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Thermodynamic dissection of the binding energetics of KNI-272, a potent HIV-1 protease inhibitor

ADRIAN VELAZQUEZ-CAMPOY,[1] IRENE LUQUE,[1] MATTHEW J. TODD,[1]
MARK MILUTINOVICH,[1] YOSHIAKI KISO,[2] AND ERNESTO FREIRE[1]

[1]Department of Biology and Biocalorimetry Center, The Johns Hopkins University, Baltimore, Maryland 21218
[2]Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan

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Abstract

KNI-272 is a powerful HIV-1 protease inhibitor with a reported inhibition constant in the picomolar range. In this paper, a complete experimental dissection of the thermodynamic forces that define the binding affinity of this inhibitor to the wild-type and drug-resistant mutant V82F/I84V is presented. Unlike other protease inhibitors, KNI-272 binds to the protease with a favorable binding enthalpy. The origin of the favorable binding enthalpy has been traced to the coupling of the binding reaction to the burial of six water molecules. These bound water molecules, previously identified by NMR studies, optimize the atomic packing at the inhibitor/protein interface enhancing van der Waals and other favorable interactions. These interactions offset the unfavorable enthalpy usually associated with the binding of hydrophobic molecules. The association constant to the drug resistant mutant is 100–500 times weaker. The decrease in binding affinity corresponds to an increase in the Gibbs energy of binding of 3–3.5 kcal/mol, which originates from less favorable enthalpy (1.7 kcal/mol more positive) and entropy changes. Calorimetric binding experiments performed as a function of pH and utilizing buffers with different ionization enthalpies have permitted the dissection of proton linkage effects. According to these experiments, the binding of the inhibitor is linked to the protonation/deprotonation of two groups. In the uncomplexed form these groups have pKs of 6.0 and 4.8, and become 6.6 and 2.9 in the complex. These groups have been identified as one of the aspartates in the catalytic aspartyl dyad in the protease and the isoquinoline nitrogen in the inhibitor molecule. The binding affinity is maximal between pH 5 and pH 6. At those pH values the affinity is close to 6×10^{10} M^{-1} ($K_d = 16$ pM). Global analysis of the data yield a buffer- and pH-independent binding enthalpy of -6.3 kcal/mol. Under conditions in which the exchange of protons is zero, the Gibbs energy of binding is -14.7 kcal/mol from which a binding entropy of 28 cal/K mol is obtained. Thus, the binding of KNI-272 is both enthalpically and entropically favorable. The structure-based thermodynamic analysis indicates that the allophenylnorstatine nucleus of KNI-272 provides an important scaffold for the design of inhibitors that are less susceptible to resistant mutations.

Keywords: binding thermodynamics; calorimetry; HIV-1 protease; HIV-1 protease inhibitors; KNI inhibitors; KNI-272

The binding affinity, $K_a = e^{-\Delta G/RT}$, is determined by the Gibbs energy of binding ΔG, which in turn is determined by the enthalpy ΔH and entropy ΔS changes ($\Delta G = \Delta H - T\Delta S$). In principle, many combinations of ΔH and ΔS values can give rise to the same ΔG value and therefore elicit the same binding affinity. In drug design, lead compounds are screened on the basis of binding affinity, and therefore the thermodynamic basis of their interactions with the target molecule are not known. However, do enthalpically dominated ligands behave the same as entropically dominated ligands? According to thermodynamics, their binding affinities will have different temperature dependences, but is this the only difference? Do they exhibit the same advantages and disadvantages as molecular scaffolds for inhibitor design? Are there any situations in which one type of inhibitors should be preferred over the other? Previously, we have measured the binding thermodynamics of protease inhibitors currently in clinical use (Indinavir, Nelfinavir, Saquinavir, and Ritonavir) by high sensitivity isothermal titration calorimetry (Todd et al., 2000; Velazquez-Campoy et al., 2000). It was found that the binding of these inhibitors is either enthalpically unfavorable or characterized by a slightly favorable enthalpy. In all cases, the dominant driving force for binding is a large positive entropy change that originates primarily from a large positive solvation entropy due to the burial of a large hydrophobic surface upon binding, and a small loss of conformational entropy due to the little flexibility of inhibitors preshaped to the geometry of the binding site.

Reprint requests to: Ernesto Freire, Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218; e-mail: ef@jhu.edu.

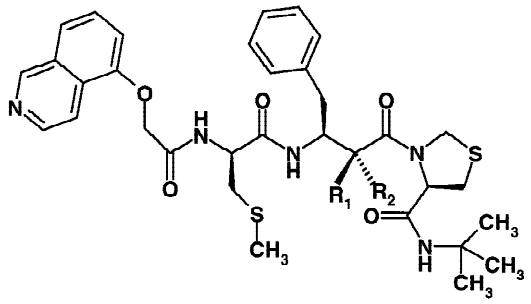

Fig. 1. Chemical structure of KNI-272 and KNI-529. For KNI-272, R_1 = OH and R_2 = H. For KNI-529, R_1 = H and R_2 = OH.

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 (Luque et al., 1998; Todd & Freire, 1999; Velazquez-Campoy et al., 2000). The difficulty of rigid inhibitors to adapt to an altered binding site geometry lowers the strength of van der Waals, hydrogen bonds, 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 higher 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 compensation 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. It is therefore desirable to identify and characterize families of compounds that exhibit favorable binding enthalpies. While enthalpic inhibitors are not intrinsically flexible, the existence of favorable interactions facilitates the introduction of flexible elements in the design process.

KNI-272 (Fig. 1) is a potent allophenylnorstatine-based HIV-1 protease inhibitor characterized by an inhibition constant in the subnanomolar range (Baldwin et al., 1995; Kiso, 1996; Kiso et al., 1999). Here, we show that KNI-272 binds to the protease with a favorable binding enthalpy and that the origin of this behavior is related to the water molecules that are immobilized at the binding interface. The high binding affinity of this inhibitor combined with the enthalpically favorable nature of its interaction with the protease suggests that the allophenylnorstatine nucleus can be used as a scaffold for the design of protease inhibitors with a lower susceptibility to resistance-causing mutations.

Results and discussion

Energetics of KNI-272 binding to HIV-1 protease

The energetics of the association between the HIV-1 protease and the powerful allophenylnorstatine-containing inhibitor KNI-272 was measured directly by isothermal titration calorimetry. For comparison, similar experiments were performed with KNI-529, a less potent diasteroisomer of KNI-272 (Baldwin et al., 1995; Katoh et al., 1999; Kiso, 1996). Figure 2A shows a calorimetric titration

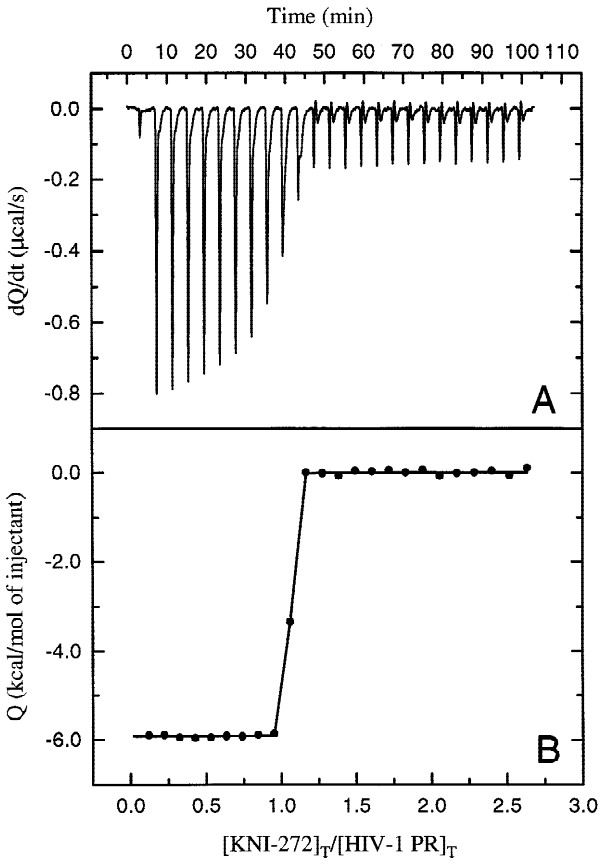

Fig. 2. Calorimetric titration of HIV-1 protease with the inhibitor KNI-272. **A:** The heat effects associated with the injection of KNI-272 (10 μL per injection of a 0.3 mM solution) into the calorimetric cell (1.4 mL) containing HIV-1 protease at a concentration of 20 μM dimer. The experiment was performed at 25 °C in 10 mM MES pH 5, 2% DMSO. **B:** The binding isotherm corresponding to the data in **A**. Under these conditions, the binding constant is beyond the reach of titration calorimetric determination and need to be determined by differential scanning calorimetry as discussed in the text.

corresponding to the binding of KNI-272 to the HIV-1 protease at 25 °C in 10 mM MES buffer, pH 5.0. The most notable feature of this experiment is that the binding of KNI-272 is exothermic, i.e., characterized by a favorable enthalpy change. The inhibitor concentration dependence of the heat released in the binding process, after normalization and correction for heats of dilution, is shown in Figure 2B. Under the experimental conditions shown in the figure, the binding affinity is too high for accurate calorimetric determination; only a lower limit of 10^9 M^{-1} could be obtained. Separate experiments using differential scanning calorimetry (see below) were required for determination of the association constant and yield a value of 6×10^{10} M^{-1} under the same conditions. The enthalpy and heat capacity changes, on the other hand, can be measured very precisely under these conditions.

The heat capacity change ΔCp_{bind} was determined by performing calorimetric experiments at different temperatures, under the same pH and buffer conditions. Figure 3 shows the temperature dependence of the binding enthalpy for KNI-272 and for the closely related diasteroisomer KNI-529. The heat capacity change associated with the binding reaction is estimated from the slope of the linear regression and the values are -430 ± 20 cal/K mol and

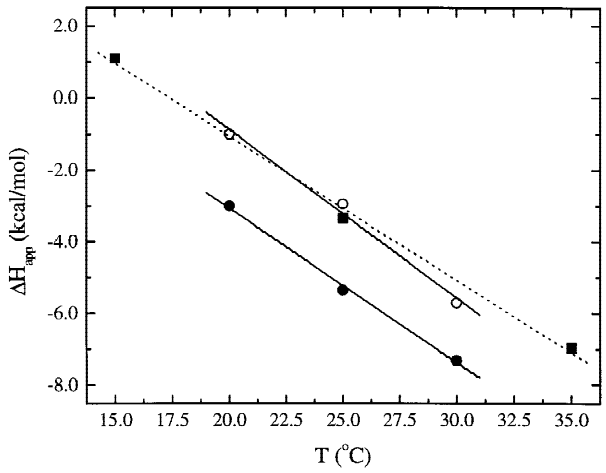

Fig. 3. The temperature dependence of the binding enthalpy of KNI-272 (filled circles) and KNI-529 (open circles) to the wild-type HIV-1 protease and KNI-272 to the inhibitor resistant mutant V82F/I84V (filled squares). All the experiments were performed at pH 5 in 10 mM acetate buffer. The heat capacity change associated with the binding reaction is estimated from the slope of the linear regression and the values are -430 ± 20, -470 ± 40, and -400 ± 20 cal/K mol, respectively. At 25 °C the binding enthalpy is -5.4 ± 0.1 kcal/mol for KNI-272 and -2.9 ± 0.1 kcal/mol for KNI-529 to the wild-type and -3.3 ± 0.1 kcal/mol for KNI-272 to the V82F/I84V mutant.

-470 ± 40 cal/K mol, respectively. At 25 °C the binding enthalpy is -5.4 ± 0.1 kcal/mol for KNI-272 and -2.9 ± 0.1 kcal/mol for KNI-529, consistent with the observation that KNI-529 has a lower affinity for the protease than KNI-272 (see below). The experiments shown in Figure 3 were performed in acetate buffer to minimize the influence of the buffer on the enthalpy change (Gomez & Freire, 1995). In addition, the net number of protons exchanged between the complex and the solution is minimal at pH 5.5 (Fig. 5).

The effect of protonation/deprotonation on the binding enthalpy (proton linkage)

In a binding process, certain ionizable groups in the protein and in the ligand may experience a change in their pK. As a result, and depending on the pH of the experiments, the binding reaction will be coupled to a protonation/deprotonation process. The physical evidence for the coupling between binding and proton transfer (proton linkage) is the pH dependence of the binding thermodynamic parameters (enthalpy, association constant, Gibbs energy, entropy) and also the dependence of the measured or apparent binding enthalpy (ΔH_{app}) on the ionization enthalpy of the buffer in which the experiments are made (Gomez & Freire, 1995). 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_{app} (buffer dependent and pH dependent) is the sum of two terms: the reaction enthalpy ΔH_{bind} independent of the buffer used in the experiment (but pH dependent) and another term representing the contribution of the proton ionization of the buffer ΔH_{ion}, which is multiplied by n_H, the number of protons that are absorbed (or released if n_H is negative) by the protein/inhibitor complex upon binding.

Figure 4 shows the dependence of the apparent binding enthalpy on the enthalpy of ionization of the buffer at pH 3.8. The slope is equal to -0.70 ± 0.06, indicating that under these conditions the binding of the inhibitor is coupled to the release of protons from the complex. The buffer-independent binding enthalpy (*y*-axis intercept) is equal to -2.5 ± 0.1 kcal/mol. Similar experiments were performed at different pH values. The results are shown in Figure 5 and provide the number of protons that are absorbed or released by the inhibitor/protease complex and the buffer-independent binding enthalpy at all pH values. These results indicate that around pH 5.5 the reaction is not coupled to a net gain or loss of protons. Below pH 5.5, a maximum of 0.7 protons (at pH 3.8) are released from the protease/inhibitor complex. Above pH 6, the binding reaction proceeds with a net gain of protons by the complex. This behavior indicates that more than one ionizable group experiences a change in pK upon binding. At all pH values, the binding enthalpy was negative, indicating that the exothermic character of the binding of this inhibitor is due to its intrinsic properties rather than to the coupling of the process to the ionization of the buffer.

The groups that protonate or deprotonate upon binding do not necessarily belong to the protease. In fact, the inhibitor KNI-272 has one ionizable group: the nitrogen in the isoquinoline aromatic ring. Isoquinoline has a pK value of 5.07 and ionization enthalpy of 5.9 kcal/mol and other analogous molecules (e.g., pyridine, benzoquinoline, acridine) have pK and ionization enthalpy values very similar (Christensen et al., 1976). Therefore, this group and at least one of the catalytic aspartic residues in the protein appear to be likely candidates to exhibit a significant change in their pK values upon binding. Previous NMR studies with this same inhibitor (Wang et al., 1996c) indicate that in the complex the catalytic

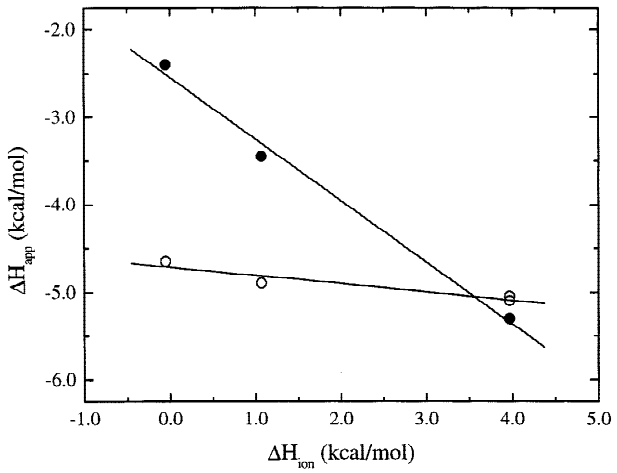

Fig. 4. The dependence of the apparent binding enthalpy of KNI-272 to the wild-type HIV-1 protease (filled circles) and the resistant mutant V82F/I84V (open circles) as a function of the ionization enthalpy of the buffer at pH 3.8. For the wild-type, the slope is equal to -0.7 ± 0.06 and for the mutant it is -0.09 ± 0.02, indicating that under these conditions the binding of the inhibitor is coupled to the release of protons from the complex and that the number is larger for the wild-type than the mutant. The buffer independent binding enthalpy was equal to -2.54 ± 0.1 kcal/mol for the wild-type and -4.7 ± 0.1 for the mutant. Similar experiments were performed at different pH values (Fig. 5).

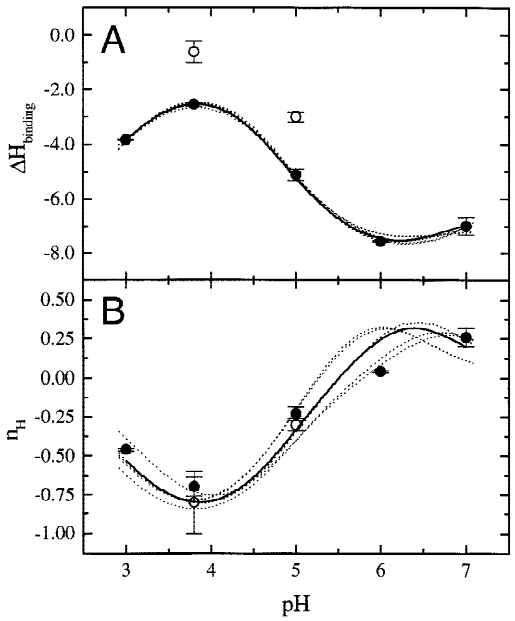

Fig. 5. The buffer-independent binding enthalpy (**A**) and the change in the number of protons associated with the complex upon KNI-272 binding (**B**) as a function of pH (solid circles). The solid lines correspond to the expected pH dependence calculated with the average values for the set of parameters obtained by global nonlinear least squares of the data (Table 1). The dotted lines correspond to the curves calculated with the parameter values obtained with individual fits. The open circles correspond to the inhibitor KNI-529.

Asp25 and Asp125 exhibit anomalous pK values of >6.2 and <2.5, consistent with Asp25 being protonated and Asp125 being deprotonated over the pH range of our studies.

Nonlinear least-squares analysis of proton linkage

The analysis described above permits determination of the number of protons that are gained or released by the protein/inhibitor complex and the buffer independent binding enthalpy. These quantities are a function of the pK and protonation enthalpies of the groups involved in both the free and bound forms, as well as the intrinsic enthalpy of the reference state, which corresponds to the fully deprotonated species. The n_H and ΔH_{bind} data were fitted globally by nonlinear least-squares regression analysis in terms of Equations 1 and 2. The results are shown in Table 1. The data could be fitted well to a minimum of two ionization sites with no improvement in the goodness of the fit if a larger number of sites were included. Several alternatives were considered in the analysis to evaluate the robustness of the fit, including fixing some of the fitting parameters to some specific values. For example, because one of the groups undergoing protonation/deprotonation appears to be an aspartic residue, the protonation enthalpy of one of the free groups was set to -1.3 kcal/mol, which corresponds to the protonation enthalpy of a solvent exposed free carboxylic group (Christensen et al., 1976).

As shown in Table 1, the analysis was consistent with the presence of two ionization sites with pK values of 6.0 and 4.8 in the free protein and 6.6 and 2.9 in the complex. Interestingly, the site characterized by the pK values of 6.0 and 6.6 is the one associated with the protonation enthalpy of a carboxylic group, strongly suggesting that this group is one of the aspartates in the aspartyl dyad. This result agrees with previous reports that one of these groups has a pK $\sim$6 in the free state, and that this pK increases to higher values when bound to KNI-272 (Trylska et al., 1999; Wang et al., 1996c). The second ionizable group has an apparent pK of 4.8 in the free state and 2.9 in the complex. This second group is estimated to have a protonation enthalpy of -6.3 kcal/mol in the uncomplexed system, suggesting that a group different than an aspartyl moiety is contributing to the protonation thermodynamics. One plausible candidate, as mentioned above, is the nitrogen in the aromatic isoquinoline ring of KNI-272 (see Fig. 1), which has a pK value and a protonation enthalpy very close to the pK and ionization enthalpy values obtained by the nonlinear least-squares fitting for the uncomplexed system. We tested this possibility by performing pH titrations on KNI-272 in solution and following the pH dependence of the absorbance at 352 nm. As shown in Figure 6, the data were consistent with a pK of 4.9 ± 0.02, which is within the range obtained from the nonlinear least-squares analysis of the

Table 1. *Global nonlinear least-squares analysis of proton linkage for binding of the inhibitor KNI-272 to the HIV-1 protease*

Quantity[a]	Fit 1	Fit 2	Fit 3	Fit 4	Fit 5	Average[b]
pK_1^c	2.87	2.75	3.17	2.92	2.89	2.92 ± 0.15
pK_1^f	4.79	4.89	4.89	4.87	4.77	4.84 ± 0.06
pK_2^c	6.24	6.92	7.00	6.72	6.26	6.63 ± 0.4
pK_2^f	5.54	6.37	6.50	6.01	5.59	6.00 ± 0.4
ΔH^0_{bind}	-7.1	-5.7	-5.3	-6.33	-7.1	-6.3 ± 0.8
ΔH_{p1}	-5.8	-6.5	-6.91	-5.9	-5.9^c	-6.3 ± 0.5
ΔH_{p2}	-1.3^c	-1.3^c	-1.3^c	-1.3^c	-1.3^c	-1.3
$\Delta\Delta H_{p1}$	0^c	0^c	2.59	1.35	0^c	—
$\Delta\Delta H_{p2}$	0^c	-2.31	-2.78	-1.22	0^c	—
SSR	0.268	0.096	0.038	0.129	0.27	—

[a] All enthalpy values are in kcal/mol.
[b] Average of all fits.
[c] These quantities were not allowed to float during fit.

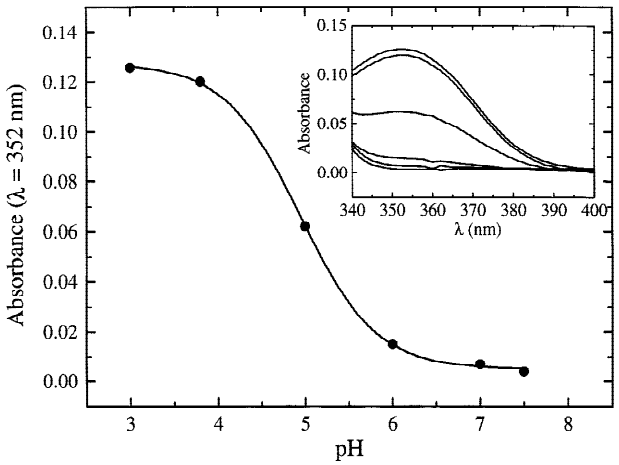

Fig. 6. pH dependence of the optical absorbance of KNI-272 at 352 nm. The data are consistent with the presence of one ionization group with a pK of 4.9 ± 0.02 (solid line).

binding data. Together, these data strongly suggest that the two groups undergoing protonation/deprotonation are one of the aspartates in the aspartyl dyad and the aromatic isoquinoline nitrogen in KNI-272.

According to the nonlinear least-squares analysis, the estimated buffer- and pH-independent binding enthalpy is -6.3 ± 0.8 kcal/mol, indicating that the binding of KNI-272 is intrinsically exothermic, and not due to the coupling of the binding process to a protonation/deprotonation reaction.

The association constant

Although the association constant of KNI-272 is too large to be accurately measured by isothermal calorimetry, it can be estimated by differential scanning calorimetry (Brandts & Lin, 1990; Todd & Freire, 1999; Velazquez-Campoy et al., 2000). In the presence of the inhibitor, the Gibbs energy of structural stabilization of the protease $\Delta G(T, \text{pH}, [I])$ is given by

$$\Delta G(T, \text{pH}, [I]) = \Delta G^0(T, \text{pH}) + RT \ln(1 + K_a[I]) \qquad (2)$$

where $\Delta G^0(T, \text{pH})$ is the Gibbs energy in the absence of inhibitor, and $[I]$ is the inhibitor concentration. The dependence of $\Delta G^0(T, \text{pH})$ on temperature and pH was established in a previous publication (Todd & Freire, 1999; Todd et al., 1998). The experimentally derived master equation for $\Delta G^0(T, \text{pH})$ is

$$\begin{aligned}\Delta G^0(T, \text{pH}) = {} & -9{,}000 + 3{,}200\cdot(T - 298.15) \\ & - T\cdot(-79.15 + 3{,}200\cdot\ln(T/298.15)) \\ & - 4\cdot R\cdot T\cdot\ln\left(\frac{(1 + 10^{4.3-\text{pH}})}{(1 + 10^{2.9-\text{pH}})}\right). \qquad (3)\end{aligned}$$

The binding of the inhibitor stabilizes the native conformation of the protein, being the shift in the denaturation temperature T_m, toward high temperatures the most evident feature. The association constant can be estimated from the dependence of T_m on inhibitor concentration in combination with the master equation for $\Delta G^0(T, \text{pH})$ and the stability equations for a dimeric protein as described before (Todd & Freire, 1999; Todd et al., 1998). In the absence of inhibitor, the T_m of the HIV-1 protease is 62.3° (15.6 μM protease, 10 mM glycine, 2% DMSO, pH 3.8). In the presence of 40 μM KNI-272, the T_m for the protease increases to 77.0 °C, consistent with an association constant for KNI-272 of $9 \cdot 10^9$ M^{-1} at pH 3.8. For KNI-529 under the same conditions, the estimated association constant is 5×10^6 M^{-1} (T_m = 68.2 °C). Also, from titration calorimetric experiments, we determined an association constant for KNI-529 of 3×10^6 M^{-1} in agreement with previous results (Baldwin et al., 1995).

With the pK values obtained from the analysis of the titration data and the estimated value for the association constant at 25 °C and pH 3.8 (the HIV-1 protease exhibits reversible thermal denaturation only at acidic pH values), it is possible to calculate the pH dependence of the association constant of the inhibitor, as shown in Figure 7. There is a region of maximal affinity between pH 5 and pH 6. At those pH values, the affinity is close to $6 \pm 1 \times 10^{10}$ M^{-1} ($K_d = 16 \pm 3$ pM). The affinity decreases at low pH (due to the deprotonation process) and at high pH (due to the protonation process). Using a value of -14.7 ± 0.1 kcal/mol for the Gibbs energy of binding under conditions in which $n_H = 0$, a buffer- and pH-independent binding entropy of 28 ± 3 cal/K mol is obtained. Thus, the binding of KNI-272 is both enthalpically and entropically favorable.

Binding of KNI-272 to V82F/I84V-resistant mutant

The double mutation V82F/I84V has been shown to lower the binding affinity of protease inhibitors in clinical use (Markowitz et al., 1995; Ala et al., 1998; Klabe et al., 1998) and also KNI-272 (Gulnik et al., 1995). This double mutation is located at the edges of the active site, distorting the dimensions and geometry of the binding site without significantly changing its polarity or chemical composition. The binding thermodynamics for the binding of KNI-272 to the mutant HIV-1 protease V82F/I84V was determined by

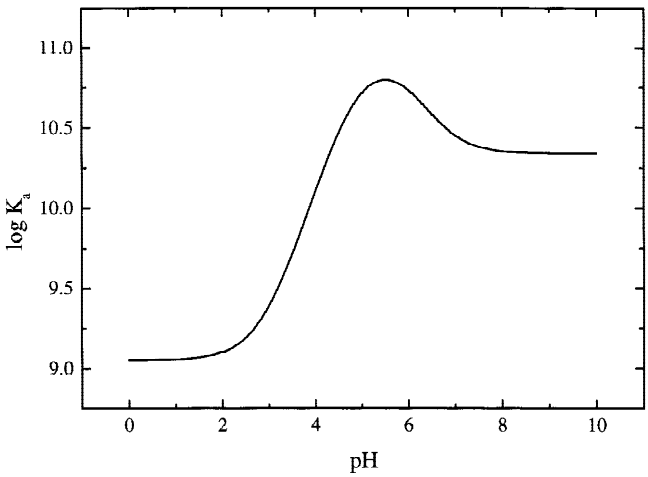

Fig. 7. The pH dependence of the logarithm of the association constant of KNI-272 to the wild-type HIV-1 protease at 25 °C and low salt concentration. The association constant is maximal between pH 5 and pH 6. At those pH values, the affinity is close to $6 \pm 1 \times 10^{10}$ M^{-1} ($K_d = 16 \pm 3$ pM).

ITC experiments performed under the same conditions used for the wild-type protease (10 mM acetate, 2% DMSO, pH 5). The heat capacity change (Fig. 3) was -400 ± 20 cal/K mol, which is very close to the value obtained for the wild-type.

At pH 3.8 (10 mM glycine, 2% DMSO), the apparent binding enthalpy was -4.5 ± 0.1 kcal/mol, which is surprisingly more favorable than the one measured for the wild-type under the same conditions (-3.4 ± 0.1 kcal/mol). Because, under these conditions, an average of -0.7 ± 0.06 protons are released from the complex upon binding to the wild-type and this release is accompanied by a positive enthalpy change, it was decided to check if the protonation/deprotonation process was different with the mutant protease. The dependence of the apparent binding enthalpy on the ionization enthalpy of the buffer yielded 0.09 ± 0.02 protons released upon binding and a buffer-independent binding enthalpy was -4.7 ± 0.1 kcal/mol (Fig. 4). From these results, it is expected that the association constant for the binding to the mutant protease should not exhibit a pH dependence as pronounced as the one observed with the wild-type. Because the release of protons from the complex at pH 3.8 is accompanied by a positive enthalpy change of 6.3 ± 0.5 kcal/mol (Table 1), the intrinsic binding enthalpy of KNI-272 to the mutant protease is estimated to be -5.3 ± 0.6 kcal/mol compared to the value of -6.9 ± 0.6 kcal/mol obtained for the wild-type protease using the same procedure. Thus, the intrinsic binding enthalpy of the inhibitor is less favorable ($\sim$1.7 kcal/mol) to the resistant mutant than to the wild-type, even though the apparent enthalpy may show a reverse trend due to differences in the protonation/deprotonation coupling. The effect of the double mutation on the binding enthalpy is consistent with a reduction in the magnitude of van der Waals and other inhibitor/protein interactions, probably as a result of the inability of KNI-272 to adapt to the distorted geometry of the mutated binding site.

The association constant for the binding of KNI-272 to the mutant protease was determined by DSC under the same conditions employed for the wild-type (10 mM glycine, 2% DMSO, pH 3.8, 11.6 μM (dimer) HIV-1 protease V82F/I84V, 40 μM KNI-272). The estimated value for K_a at 25 °C was 10^8 M^{-1}, nearly 100 times less than that obtained for the binding to the wild-type ($9 \cdot 10^9$ M^{-1}) under the same conditions. Due to the protonation coupling of the binding reaction, the binding constant to the wild-type increases by a factor of 5 to 6 at the optimal pH range. Because the mutant protease shows a much weaker protonation coupling, the association constant is expected to be more than 500-fold weaker than that of the wild-type in the optimal pH range (between pH 5 and 6). These results indicate that mutations in the protease molecule are able to affect the binding energetics of inhibitors not only through changes in direct interactions but also through indirect means like a change in the magnitude of the protonation/deprotonation coupling.

Structure-based thermodynamic analysis

The most salient feature in the binding thermodynamics of KNI-272 is its strongly exothermic character, which contrasts sharply with the endothermic behavior of other inhibitors, including those in clinical use (Hoog et al., 1996; Luque et al., 1998; Todd & Freire, 1999; Todd et al., 2000; Velazquez-Campoy et al., 2000). The structure of the complex of KNI-272 and the HIV-1 protease has been solved crystallographically at 2.0 Å resolution (Protein Data Bank file 1HPX) (Baldwin et al., 1995).

Although KNI-272 is hydrophobic, its binding involves the presence of long-lived hydration water molecules at the protein/inhibitor interface (Wang et al., 1996b). Without considering the water molecules 869 Å^2 of nonpolar and 372 Å^2 of polar surface area buried upon binding (i.e., a 2.33 nonpolar/polar ratio). This ratio is similar to that observed for inhibitors in clinical use and would be consistent with an unfavorable enthalpy change. Saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir (VX478) bury a larger proportion of nonpolar area (2.9, 2.4, 3.4, 3.2, and 2.6 nonpolar/polar ratios, respectively), and their binding is characterized by positive enthalpies at 20 °C (Todd et al., 2000; Velazquez-Campoy et al., 2000). The binding of KNI-272, on the other hand, is exothermic at that temperature. The presence of these long-lived water molecules at the binding interface has been observed both in the crystal and by NMR measurements in solution (Wang et al., 1996b). Four of these waters (301, 566, 607, and 608 in the crystal structure) are in direct contact with the inhibitor (distances smaller that 5 Å); the other two (waters 406 and 426) are located at longer distances (7 Å), but are still completely buried and tightly bound to the protease/KNI272 complex (Wang et al., 1996b). The burial of all these water molecules increases the overall polarity of the change in solvent accessible surface area. When all the tightly bound water molecules are included in the calculation, the surface areas buried upon binding become 833 Å of nonpolar and 612 Å of polar surface area, i.e., a 1.36 nonpolar/polar ratio. Considering only the waters in direct contact with the inhibitor, the values for the surface area buried upon formation of the complex are 829 Å of nonpolar area and 526 Å of polar area, i.e., a 1.56 nonpolar/polar ratio. The potential role of this bound water in the potency of the inhibitor KNI-272 has been hypothesized before (Wang et al., 1996b). Similar conclusions have been reached in other cases (Renzoni et al., 1997). The increased polarity, atomic packing, and van der Waals interactions provided by these water molecules appears to contribute to the favorable binding enthalpy as observed experimentally. Analysis of the complex using the structural parameterization of the energetics published previously (Murphy & Freire, 1992; Lee et al., 1994; Gomez et al., 1995; D'Aquino et al., 1996; Hilser et al., 1996; Luque et al., 1996; Luque & Freire, 1998) yields a binding enthalpy of -4.9 kcal/mol at 25 °C in good agreement with the experimental data. If the tight water molecules were not present, the calculated enthalpy change would become positive and close to $+6$ kcal/mol.

This behavior should be contrasted with the situation existing for other inhibitors. Direct observation of long-lived water molecules in solution has only been reported for the cyclic urea inhibitor DMP323, which was designed to displace the ubiquitous water molecule (301 in the structure of KNI-272) that bridges the inhibitor and the flaps (Wang et al., 1996a). In this case, however, only two long-lived water molecules (equivalent to waters 566 and 608 in the KNI-272 structure) were observed in the binding site (Wang et al., 1996b). The crystal structures of inhibitors in clinical use (Indinavir, Nelfinafir, Ritonavir, Saquinavir) show only three buried water molecules (equivalent to waters 301, 566, and 608 in the KNI-272 structure) at distances smaller that 5 Å of the inhibitor. On average, the ratio of nonpolar to polar surface area buried upon binding for these inhibitors is 2.96 without considering the water molecules and 2.11 considering them. For KNI-272 the tightly bound water molecules lower the ratio to 1.56 as discussed above. The presence of the water molecules increases the effective polarity of the inhibitor, making it comparable to that observed for polar

peptide inhibitors (e.g., Glu-Asp-Leu) that bind to the protease with a favorable binding enthalpy (Velazquez-Campoy et al., 2000).

The buffer- and pH-independent binding entropy of 28 cal/K mol is composed primarily of the solvation entropy change, the loss of conformational degrees of freedom, and the loss of translational entropy. The entropy change contributes −7.8 kcal/mol to the binding Gibbs energy at 25 °C, which is on the same order as the enthalpy contribution. The presence of six bound water molecules in the KNI-272 complex indicates that the dehydration at the inhibitor/protein interface is not complete. Energetically, the presence of these water molecules reduces the magnitude of the desolvation entropy while making the enthalpy change more favorable. From a structural point of view, KNI-272 is a conformationally constrained molecule (Kiso, 1996), and as such, it losses little conformational entropy upon binding. The small conformational entropy penalty contributes to the high binding affinity of this inhibitor. On the other hand, these same conformational constraints contribute to the loss of binding affinity against protease mutations that distort the geometry of the binding site.

Conclusions

KNI-272 binds to the wild-type HIV-1 protease with high-affinity ($K_i \sim 16$ pM at pH 5–6, low-salt concentration). The origin of the extremely high affinity of this inhibitor is the combination of favorable binding enthalpy and favorable binding entropy changes. In addition, these experiments also emphasize the favorable contribution of buried water molecules to the binding energetics. Previously, we have concluded that the conformational rigidity of inhibitors in which high binding affinity has been achieved by significant conformational restraints that pre-shape the inhibitor to the binding site are especially susceptible to resistant mutations (Luque et al., 1998; Todd & Freire, 1999; Velazquez-Campoy et al., 2000). Highly constrained molecules are unable to adapt to a distorted binding site geometry, and consequently lose van der Waals and other favorable interactions, resulting in a significant loss of binding affinity. Even though KNI-272 binds with a favorable enthalpy change, it is still highly constrained, and therefore susceptible to the effects of resistant mutations like the one reported in this paper.

We have suggested previously that an increased flexibility of the inhibitors should be a desirable feature (Luque et al., 1998; Todd & Freire, 1999; Velazquez-Campoy et al., 2000). However, increased flexibility alone will bring about a lower binding affinity and specificity unless it is compensated by additional interactions of an enthalpic nature (Velazquez-Campoy et al., 2000). The fact that KNI-272 and other allophenylnorstatine-containing inhibitors bind to the protease molecule with a favorable enthalpy change provides an important chemical scaffold for the design of more conformationally relaxed inhibitors. KNI-272 has a binding affinity in the picomolar range, which is much higher than that of other inhibitors. Against this background, there is room for the introduction of certain amount of flexibility at critical points without lowering the affinity below the level of existing inhibitors. This appears to be the situation with JE-2147, another allophenylnorstatine-containing inhibitor that has been shown to maintain high potency against several inhibitor resistant mutants of the HIV-1 protease due to the introduction of flexible regions in the inhibitor (Kiso, 1998; Mimoto et al., 1999; Yoshimura et al., 1999). Preliminary calorimetric measurements with JE-2147 (not published) indicate that its binding to the protease is also strongly exothermic (enthalpically favorable). The results of these studies will be the subject of a future communication.

Materials and methods

Protease purification

HIV-1 protease was prepared according to the following procedure optimized for the high yield, activity, and stability required for calorimetric analysis. Plasmid-encoded mutant protease (Q7K/L33I/L63I designed to remove three hypersensitive autolytic sites) was expressed as inclusion bodies in *Escherichia coli* 1458. A mutant protease (Q7K/L33I/L63I/V82F/I84V) was created using site-directed mutagenesis (Stratagene QuickChange) according to the manufacturer's instructions. Oligonucleotides (IDT DNA, Inc., Coralville, Iowa) that encoded for the desired mutations (V82F/I84V) were designed, annealed to the Q7K/L33I/L63I plasmid DNA, then amplified using Pfu Turbo DNA polymerase. The DNA sequence of expression plasmids containing these unique mutations was confirmed; the only amino acid changes were those intentionally introduced using the designed oligonucleotide. Drug-resistant, mutant protease was also expressed as inclusion bodies. 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 (20,000 × *g* for 20 min at 4 °C). Inclusion bodies were washed with three buffers. Each wash consisted of resuspension (glass homogenizer, sonication) and centrifugation (20,000 × *g* for 20 min at 4 °C). In each step a different washing buffer was employed: Wash-1 (25 mM Tris, 2.5 mM EDTA, 0.5 M NaCl, 1 mM Gly-Gly, 50 mM 2-ME, pH 7.0), Wash-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), Wash-3 (25 mM Tris, 1 mM EDTA, 1 mM Gly-Gly, 50 mM 2-ME, pH 7.0). Protease was solubilized in 25 mM Tris, 1 mM EDTA, 5 mM NaCl, 1 mM Gly-Gly, 50 mM 2-ME, 9 M Urea, pH 8.0, clarified by centrifugation and applied directly to an anion exchange Q-Sepharose column (Q-Sepharose HP, Pharmacia, Uppsala, Sweden) previously equilibrated with the same buffer. 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 remaining contaminants occurred upon acidification. Protease-containing fractions were concentrated and stored at 4 °C at 5–10 mg/mL.

The HIV-1 protease was folded by 10-fold dilution into 10 mM formic acid at 0 °C. The pH was gradually increased to 3.8, then the temperature was raised to 30 °C. Sodium acetate pH 5.0 was added up to 100 mM and protein was concentrated. Folded protease was desalted into 1 mM sodium acetate, 2 mM NaCl at pH 5.0 using a gel filtration column (PD-10, Pharmacia) and stored at either 4 °C or −20 °C (≥2.5 mg/mL) without loss of activity in several weeks. After folding, the 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 (California Peptide Research Inc., Napa, California). 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 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 (1,800 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 unhydrolyzed. Typical protease preparations hydrolyzed chromogenic substrate at 5–6 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, Massachusetts). 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, 2% DMSO, pH 3.8. Solutions containing 17 μM (dimer) HIV-1 protease and 0–50 μM KNI-272 were 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 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 carried out using a high-precision VP-ITC titration calorimeter (Microcal Inc., Northampton, Massachusetts). The enzyme solution (20 μM dimer) in the calorimetric cell was titrated with KNI-272 or KNI-529 dissolved in the same buffer (at a concentration of 0.3 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. The binding enthalpy was measured at different pH values using several buffers (containing 2% DMSO) differing in ionization enthalpy. The extension and contribution of protonation/deprotonation processes to the binding were assessed by the determination of the pH-dependence of the binding enthalpy and by the use of buffers with different enthalpies of ionization (Gomez & Freire, 1995; Baker & Murphy, 1996). Analysis of the data was performed using software developed in this laboratory. The reported enthalpy errors correspond to the standard deviation of the areas under the calorimetric peaks, and those in heat capacity to the errors in the regression analysis of the temperature of the enthalpy change.

Analysis of proton linkage

For a binding reaction in which the association of the ligand is coupled to the absorption or release of protons, the experimental thermodynamic parameters become a function of pH and some of them also a function of the buffer in which the reaction is performed. These dependencies provide the information necessary to dissect intrinsic binding effects from protonation effects. The relevant parameters in the analysis are the number of protons that are absorbed or released, n_H, the measured binding enthalpy, ΔH_{app}, which is pH and buffer dependent, ΔH_{bind}, which is pH dependent but buffer independent, and the association constant itself, K_a. The pH dependence of the thermodynamic parameters is given by the following proton-linkage equations (Baker & Murphy, 1996) and can be extracted from the data by global nonlinear least-squares analysis:

$$n_H = \sum_i \left(\frac{10^{\mathrm{pK}_i^c - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^c - \mathrm{pH}}} - \frac{10^{\mathrm{pK}_i^f - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^f - \mathrm{pH}}} \right) \quad (4)$$

$$\Delta H_{bind} = \Delta H_{bind}^0 + \sum_i \left(\left(\frac{10^{\mathrm{pK}_i^c - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^c - \mathrm{pH}}} - \frac{10^{\mathrm{pK}_i^f - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^f - \mathrm{pH}}} \right) \Delta H_{p,i} + \frac{10^{\mathrm{pK}_i^c - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^c - \mathrm{pH}}} \Delta\Delta H_{p,i} \right) \quad (5)$$

$$K_a = K_a^0 \prod_i \left(\frac{1 + 10^{\mathrm{pK}_i^c - \mathrm{pH}}}{1 + 10^{\mathrm{pK}_i^f - \mathrm{pH}}} \right) \quad (6)$$

where pK_i^c and pK_i^f are the pK values for the i ionizable group in the ligated (complex) and in the unligated species (free), ΔH_{bind}^0 is the buffer-independent and pH-independent binding enthalpy (fully deprotonated species), $\Delta H_{p,i}$ and $\Delta\Delta H_{p,i}$ are the protonation enthalpy and change in the protonation enthalpy for the i ionizable group in the protein or in the ligand, respectively, and K_a^0 is the pH-independent association constant (fully deprotonated species). The difference between the pK values after, pK_i^c, and before the binding, pK_i^f, reflects the change in the population of the protonated and deprotonated species for each ionizable group upon binding and the character of the contribution (favorable or unfavorable) to the global affinity and enthalpy of each single proton transfer. The reported errors in n_H and ΔH_{bind} correspond to the errors in the lineal regression of the dependence of the binding enthalpy on the enthalpy of ionization of the buffer.

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