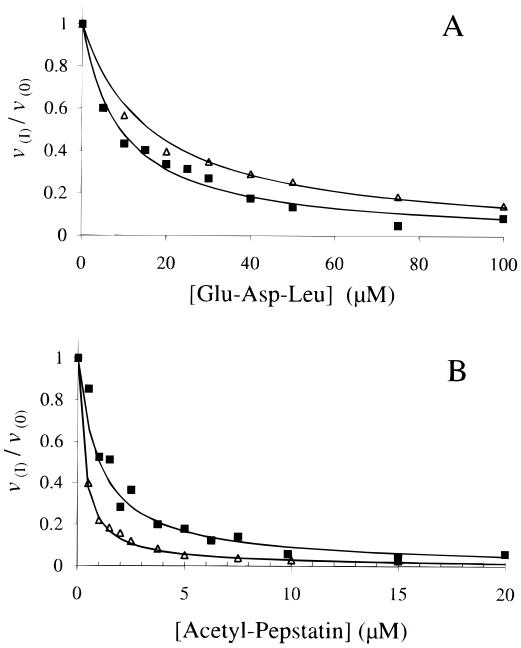

FIGURE 3: Temperature dependence of HIV protease inhibition for an exothermic and endothermic inhibitor. Fractional activities, measured at increasing concentrations of Glu-Asp-Leu (A) or acetyl pepstatin (B), were determined at 15 (■) or 35 °C (△), as described in Experimental Procedures. Inhibitors with favorable enthalpy (Glu-Asp-Leu) show decreased affinity as the temperature is increased. Inhibitors with unfavorable enthalpy (acetyl pepstatin) show increased affinity with increasing temperature. The inhibition by acetyl pepstatin was measured in 10 mM NaOAc (pH 5.0) using 50 μM chromogenic substrate. The inhibition by Glu-Asp-Leu was assessed in 10 mM sodium formate (pH 3.5) using 40 μM substrate. In both cases, [S] $\ll K_m$ ~2.5 mM.

other inhibitors. Acetyl pepstatin is significantly more hydrophobic (Ac-Val-Val-Sta-Ala-Sta), and its binding at 25 °C is dominated by a large solvation entropy (82 cal K^{-1} mol^{-1}) and an unfavorable enthalpy change (7.3 kcal/mol) (*2*, *3*). For acetyl pepstatin, 854 Å^2 of nonpolar and 464 Å^2 of polar surface area are buried upon binding (i.e., a 1.84 nonpolar/polar ratio). The nonpolar/polar ratio is even larger for inhibitors in clinical use, saquinavir, ritonavir, indinavir, nelfinavir, and VX478 (2.9, 2.4, 3.4, 3.2, and 2.6, respectively). For the binding of SB203386 to the SIV protease, which is also characterized by an unfavorable enthalpy (*6*), 666 Å^2 of nonpolar and 295 Å^2 of polar surface area are buried upon binding, i.e., a 2.25 nonpolar/polar ratio (PDB file 1TCW). As expected, the higher the proportion of nonpolar surface area buried upon binding, the more unfavorable the enthalpy change.

In addition to the loss of translational entropy, the major force opposing binding is the loss of conformational entropy. In solution, Glu-Asp-Leu exhibits the characteristic degrees of freedom associated with linear peptides (*11*, *13*). Upon binding, the backbone and side chains lose conformational degrees of freedom, resulting in a conformational entropy loss of close to −20 cal K^{-1} mol^{-1}. Additionally, side chains in the binding site and part of the flap in the protease also lose conformational entropy. The total conformational entropy loss is close to −25 cal K^{-1} mol^{-1} which is compensated by a favorable gain in solvation entropy of 34 cal K^{-1} mol^{-1} and a favorable enthalpy change.

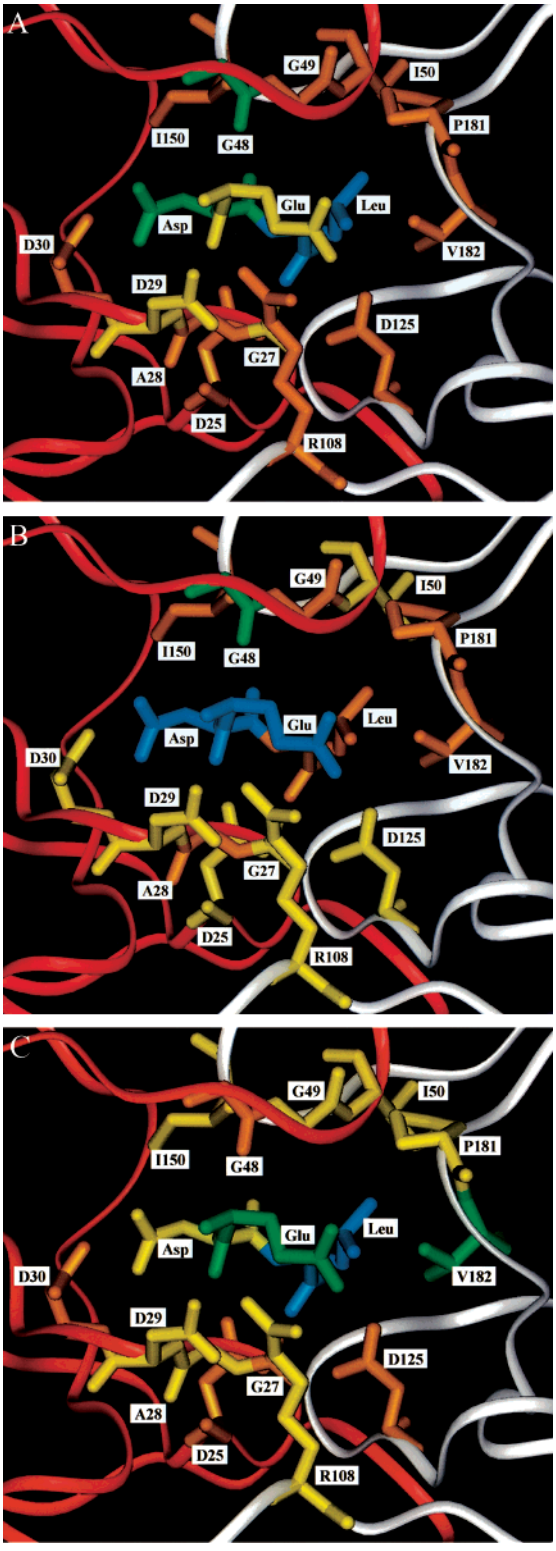

FIGURE 4: Structural distribution of the interactions of Glu-Asp-Leu with the HIV-1 protease. The residues that participate in binding have been color-coded according to the magnitude of the intrinsic interactions. Panel A shows the Gibbs energy distribution: orange (0 to −0.5 kcal/mol), yellow (−0.5 to −1.0 kcal/mol), green (−1.0 to −1.5 kcal/mol), and blue (−1.5 to −2.0 kcal/mol). Panel B shows the enthalpy distribution: orange (1.0−0 kcal/mol), yellow (0 to −1.0 kcal/mol), green (−1.0 to −2.0 kcal/mol), and blue (−2.0 to −3.0 kcal/mol). Panel C shows the solvation entropy distribution: orange (<0 cal K^{-1} mol^{-1}), yellow (0−3.0 cal K^{-1} mol^{-1}), green (3.0−6.0 cal K^{-1} mol^{-1}), and blue (>6 cal K^{-1} mol^{-1}). In all cases, the two subunits of the HIV-1 protease are represented by red and white ribbons, respectively.

Another significant difference between Glu-Asp-Leu and other inhibitors, especially the so-called symmetric inhibitors, is that they span the entire binding site and interact almost equally with both protease subunits, inducing a larger rearrangement in the flap region. Previous studies have suggested that the rearrangement of the flap is enthalpically and entropically unfavorable (*2*, *4*). Thus, the extent of flap rearrangement induced by an inhibitor also contributes to the overall binding energetics.

Exothermic versus Endothermic Binding. There are significant differences in the behavior of exothermic and endothermic inhibitors. Exothermic inhibitors require smaller entropy changes for equally high affinity binding. An endothermic inhibitor, on the other hand, requires large positive entropy changes; i.e., the entropy change not only has to compensate for the unfavorable enthalpy change but also has to provide the necessary binding energy to achieve the observed binding affinity. For example, while the binding of Glu-Asp-Leu is characterized by an entropy change of 9.2 cal K^{-1} mol^{-1}, acetyl pepstatin requires an entropy change of 44.7 cal K^{-1} mol^{-1} to achieve the same binding affinity. In structure-based drug design, high affinity is achieved by optimizing lead compounds that show some reasonable basal affinity. Optimization is accomplished by introducing chemical modifications which in most cases increase the binding affinity by increasing the entropy change. This is especially true if the lead compound has an endothermic character. Where does all that engineered favorable entropy come from?

Two main forces contribute to the entropy change: (1) an unfavorable term which originates from the loss in conformational degrees of freedom from the ligand and from some residues in the protein (conformational entropy change) and (2) a favorable term which originates from the release of water molecules upon binding (solvation entropy). Other entropic effects, like the loss of translational degrees of freedom, are common to all binding processes and cannot be modified in molecular design. It is evident that the most favorable binding entropy will be obtained if the conformational entropy losses are minimized and the solvation entropy gain is maximized. The conformational entropy losses are minimized if the inhibitor is engineered to be relatively rigid in solution and preshaped to the geometry of the binding site. The solvation entropy, on the other hand, is maximized if the inhibitor is made extremely hydrophobic. These two strategies are routinely used, explicitly or implicitly, in drug design. One of the major thermodynamic consequences of the principle of shape complementarity, for example, is the minimization of the conformational entropy loss, and one of the thermodynamic consequences of the enhanced hydrophobicity of designed inhibitors is the maximization of the solvation entropy gain.

The normalized conformational entropy loss for Glu-Asp-Leu is about -0.85 cal K^{-1} mol^{-1} per atom compared to a value of -0.55 cal K^{-1} mol^{-1} per atom for acetyl pepstatin, and values on the order of -0.35 cal K^{-1} mol^{-1} per atom for engineered synthetic inhibitors. These differences reflect the conformational constraints of the free inhibitors in solution. If Glu-Asp-Leu were as conformationally constrained as engineered synthetic inhibitors (i.e., a conformational entropy loss on the order of -0.35 cal K^{-1} mol^{-1}), it would have a binding affinity of 2.8×10^7 M^{-1} ($K_D = 35$ nM) which is close to that reported for most clinical inhibitors. This calculation indicates that, at least in certain situations, enthalpically favorable polar interactions can effectively replace the favorable solvation entropy generated by the burial of nonpolar hydrophobic groups.

Drug Resistance. The response of an inhibitor to different perturbations depends on the nature of the forces that determine its binding affinity. An inhibitor that exhibits little conformational entropy and is preshaped to the geometry of the binding site has limited capacity to adapt to a geometric distortion in the binding site. In fact, inhibitor rigidity has been linked to susceptibility to mutations causing drug resistance in HIV-1 protease as well as reverse transcriptase (*2*, *16*). On that basis, it has been concluded that inhibitor flexibility is a desirable feature. However, a flexible inhibitor will exhibit low affinity unless the unfavorable increase in conformational entropy is compensated by another interaction. The results presented here suggest that an attractive alternative is to compensate the entropic loss enthalpically, by introducing polar groups at locations where strong hydrogen bonds, polar van der Waals interactions, or salt bridges can be established. Moreover, these interactions will also contribute to specificity, and the presence of additional polar groups will provide better water solubility.

Since polar interactions have a higher degree of specificity, a plausible design approach suggested by the analysis presented in this paper would be to satisfy polar interactions first and only then to increase the hydrophobicity of lead compounds. Within this context, the identification of exothermic lead compounds becomes the first step in the design process. Prior to optimization, a compound like Glu-Asp-Leu has an intrinsic inhibition constant close to 20 μM which is considered acceptable for an initial lead compound and a good candidate for subsequent optimization. The identification and optimization of enthalpically favorable inhibitors would require revised experimental and computational strategies since existing databases are usually biased toward highly hydrophobic compounds and docking algorithms do not usually screen jointly for enthalpy and Gibbs energy.

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BI992399D