Thermodynamic Basis of Resistance to HIV-1 Protease Inhibition: Calorimetric Analysis of the V82F/I84V Active Site Resistant Mutant†

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ABSTRACT: One of the most serious side effects associated with the therapy of HIV-1 infection is the appearance of viral strains that exhibit resistance to protease inhibitors. The active site mutant V82F/I84V has been shown to lower the binding affinity of protease inhibitors in clinical use. To identify the origin of this effect, we have investigated the binding thermodynamics of the protease inhibitors indinavir, ritonavir, saquinavir, and nelfinavir to the wild-type HIV-1 protease and to the V82F/I84V resistant mutant. The main driving force for the binding of all four inhibitors is a large positive entropy change originating from the burial of a significant hydrophobic surface upon binding. At 25 °C, the binding enthalpy is unfavorable for all inhibitors except ritonavir, for which it is slightly favorable (−2.3 kcal/mol). Since the inhibitors are preshaped to the geometry of the binding site, their conformational entropy loss upon binding is small, a property that contributes to their high binding affinity. The V82F/I84V active site mutation lowers the affinity of the inhibitors by making the binding enthalpy more positive and making the entropy change slightly less favorable. The effect on the enthalpy change is, however, the major one. The predominantly enthalpic effect of the V82F/I84V mutation is consistent with the idea that the introduction of the bulkier Phe side chain at position 82 and the Val side chain at position 84 distort the binding site and weaken van der Waals and other favorable interactions with inhibitors preshaped to the wild-type binding site. Another contribution of the V82F/I84V to binding affinity originates from an increase in the energy penalty associated with the conformational change of the protease upon binding. The V82F/I84V mutant is structurally more stable than the wild-type protease by about 1.4 kcal/mol. This effect, however, affects equally the binding affinity of substrate and inhibitors.

The HIV-1 protease has been the most important target for drug development against HIV-1 infection due to its key role in viral maturation. The HIV-1 protease is a dimer composed of identical subunits of 99 residues each. The crystallographic structure of the free enzyme as well as the enzyme bound to many inhibitors, including those in clinical use, have been obtained at high resolution (see for example, refs *1*−*11*). Several HIV-1 protease inhibitors are used in antiretroviral therapies and have shown significant promise in combination regimes that include reverse transcriptase inhibitors or several protease inhibitors. A major limiting factor in the treatment of HIV-1 infection has been the emergence of viral strains that exhibit resistance to protease inhibitors (*5*, *12*−*18*). The loss of sensitivity usually occurs because the resistant viral strains encode for protease molecules containing specific amino acid mutations that lower the affinity for the inhibitors, yet maintain sufficient affinity for the substrate. For some mutations, the affinity toward the inhibitor might decrease by up to 3 orders of magnitude, while the K_m for the substrate changes by less than 1 order of magnitude (*5*, *7*, *19*). More than 87 mutations have been observed in at least 47 positions within the HIV-1 protease monomer and shown to express resistance toward one or more inhibitors (*20*). These mutations have been classified as active site and nonactive site (*21*). Active site mutations usually do not involve residues that are directly involved in catalysis, whereas nonactive site mutations are usually located at the hinge of the flap region, the dimer interface, and the beta sheet region (*20*, *22*). Nonactive site mutations may interfere with binding through long-range interactions rather than direct interactions with inhibitors.

The double mutation V82F/I84V has been shown to affect the protease inhibitors in clinical use: ritonavir, saquinavir, nelfinavir, indinavir, and amprenavir (*22*−*24*). This double mutation is located at the edges of the active site, distorting its wild-type geometry without changing its polarity or chemical composition. It is therefore important to investigate the thermodynamic origin of the mutation effect on the binding affinity of inhibitors. A detailed knowledge of this effect will provide important information and general rules for the design of inhibitors that are less susceptible to the effects of mutations.

EXPERIMENTAL PROCEDURES

Protease Purification. HIV-1 protease was prepared according to the following procedure optimized for the high yield, activity, and stability required for calorimetric analysis.

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Plasmid-encoded mutant protease (Q7K/L33I/L63I 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, 10 mM 2-ME, pH 7.5) and broken with two passes through a French pressure cell (≥16 000 psi). Cell debris and protease-containing inclusion bodies were collected by centrifugation (20000*g* for 20 min at 4 °C). Inclusion bodies were subjected to a sequence of four steps, each one consisting of resuspension (glass homogenizer, sonication) and centrifugation (20000*g* for 20 min at 4 °C). In each step, a different washing buffer was employed: buffer 1 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly−Gly, 50 mM 2-ME, pH 7.0), buffer 2 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly−Gly, 50 mM 2-ME, 1 M urea, pH 7.0), buffer 3 (25 mM Tris, 1 mM EDTA, 1 mM Gly−Gly, 50 mM 2-ME, pH 7.0), buffer 4 (25 mM Tris, 1 mM EDTA, 1 mM Gly−Gly, 50 mM 2-ME, 9 M urea, pH 8.0). After the last centrifugation (at 25 °C), protease was recovered in the supernatant and purified using one ion exchange chromatographic step. Solubilized, unfolded protease was applied directly to an anion exchange Q-Sepharose column (Q-Sepharose HP, Pharmacia) previously equilibrated with buffer 4. The protease was passed through the column and was acidified by adding formic acid to 25 mM immediately upon elution from the column. Precipitation of a significant amount of contaminants occurred upon acidification. 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 100 mM, and protein was concentrated. Folded protease was desalted into 1 mM sodium acetate and 2 mM NaCl at pH 5.0 using a buffer exchange column (PD-10, Pharmacia) and stored at either 4 or −20 °C (≥2.5 mg/mL) without loss of activity. After folding, the protease was homogeneous by SDS−PAGE.

The gene encoding mutant HIV-1 protease (containing three mutations designed to remove autolytic sites, Q7K/L33I/L63I) was transferred to the pET24a vector (Novagen), where the expression is under control of the T7 promoter. Briefly, a *Mae*II−*Hin*dIII fragment from the original plasmid (*25*) was purified, then ligated with purified *Hin*dIII−*Nde*1 fragment of pET24a plus a synthetic oligonucleotide (Integrated DNA Technologies, Inc. Coralville, Iowa) designed to span the two oligonucleotide fragments and regenerate the N-terminus of the HIV-1 protease. The sequence of the construct in pET24a was confirmed. Mutations at positions 82 and 84 were introduced using an in vitro site-directed mutagenesis kit (Stratagene), and mutations were confirmed by DNA sequencing. Mutant protease was expressed in BL21/DE3 cells by adding IPTG to 0.5 mM once culture density (as determined by absorbance at 600 nm) was 1.5 or greater. Cells were harvested by centrifugation, and protease was purified from washed inclusion bodies and folded using the same procedure as in the wild-type protease.

Clinical inhibitors, indinavir, nelfinavir, saquinavir, and ritonavir, were purified from commercial capsules by HPLC (Waters, Inc.) using a semipreparative C-18 reversed-phase column developed with 0−100% acetonitrile in 0.05% TFA. Purified inhibitors were lyophilized and stored at −20 °C in the crystalline form (indinavir, nelfinavir) or as suspensions in DMSO (saquinavir, ritonavir).

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 stands for norleucine and nPhe stands for *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 100 nM protease, 100 μM substrate, 10 mM sodium acetate, and 1 M NaCl (pH 5.0). The decrease in absorbance was monitored at 300 nm using a Cary 100 spectrophotometer (Varian Instruments). An extinction coefficient for the absorbance difference 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 5−6 s^{-1} (per dimer) at 25 °C.

Inhibition constants (K_i) for the inhibitors were obtained at 25 °C by measuring the rate of substrate hydrolysis using 20 nM protease in 10 mM sodium acetate, pH 5.0, and 100 μM substrate plus increasing amounts of inhibitor. Under these conditions, the K_m for the chromogenic substrate is ∼100 μM. Inhibition constants (K_i) were obtained by fitting the data to standard equations for tight binding inhibitors (*26*) and considering the decrease in free inhibitor concentration when a nonnegligible portion of the total is bound. The K_m and K_i were also assayed at different NaCl concentrations.

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 buffer-exchanged protease solutions in 10 mM glycine, pH 3.6. Protease specific activity was determined in unheated samples and compared to 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 MCS or a VP-ITC titration calorimetric system (Microcal Inc., Northampton, MA). The enzyme solution (20 μM dimer) in the calorimetric cell was titrated with the appropriate inhibitor dissolved in the same buffer (at a concentration of 0.4 mM in the injection syringe). Because of its low solubility, the binding of ritonavir was measured by titrating from 200 μM protease solution into 20 μM ritonavir in the calorimetric cell. 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. The extension and contribution of protonation/deprotonation processes to the binding was assessed by measuring the binding enthalpy in buffers with different

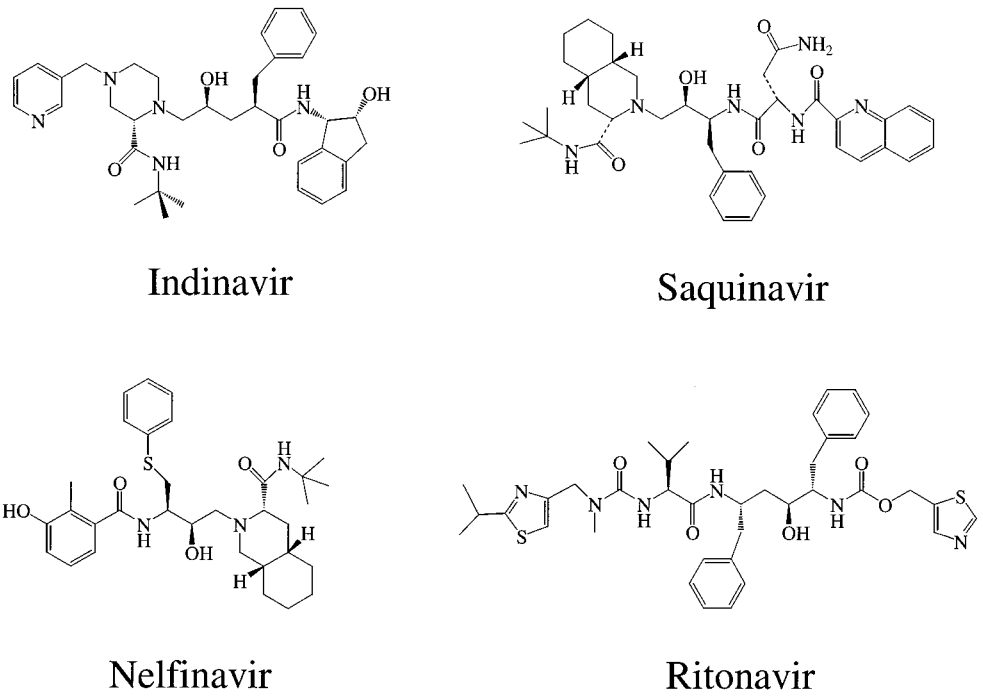

FIGURE 1: Chemical structures of the HIV-1 protease inhibitors indinavir, nelfinavir, saquinavir, and ritonavir.

enthalpies of ionization (*27*, *28*). Analysis of the data was performed using software developed in this laboratory.

RESULTS AND DISCUSSION

Binding of Clinical Inhibitors to Wild-Type HIV-1 Protease. The HIV-1 protease inhibitors indinavir, saquinavir, nelfinavir, and ritonavir (Figure 1) bind to the wild-type protease molecule with high affinity and with a stoichiometry of 1 inhibitor molecule/protease dimer. At pH 4.5−5.5, the pH range at which inhibition data are usually reported (*4*, *6*, *7*, *29*−*34*), the binding affinities for indinavir, saquinavir, nelfinavir, and ritonavir range between 10^{-10}−10^{-9} M with only small differences among the inhibitors when assayed under identical conditions (*24*). Until today, however, the thermodynamic origin of the forces that determine inhibitor binding has not been experimentally elucidated, and therefore, the distribution of enthalpic and entropic contributions to the Gibbs energy of binding is not known. Figure 2, panel A shows the results of a calorimetric titration of indinavir into the HIV-1 protease at 20 °C, pH 5.0, 10 mM acetate buffer. Under these conditions, the binding affinity is beyond the range required for accurate determination by isothermal titration calorimetry; however, the enthalpy change can be measured with high accuracy. Consequently, the Gibbs energies of binding were estimated from the measured K_i's in inhibition experiments as described in Experimental Procedures. Under the experimental conditions used in the calorimetric experiments, we measured K_i's of 2, 4, 2, and 0.3 nM for indinavir, nelfinavir, saquinavir, and ritonavir, respectively. We observed an increase in binding affinities at very high salt concentrations. At 1 M, NaCl the binding affinities were characterized by K_i's of 0.2, 1, 0.3, and 0.02 nM, respectively, corresponding to an average overall effect on ΔG of 1.2 ± 0.3 kcal/mol. The change is small within the physiological range of salt concentrations. The increase in inhibitor binding affinity at high salt concentration has been noticed before (*20*).

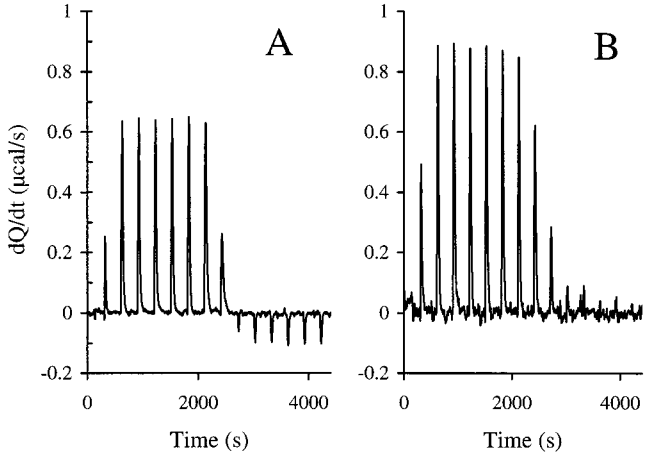

FIGURE 2: Panel A: isothermal calorimetric titration of indinavir into the wild-type HIV-1 protease at 20 °C, pH 5.0, and 10 mM acetate buffer. Panel B: isothermal calorimetric titration of indinavir into the V82F/I84V mutant HIV-1 protease at 20 °C, pH 5.0, and 10 mM acetate buffer. These experiments clearly indicate the endothermic (unfavorable) character of the binding of the inhibitor and that the binding enthalpy to the resistant mutant is even more unfavorable than to the wild type.

As shown in Figure 2, the isothermal titration calorimetry experiments clearly indicate that the binding of indinavir is endothermic at 20 °C and characterized by a positive (unfavorable) enthalpy change of 6.2 kcal/mol. Under the conditions used in calorimetric experiments, the Gibbs energy of binding is −12.0 kcal/mol, indicating that the entropy change contributes nearly −18 kcal/mol to the Gibbs energy to compensate for the unfavorable enthalpy change and provide the favorable binding affinity. Additional titrations performed at different temperatures indicate that the binding of indinavir is also characterized by a negative change in heat capacity of −450 ± 50 cal/K·mol. Similar calorimetric experiments were conducted with the other inhibitors considered in this paper.

Table 1: Binding Thermodynamics of Clinical Inhibitors to HIV-1 Protease[a]

inhibitor	ΔH (25) cal/mol	ΔC_p cal/K·mol	$-T\cdot\Delta S(25)$ cal/mol	ΔG (25) cal/mol	n_H
indinavir	3900 ± 150	-450 ± 50	$-15\,700$	$-11\,800$	-0.6 ± 0.2
nelfinavir	2800 ± 100	-400 ± 40	$-14\,200$	$-11\,400$	-0.4 ± 0.1
saquinavir	2200 ± 200	-340 ± 20	$-14\,000$	$-11\,800$	-0.6 ± 0.3
ritonavir	-2300 ± 300	-380 ± 30	$-11\,200$	$-13\,500$	-0.2 ± 0.2

[a] The thermodynamic parameters in the table correspond to pH 5.0 and 10 mM acetate buffer. The ΔG values were calculated from K_i values obtained under the same conditions. A negative n_H value indicates that the reaction is coupled to the release of protons under the specified conditions.

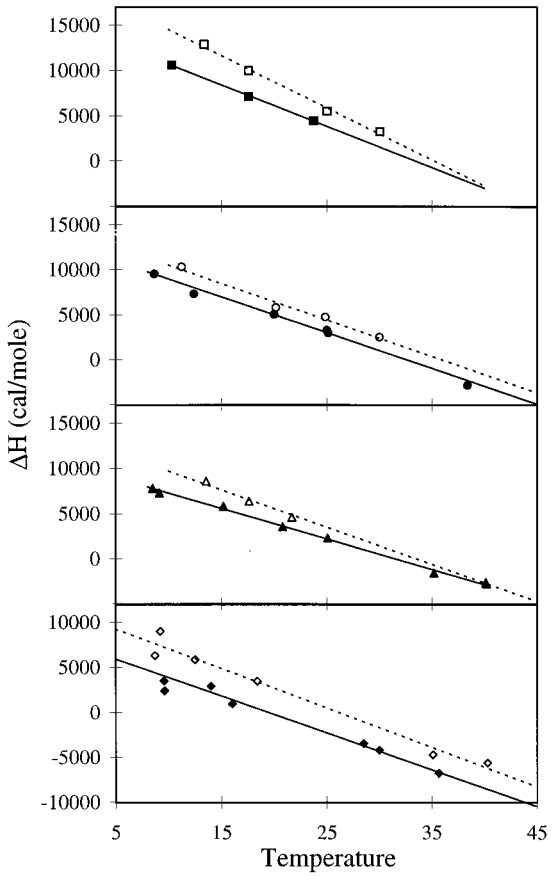

FIGURE 3: Temperature dependence of the measured binding enthalpy change for (from top to bottom) indinavir, nelfinavir, saquinavir and Ritonavir. The experiments were performed at pH 5.0 and 10 mM acetate buffer to minimize the temperature dependence of protonation effects. The solid symbols refer to the wild-type protease, and the open circles refer to the resistant mutant V82F/I84V.

Figure 3 shows the measured enthalpy change for indinavir, saquinavir, nelfinavir, and ritonavir as a function of temperature. The binding enthalpy for all protease inhibitors except ritonavir is positive at 25 °C. Ritonavir yields a slightly negative binding enthalpy (−2.3 kcal/mol) at 25 °C. Also, all inhibitors exhibit negative heat capacities for binding ranging between −350 and −450 cal/K·mol (Table 1). Since the binding enthalpy is unfavorable or only slightly negative, it follows that the main driving force for inhibitor binding is in all cases a large positive entropy change. The thermodynamic results are summarized Table 1 and clearly indicates that in all cases the entropy change is the main driving force for binding, contributing on the order of −11 to −16 kcal/mol to the Gibbs energy of binding at 25 °C.

Inhibitor Binding to V82F/I84V Resistant Mutant. The double mutant V82F/I84V has been shown to lower the binding affinity of protease inhibitors currently in clinical use (*24*). For indinavir, nelfinavir, saquinavir, and ritonavir, we found that the V82F/I84V mutation lowers the Gibbs energy of binding by 2.6, 1.7, 1.4, and 3.5 kcal/mol, respectively (Table 2), which are close to values reported previously (*24*). The double mutation V82F/I84V is structurally located at both sides of the binding site as indicated in Figure 4. From a chemical point of view, the V82F/I84V mutation changes the geometry of the binding site without introducing a significant change in its chemical character. Figure 2, panel B, shows the results of a calorimetric titration of indinavir into the V82F/I84V resistant mutant under the same conditions shown in panel A for the wild-type protease. The binding enthalpy is about 2 kcal/mol more positive (less favorable) than for the wild type. Less favorable enthalpies were also obtained with the other inhibitors as shown in Figure 3. For nelfinavir and ritonavir, the heat capacity changes were similar for the wild-type and mutant enzymes. For indinavir and saquinavir (see Figure 3), the heat capacity changes were slightly larger for the mutant enzyme, suggesting that for the latter two inhibitors the double mutation induces an increase in the magnitude of the nonpolar surface area that becomes buried upon binding.

Even though the effect of the mutation on the enthalpy change is the predominant one, it does not completely account for the decrease in binding affinity. The effect of the V82F/I84V mutation on ΔH amounts to 2 kcal/mol for indinavir, 1.6 kcal/mol for nelfinavir, 1.3 kcal/mol for saquinavir, and 2.8 kcal/mol for ritonavir. On the other hand, the effect on $-T\Delta S$ amounts to 0.6, 0.1, 0.1, and 0.7 kcal/mol, respectively. It is clear from these results that the V82F/I84V mutant lowers the binding affinity of the inhibitors by making the binding enthalpy more positive and making the entropy change slightly less favorable.

Protonation Effects. The coupling of a binding reaction to the absorption or release of protons by the protein complex can be evaluated by performing the calorimetric titrations in buffers with different ionization enthalpies (*27*). If the binding reaction absorbs or releases protons, those protons will be given or taken by the buffer used in the experiments, and the measured enthalpy change (ΔH_{app}) will be a function of the ionization enthalpy of the buffer:

$$\Delta H_{app} = \Delta H_{bind} + n_H \Delta H_{ion} \quad (1)$$

where ΔH_{bind} is the buffer-independent binding enthalpy, ΔH_{ion} is the ionization enthalpy of the buffer, and n_H is the number of protons that are absorbed (or released if n_H is negative) by the protein/inhibitor complex upon binding. Figure 5 shows a calorimetric titration of indinavir at pH 5.0, 25 °C performed in three different buffers, acetate, MES, and ACES, which are characterized by ionization enthalpies

Table 2: Binding Thermodynamics of Clinical Inhibitors to Resistant Mutant V82F/I84V[a]

inhibitor	ΔH (25) cal/mol	ΔC_p cal/K · mol	$-T\cdot\Delta S$ (25) cal/mol	ΔG (25) cal/mol	n_H
indinavir	5900 ± 200	−580 ± 60	−15 100	−9 200	−0.5 ± 0.2
nelfinavir	4400 ± 200	−410 ± 40	−14 100	−9 700	−0.4 ± 0.3
saquinavir	3500 ± 150	−410 ± 40	−13 900	−10 400	−0.4 ± 0.2
ritonavir	500 ± 100	−440 ± 40	−10 500	−10 000	−0.1 ± 0.2

[a] The thermodynamic parameters in the table correspond to pH 5.0 and 10 mM acetate buffer. The ΔG values were calculated from K_i values obtained under the same conditions. A negative n_H value indicates that the reaction is coupled to the release of protons under the specified conditions.

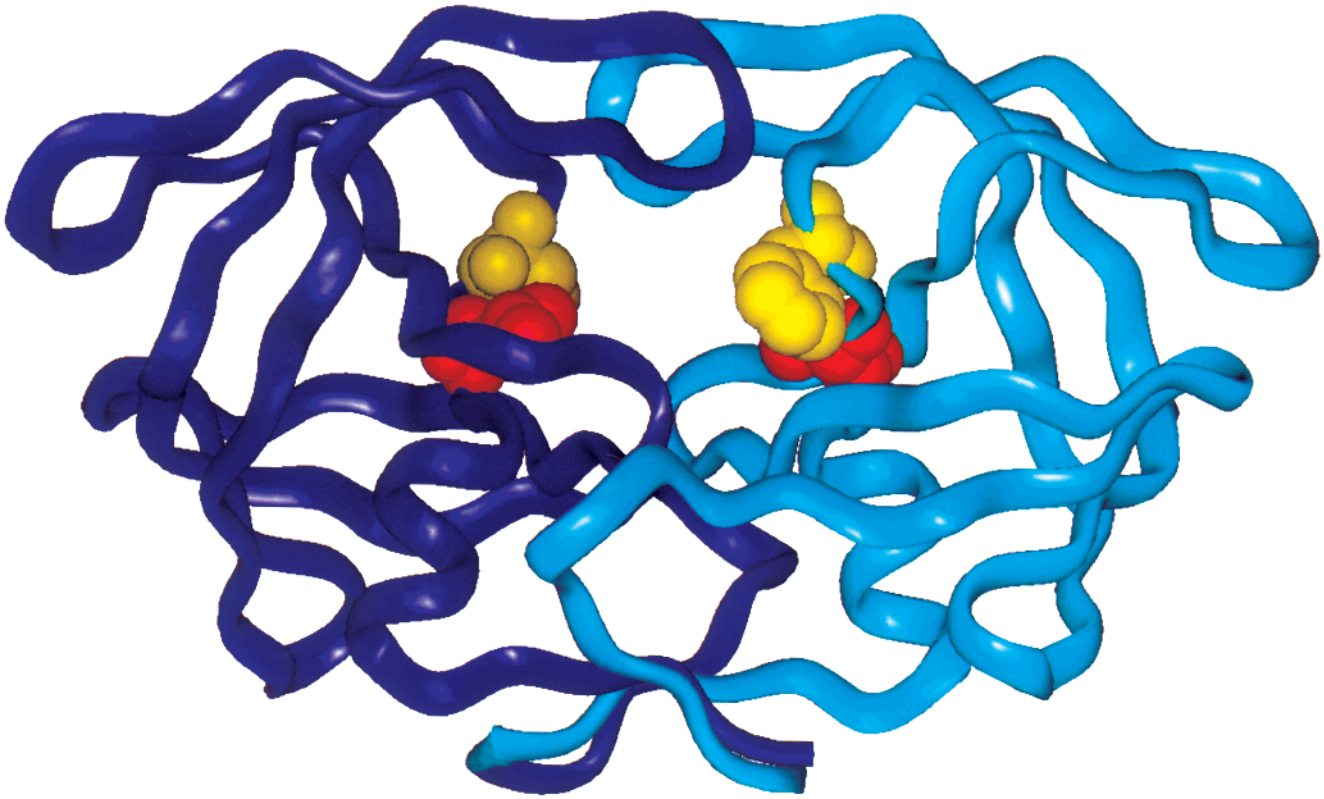

FIGURE 4: Structure of the HIV-1 protease showing the location of the drug resistant mutation V82F/I84V. In both monomers, the V82F mutation is shown in yellow and the I84V mutation is shown in red.

of 0.12, 3.72, and 7.51 kcal/mol, respectively. It is evident that the apparent binding enthalpy decreases at increasing ionization enthalpies of the buffer, indicating that the binding reaction is coupled to the release of protons from the protease/inhibitor complex. The slope of the data shown in Figure 5 indicates that at pH 5.0 the binding of indinavir is coupled to the release of 0.6 ± 0.2 protons. Similar experiments were performed with the other inhibitors and indicate that the binding of nelfinavir, saquinavir, and ritonavir is coupled to the release of 0.4 ± 0.1, 0.6 ± 0.3, and 0.2 ± 0.2 protons, respectively (Table 1). The buffer-independent binding enthalpies, ΔH_{bind}, correspond to the values obtained in a buffer with zero ionization enthalpy and fall within the experimental error of the values obtained in acetate buffer ($\Delta H_{ion} = 0.12$ kcal/mol) and are summarized in Tables 1 and 2. The results obtained with the V82F/I84V resistant mutant indicate that, within the experimental error, the protonation effects are not significantly different for the mutant and the wild-type proteases. In this situation, any differences in the binding enthalpy for the wild type and mutant cannot be attributed to pK differences in the groups undergoing protonation/deprotonation.

The release of protons from the complexes formed by indinavir, nelfinavir, saquinavir, and to a lesser extent, ritonavir indicates that the binding affinity is also a function of pH. In particular, the binding affinity is expected to increase at higher pH values according to the standard linkage equation $\partial \log K_a/\partial \mathrm{pH} = -n_H$ (*35*). Previously, other investigators have reported that the affinity of some HIV-1 protease inhibitors does indeed increase with pH (*7*, *36*) consistent with the observations reported here. Other inhibitors show different trends, notably, the allopenylnorstatine-based inhibitors also investigated in this laboratory (*37*). However, the identity of the groups that undergo protonation/deprotonation during inhibitor binding is not well-established (*20*), a situation compounded by the fact that groups in the inhibitors themselves might also undergo changes in protonation upon binding (*37*). For the V82F/I84V mutation, any potential changes in pK of the ionizable groups involved in binding appear small and do not affect binding affinities to a significant extent; however, it must be noted that it is feasible for a mutation to affect the binding affinity of inhibitors by modifying the pK of groups that become protonated or deprotonated upon binding.

The Origin of The Favorable Entropy Change. The entropy contribution ($-T\Delta S$) to the Gibbs energy is composed of two major terms: (i) a large favorable solvation entropy change that originates from the release of water

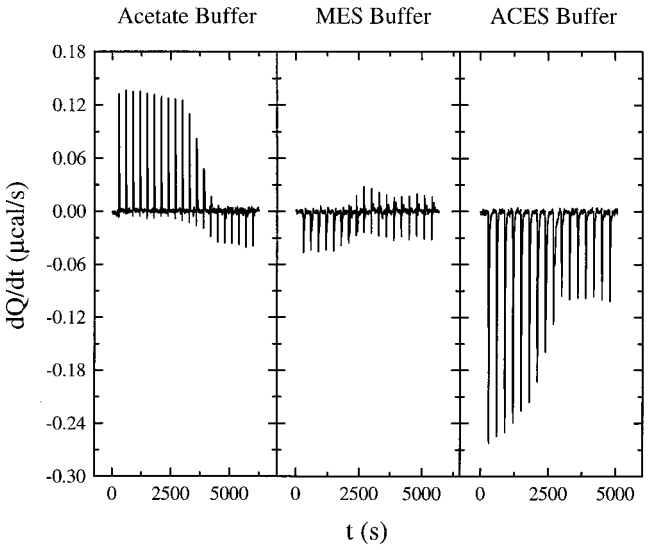

FIGURE 5: Isothermal calorimetric titration of indinavir at pH 5.0, 25 °C, performed in three different buffers, acetate, MES, and ACES, which are characterized by ionization enthalpies of 0.12, 3.72, and 7.51 kcal/mol, respectively. The negative dependence of the enthalpy change on the enthalpy of ionization of the buffer indicates that the binding of the inhibitor is coupled to the release of protons from the protein/inhibitor complex.

molecules associated with the burial of a significant hydrophobic surface upon binding and (ii) an unfavorable conformational entropy change due to the loss of conformational degrees of freedom by the inhibitor and some residues in the protein. Upon binding, indinavir, nelfinavir, saquinavir, and ritonavir bury a large surface area from the solvent amounting to −1361, −1142, −1259, and −1374 Å^2, respectively; however, most of the area buried is hydrophobic (77, 76, 74, and 70% for indinavir, nelfinavir, saquinavir, and ritonavir, respectively). These structural characteristics are consistent with a large contribution from the hydrophobic effect to the binding affinity. In fact, a structure-based thermodynamic analysis of the inhibitor/protease complexes as described by Luque et al. (*38*) indicates that the solvation entropy changes are on the order of 90−100 cal/K·mol, amounting to a favorable contribution of −27 to −30 kcal/mol to the Gibbs energy and providing the main driving force for binding.

The favorable solvation entropy is opposed by the loss of conformational entropy and to a lesser extent by the loss of translational entropy upon binding. The loss of conformational entropy is an unfavorable contribution to the binding affinity; thus, a common goal in molecular design is to minimize this entropy loss. Given that the inhibitor is the only molecule under designer control, this goal is usually accomplished by introducing conformational constraints in the inhibitor molecule (i.e., if the free inhibitor has few conformational degrees of freedom, the loss upon binding will be small). The protease inhibitors discussed in this paper show significant conformational constraints when compared with peptide inhibitors of similar molecular weights. One way of estimating the degree to which a molecule is conformationally constrained is by calculating the ratio of rotatable bonds to the total number of non-hydrogen atoms in the molecule (here denoted ϵ). Highly constrained molecules have very low ϵ ratios. For example, the peptide inhibitor acetyl pepstatin or the tripeptide inhibitor derived from the trans-frame region of Gag−Pol (GluAspLeu) are characterized by ϵ values of 0.44 and 0.5. Indinavir, nelfinavir, saquinavir, and ritonavir, on the other hand, have ϵ values of 0.27, 0.26, 0.27, and 0.38, respectively. The total loss in conformational entropy (protease plus inhibitor) is on the order of −45 cal/K·mol for the binding of indinavir, nelfinavir, and saquinavir, and somewhat larger for ritonavir ($\sim$ −55 cal/K·mol). In addition to the loss of conformational entropy by the inhibitors themselves, the protease also experiences a loss in the conformational entropy of the side chains that interact directly with the inhibitors and also the loss of conformational entropy by the flaps, which exhibit low structural stability in the unbound state. The changes experienced by the protease itself are expected to be largely independent of the inhibitor. The higher entropy loss for ritonavir is consistent with its higher ϵ value. Despite its higher overall flexibility, ritonavir is the inhibitor most affected by the V82F/I84V mutation. This observation indicates that average flexibility alone is not enough and that inhibitor flexibility needs to be located at critical points in relation to the target molecule.

The observed entropy changes for the protease inhibitors are inherited from a molecular design process in which the solvation entropy gain is maximized and the conformational entropy loss is minimized. From a chemical point of view, these solvation/conformational entropy ratios are achieved by preshaping the inhibitor molecules to the binding site and by increasing their hydrophobic character. Molecules that exhibit these binding properties are fairly rigid (conformationally constrained) and highly hydrophobic.

The Structural Stability of the Wild-Type and V82F/I84V Mutant HIV-1 Protease. From a thermodynamic standpoint, the V82F/I84V mutation lowers the binding affinity of the four inhibitors studied here by affecting the binding enthalpy and to a lesser extent the binding entropy. From a structural point of view, two different effects account for the less favorable binding thermodynamics: direct effects that alter the interactions between inhibitors and protein and indirect effects that alter the energetics of the conformational rearrangement of the protease upon inhibitor binding. The higher binding enthalpy observed with the V82F/I84V mutant is consistent with the idea that the introduction of the bulkier Phe side chain at position 82 and the Val side chain at position 84 distort the binding site geometry and weaken van der Waals and other favorable interactions with the inhibitors. On the other hand, upon inhibitor binding the HIV-1 protease undergoes a conformational change that involves the movement of the flaps, which close the binding site, and a minor rotation of each monomer, as illustrated in Figure 6 (*1*, *39*). The energetics associated with this change in protein conformation needs to be included in any computation of the binding affinity of any inhibitor or substrate to the HIV-1 protease. Mutations that stabilize the free conformation in relation to the bound conformation of the protease will lower the binding affinity of substrates and inhibitors by an amount equal to the difference in the free energy required to achieve the bound conformation. For example, in the absence of any other effect, a mutation that stabilizes the flaps in the open conformation will exhibit a diminished binding affinity due to the increased energy penalty that will be required to bring the flaps into their bound conformation.

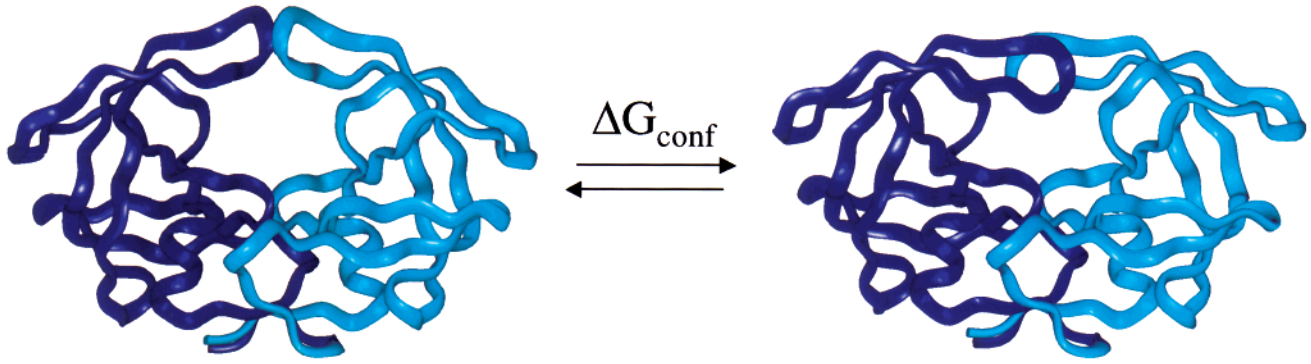

FIGURE 6: Illustration of the conformational change associated with inhibitor binding to the HIV-1 protease.

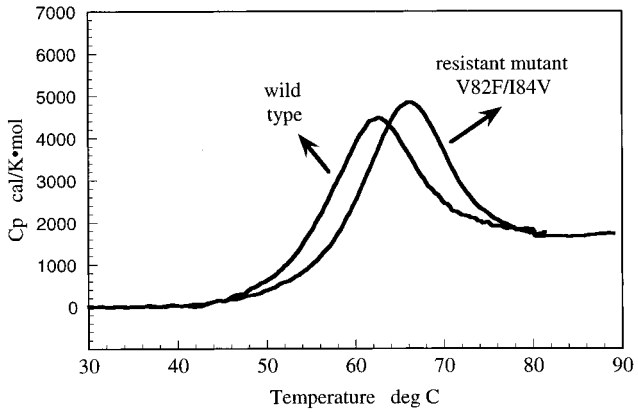

FIGURE 7: Differential scanning calorimetry of the wild type and resistant mutant V82F/I84V under identical conditions and in the absence of inhibitors. These experiments were performed at pH 3.6 and 10 mM glycine, conditions under which thermal denaturation is fully reversible. These experiments indicate that the resistant mutant is more stable than the wild-type protein.

The Gibbs energy of binding can be divided into an intrinsic part (ΔG_{int}) that includes the inhibitor/protein interactions and a term (ΔG_{conf}) that includes the Gibbs energy change associated with the conformational change in the protein. The effect of a mutation on the binding affinity ($\Delta\Delta G \equiv \Delta G_{mut} - \Delta G_{wild}$) is given by

$$\Delta\Delta G = \Delta\Delta G_{int} + \Delta\Delta G_{conf}$$

The stabilization of the free conformation of the protease will result in an increased stability toward denaturation. We have characterized the structural stability of the wild type and the V82F/I84V mutant by high sensitivity differential scanning calorimetry. The results are shown in Figure 7. Under similar conditions, the resistant mutant V82F/I84V is more stable and undergoes thermal denaturation at 4 °C higher than the wild type. Potential differences in denaturation enthalpies were too small to be distinguished by differential scanning calorimetry. Analysis of the calorimetric data indicates that the mutant V82F/I84V is 1.4 kcal/mol-dimer more stable than the wild type. If all this difference in Gibbs energy were associated with a higher stability of the free conformation of the mutant binding site, then the binding affinities would be expected to decrease by a factor of ~10. The changes in binding affinity due to these stability changes are not expected to discriminate significantly between inhibitors and substrate, i.e., this term will lower the affinity of both by similar amounts and will not, per se, generate resistance unless changes in the catalytic efficiency of the enzyme are also induced. In fact, the experimental observation is that for most inhibitors, the binding affinities decrease by a larger factor indicating that additional specific effects such as those discussed above are involved. The affinity of the peptide substrate, on the other hand, judging from the published changes in K_m (*24*), decreases by about 1.1 kcal/mol, which is close to the value expected from the conformational differences. These results offer a plausible explanation to the observation that the binding affinity of the substrate is affected less than the binding affinity of the inhibitors.

CONCLUSIONS

The experiments presented here indicate that the binding strength of current HIV-1 protease inhibitors is generated by a large positive entropy change with little or no favorable contribution from the enthalpy change. The large favorable entropy change is the result of a large desolvation entropy combined with a small loss of conformational entropy upon binding. Two major terms contribute to the binding enthalpy: a negative (favorable) contribution arising from the creation of specific interactions between protein and inhibitor and a positive (unfavorable) contribution arising from the desolvation of the protein and inhibitor groups that contribute to the binding process (*38*). The overall enthalpic balance is unfavorable or only slightly favorable indicating that the interactions between protein and inhibitors are not sufficient to overcome the unfavorable enthalpy of dehydration. These properties can be considered as the thermodynamic signatures of conformationally constrained hydrophobic inhibitors. The V82F/I84V mutation lowers the binding affinity by making the binding enthalpy even less favorable, reflecting in part the inability of these compounds to adapt to a geometrically distorted binding site. The ability of an inhibitor to adapt to a changed geometry is related to its flexibility; however, molecular flexibility cannot be introduced anywhere in the molecule. It needs to be introduced at locations that will allow them to reach observed or predicted van der Waals voids created by mutations in the target molecule.

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