A synergy of activity, stability, and inhibitor-interaction of HIV-1 protease mutants evolved under drug-pressure

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Abstract
A clinically-relevant, drug-resistant mutant of HIV-1 protease (PR), termed Flap+(I54V) and containing L10I, G48V, I54V and V82A mutations, is known to produce significant changes in the entropy and enthalpy balance of drug-PR interactions, compared to wild-type PR. A similar mutant, Flap+(I54A), which evolves from Flap+(I54V) and contains the single change at residue 54 relative to Flap+(I54V), does not. Yet, how Flap+(I54A) behaves in solution is not known. To understand the molecular basis of V54A evolution, we compared nuclear magnetic resonance (NMR) spectroscopy, fluorescence spectroscopy, isothermal titration calorimetry, and enzymatic assay data from four PR proteins: PR (pWT), Flap+(I54V), Flap+(I54A), and Flap+(I54), a control mutant that contains only L10I, G48V and V82A mutations. Our data consistently show that selection to the smaller side chain at residue 54, not only decreases inhibitor affinity, but also restores the catalytic activity.

KEYWORDS
calorimetry, HIV-1, inhibitor, NMR, protease, thermodynamics

1 | INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) protease (PR) is an enzyme essential for HIV-1 replication.1–5 Although structure-based drug design has resulted in the development of various PR inhibitors, the long-term effectiveness of these inhibitors is hampered by the generation of drug-resistance mutations.6–20 The structure of HIV-1 PR has been well characterized (Figure 1): it is a homodimer, with the two subunits interfacing via residues located in the flaps (residues 45 to 55), in the active-site fireman’s grip (residues 25 to 27) and in the N- and C-terminal β-sheet.21–23 They also indirectly interact with each other through inhibitors, at the P1 loops that include one of the Flap mutation sites, residue 82 (Figure 1). To understand the mechanism of drug-resistance evolution, thermodynamic studies of inhibitor interactions with PR and with various drug-resistant mutants have been conducted for the past two decades.24–36

Flap+(I54V), which contains a set of clinically-relevant drug-resistant PR mutations, L10I, G48V, I54V and V82A (Figure 1), is known to interact with inhibitors, such as saquinavir, amprenavir and darunavir (DRV), with a balance of entropy and enthalpy distinct from wildtype (WT) PR.35 Specifically, based on isothermal titration calorimetry (ITC) data, inhibitor interaction with WT PR is...
We explore two questions, what feature(s) of Flap+\textsubscript{(I54)} might force a change at position 54 (to V) to escape drug-pressure, and why does further evolution at this site, from I54V to I54A, occur in the continued presence of drug?

We demonstrate that Flap+ with I54A binds to the substrate-analogue inhibitor, pepstatin, similar to pWT, and stronger than Flap+\textsubscript{(I54V)} or Flap+\textsubscript{(I54)}. Further, Flap+\textsubscript{(I54A)} exhibits a higher catalytic activity, closer to the level of pWT, than Flap+\textsubscript{(I54V)} or Flap+\textsubscript{(I54)}. The Flap+\textsubscript{(I54A)} fold is similar to that of Flap+\textsubscript{(I54V)} and Flap+\textsubscript{(I54)}, but with lower dimerization affinity and less stability. Overall, the observations indicate that the I54V change, to generate Flap+\textsubscript{(I54V)}, results in decreased affinity to DRV but also leads to a decrease in catalytic activity of the Flap+ mutant, while the subsequent change to A at position 54 improves catalytic activity but with a drawback of reduced protein stability.

2 RESULTS

2.1 Overall NMR spectral pattern, indicating similarity of the flap+ mutants’ conformation

We expressed pWT, Flap+\textsubscript{(I54V)}, Flap+\textsubscript{(I54A)}, and Flap+\textsubscript{(I54)} and confirmed the purified proteins by mass spectrometry (Figure S1). To understand whether amino acid substitution at position 54 has an effect on folding, we recorded NMR spectra for each of the four proteins. We assigned the backbone chemical shifts of Flap+\textsubscript{(I54A)} and Flap+\textsubscript{(I54)}, which we previously did not study,\textsuperscript{40,41} in their DRV-bound forms. In the absence of inhibitors, spectral features above \textsuperscript{1}H 8.7 ppm and below \textsuperscript{1}H 7.8 ppm were similar among the Flap+ mutants and pWT, even at 3 \textmu M protein concentration (Figure 2a), indicating that the mutants form dimer at this concentration. Similarly, when DRV was added, many of the resonance positions in these regions of the spectra changed in a similar manner (Figure 2b).\textsuperscript{40,41} These data suggest that dimerization occurs for each of the Flap+ mutants, however, the more centrally located resonances in the spectra suggest a potential folding issue for Flap+\textsubscript{(I54A)} (see next paragraph).

When resonances at the random coil region (marked by dashed rectangle in Figure 2a) were compared, only the inhibitor-free state of Flap+\textsubscript{(I54A)} exhibited an unfolded component (\textasciitilde29\%), estimated from the signal intensity of indole NH resonances, whereas other proteins did not. The unfolded fraction in Flap+\textsubscript{(I54A)}, \textasciitilde19\% at 3 \textmu M protein concentration, did not significantly decrease upon addition of DRV (Figure 2b). To assess

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{PR structure, showing the locations of the residues that are mutated in Flap+\textsubscript{(I54V)} and Flap+\textsubscript{(I54A)}: L10, G48 and V82 (yellow spheres) and I54 (red spheres). Two subunits, A and B, are depicted in green and light blue, respectively. The flap region (residues 45–55) and the active-site fireman’s grip (residues 25 to 27) are highlighted in pink with the residue numbers in a small font. Terminal β-sheet regions, residues from 1 to 4 and 96 to 99, are marked in a small font. The structure was generated using PDB: 1T3R.\textsuperscript{54}}
\end{figure}
whether the unfolded fraction was due to dimer dissociation, Flap+(I54A) protein was folded at a higher protein concentration, 40 μM. However, the unfolded fraction did not decrease; nor did it decrease upon pepstatin titration to the solution (Figure 2c). Additional DRV titration did not decrease the unfolded fraction, either (Figure 2d). Only when Flap+(I54A) was folded in the presence of inhibitors did the unfolded fraction decrease (as a peak volume, Figure 2e, Table S1). Thus, the unfolded fraction of Flap+(I54A) is not protein-concentration dependent and is most likely due to mis-folding at the folding step during sample preparation (discussed later). These observations informed our design of ITC experiments. In particular, for the competitive experiments using both pepstatin and DRV, we decided to use proteins that were folded in the presence of pepstatin instead of proteins to which pepstatin was added after sample preparation.

2.2 | Pepstatin and DRV interaction with PRs monitored by ITC

Given the known effect of the Flap+(I54V) mutations on the thermodynamics of PR-inhibitor interactions,35 we examined the thermodynamic parameters of pWT and Flap+ interactions with both a weak binder, pepstatin, and a strong binder, DRV (described in the next section). ITC data of the pepstatin to PR interactions were recorded using PR protein concentrations between 20 and 30 μM (Figure 3a-3h). Thermodynamic...
parameters of pepstatin binding to pWT at 20°C ($\Delta G$, $-8.7 \pm 0.14$ kcal/mol; $\Delta H$, $8.7 \pm 0.24$ kcal/mol; $-T\Delta S$, $-17.4 \pm 0.28$ kcal/mol) were consistent with those obtained at 25°C by Freire’s group ($\Delta G$, $-8.4 \pm 0.9$ kcal/mol; $\Delta H$, $10.1 \pm 0.7$ kcal/mol; $-T\Delta S$, $-18.4 \pm 0.06$ kcal/mol) (Figure 3a, e, Table 1a). Here, we clearly demonstrate that thermodynamic parameters of pepstatin binding to Flap+ (I54A) were similar to that of pWT, showing small $\Delta \Delta G$, $\Delta \Delta H$ and $\Delta (-T\Delta S)$, while pepstatin binding to Flap+(I54) and Flap+(I54V) exhibited unfavorable $\Delta H$ and favorable $(-T\Delta S)$ upon pepstatin binding, showing positive $\Delta \Delta H$ and negative $\Delta (-T\Delta S)$ against pWT (Figure 4a-4c, Table 1a).

The thermodynamics of DRV binding to pWT and the Flap+ mutant proteins were determined using a competition assay, by titrating DRV in the presence of weak binder, pepstatin (Figure 3i-3p, Table 1b). Ideally, the optimal weak binder for competitive ITC experiments

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**Figure 3** Calorimetric titration of HIV-1 pWT and the Flap+ mutants, at 20°C, with (a–h) pepstatin or (i–p) DRV in the presence of excess pepstatin. The heat effects associated with the injection of the inhibitors (a–d and i–l) and corresponding isotherms (e–h and m–p) are shown.
would be chosen from a panel of weak binders based on the $K_D$ of the strong binder under study. However, we used a single weak binder, pepstatin, for all PR-DRV interaction studies, to avoid any biases caused by differences in the solubility of weak inhibitors in aqueous solution or differences in the mechanisms of PR interactions with weak inhibitors. In addition, to reduce the unfolded fraction of PRs, we used PRs that were folded in the presence of pepstatin.

In the competitive experiments, all the DRV titrations in the presence of pepstatin showed apparent favorable $\Delta H$ changes (Figure 3i-3p, Table 1b), which is consistent with previous results. Using $\Delta H$ and $\Delta G$ for the pepstatin titration alone and those of the competitive data (Table 1a,b), we calculated the thermodynamic parameters of DRV binding to PRs (Table 1c). Overall, thermodynamic parameters of Flap+(I54A) and Flap+(I54V) were similar to those published previously, showing a difference in the entropy-enthalpy balance of Flap+(I54V) relative to pWT but not Flap+(I54A) (Figure 4d-4f, Table 1c). We found that thermodynamic parameters of Flap+(I54) were more similar to those of Flap+(I54V) than pWT and Flap+(I54A) (Figure 3i-3p, Table 1c). Data reproducibility was confirmed by repeating the ITC experiments (Table S2, S3).

The poor heat flow of Flap+(I54A) upon titration with DRV in the presence of pepstatin is notable (Figure 3j). Although the injectant heat change of Flap+(I54A) (Figure 3j) seemed to be almost half of other isotherms (Figure 3i, k, l), it was not: the heat flow at each injectant in Figure 3j was just slow, and the integration of the heat change was not necessarily small. Comparison of the heat flows of these DRV titrations at the second and sixth injections are shown (which corresponds to the $/C24$ and $/C24$ points in Figure S2a, S2b, respectively). Such heat flow, not explained by a first-order reaction model (Figure S2a, S2b), even at the early titration point of the competitive titration (Figure S2c), is indicative of slow on- or off- rate of the inhibitor, or may involve an intermediate step. Due to limited inhibitor solubility, we could not vary the parameters widely to obtain detailed kinetic parameters.

### 2.3 PR dimer formation and stability

Since Flap+(I54A) exhibited slow heat flow in the isotherm, the folding and dimerization of the proteins were more quantitatively characterized using intrinsic Trp fluorescence spectroscopy at different protein

### TABLE 1 Thermodynamic parameters obtained from calorimetric titration of PR with inhibitors: (a) pepstatin-binding parameters, (b) DRV-binding parameters in the presence of excess pepstatin, and (c) DRV-binding parameters extracted from (a) and (b)

|                        | WT | Flap+(I54A) | Flap+(I54) | Flap+(I54V) |
|------------------------|----|------------|------------|------------|
| (a) Pepstatin titration |    |            |            |            |
| $K_D$ (nM)             | 315| 218        | 814        | 819        |
| $\Delta G$ (kcal/mol)  | $\pm$8.72| $\pm$8.94  | $\pm$8.17  | $\pm$8.01  |
| $\Delta H$ (kcal/mol)  | 8.67| 7.58       | 13.5       | 16.6       |
| $-T\Delta S$ (kcal/mol)| $\pm$17.4| $\pm$16.5  | $\pm$21.6  | $\pm$24.8  |
| $N$                    | 0.905| 0.728      | 1.03       | 1.14       |
| (b) DRV titration in the presence of pepstatin |    |            |            |            |
| $K_D$ (nM)             | 0.70| 159        | 7.77       | 4.39       |
| $\Delta G$ (kcal/mol)  | $\pm$12.3| $\pm$9.12  | $\pm$11.7  | $\pm$11.2  |
| $\Delta H$ (kcal/mol)  | $\pm$19.2| $\pm$14.6  | $\pm$16.   | $\pm$15.9  |
| $-T\Delta S$ (kcal/mol)| 6.96| 5.49       | 4.30       | 4.73       |
| $N$                    | 1.16| 1.26       | 1.02       | 1.18       |
| (c) DRV binding parameters |    |            |            |            |
| $K_D$ (pM)             | 1.33| 177.       | 7.20       | 18.5       |
| $\Delta G$ (kcal/mol)  | $\pm$15.9| $\pm$13.1  | $\pm$15.0  | $\pm$14.4  |
| $\Delta H$ (kcal/mol)  | $\pm$10.7| $\pm$7.09  | $\pm$2.67  | $\pm$0.513 |
| $-T\Delta S$ (kcal/mol)| $\pm$5.26| $\pm$6.00  | $\pm$12.3  | $\pm$14.4  |

Abbreviation: DRV, darunavir.
concentrations and denaturant. One of the two Trp residues in PR, residue six, is located at the dimer interface and is exposed to solution upon dimer dissociation, thus the fluorescence emission of this residue is known to be reduced by dimer dissociation. In pWT and Flap+ mutants, intrinsic Trp fluorescence exhibited linear dependence at protein concentrations above 0.25 μM (Figure 5a). Through this analysis, dimer dissociation constants for pWT and the Flap+ mutants were determined to be <0.25 μM, consistent with previous results obtained for PR and other mutants.

In-line with this observation, urea denaturation produced similar fluorescence profiles among the proteins (Figure 5b). A slightly lower chemical stability was observed only for Flap+(I54A), with a half urea denaturation concentration of 1.8 ± 0.1 M, compared to the others (2.3–2.5 M, respectively) (Figure 5b). The fluorescence emission is lower in Flap+(I54A) compared to pWT, Flap+(I54) and Flap+(I54V), presumably reflecting greater monomer unfolding compared to the other proteins. This is qualitatively consistent with the above NMR results indicating the presence of an unfolded fraction in the Flap+(I54A) samples.

2.4 | PR activity changes upon I54 mutations in flap+

Viral evolution in response to inhibition is not only dependent on drug-pressure but also depends on adaptation of the enzyme activity. Thus, we assessed the catalytic activity of PRs at three protein concentrations ranging from 50 nM to 0.5 μM by measuring the emission of a fluorescent substrate one product (Figure 5c, S2). Assays lower than 50 nM PR concentration were not conducted because differences in dimer dissociation constants may affect the activity. This requirement for a high PR protein concentration and, therefore, requirement for
much higher substrate concentration, practically prevented us from determining the substrate concentration dependence of the activity. Nevertheless, the results indicate that the activity of Flap+(I54A) is higher and more similar to that of pWT than the activity of Flap+(I54) and Flap+(I54V), suggesting that I54A may occur because it benefits the virus by improving PR proteolytic activity. Also, we found that Flap+(I54) has the lowest activity, which may be a reason why Flap+ mutation contains I54V or I54A mutation despite Flap+(I54) having a higher DRV dissociation constant compared to pWT (Table 1c).

2.5 | Flap+(I54A) structural characteristics, monitored by HD-exchange

Since Flap+(I54A) exhibited a lower stability in the fluorescence spectroscopy data (Figure 5a, b), we investigated slow conformational changes by HD-exchange experiments for Flap+(I54A), in comparison with Flap+(I54). The proteins were folded in the presence of pepstatin in a phosphate at pH 3.5. After lyophilization, proteins were solubilized by adding D2O, followed by addition of excess DRV to replace the pepstatin with DRV (Figure 6a, b). In this HD-exchange spectrum of Flap+(I54) -DRV, some of backbone amide protons were exchanged to deuteron (Figure 6a), which were basically those in the loop and turn regions, such as G16, G17, M36, L38, and G40 (Figure 6c, marked with black labels). In contrast, residues in the flap region, such as G49, G51, G52 and I54, were observed in the HD-exchange spectrum, indicating that the flaps are tightly closed in the DRV-bound form (Figure 6a, marked with blue labels). An almost identical HD-exchange feature was observed in the spectra of Flap+(I54A) spectrum (Figure 6b).

Interestingly, the amide protons of the T80 residues in Flap+(I54A) were clearly exchanged to deuterons, while Flap+(I54) were not (Figure 6a, b, marked by green). These appear as a split signal due to the asymmetric structure of DRV-bound protein. As shown in the rotated view at the I54-T80 region (Figure 6d), the δ1-methyl group of I54 is located within 4 Å from T80 Co. Thus, mutation from a bulky side chain to a smaller sidechain, I54A, must increase the flexibility of this region.

3 | DISCUSSION

The thermodynamic changes observed for Flap+ mutants are known to not result from simple addition of each individual mutation effect on the proteins' thermodynamics. Yet, the mechanistic drivers of Flap+(I54V) evolution to Flap+(I54A) under drug pressure are unknown. In this study, we aimed to understand the molecular mechanism underlying such evolution by monitoring PR-inhibitor interaction, PR folding and stability, as well as the PR activity. In this in vitro study, Flap+(I54A) exhibited a significant unfolding fraction. Due to this unfolding fraction of Flap+(I54A), NMR signal assignment of the free form could not be performed. Although we could assign backbone signals of the DRV-bound form of the protein, we refrained from performing NMR relaxation experiments of this protein, since the parameters of these are sensitive to signal overlap. Nevertheless, comprehensive experiments using ITC, NMR, fluorescence spectroscopy and
enzymatic assay of PRs elucidated characteristics of inhibitor binding, substrate processing, folding and protein conformations.

Flap+(I54) exhibited a similar thermodynamics profile to that of Flap+(I54V), including weaker pepstatin binding compared to pWT and Flap+(I54A), as well as favorable -\( T\Delta S \) change upon DRV interaction (Table 1a, c). We could not find any structural or folding stability change between Flap+(I54) and Flap+(I54V) (Figure 5a, b). However, the catalytic activity of Flap+(I54) was significantly lower than that of pWT (Figure 5c), which may be a reason for I54V mutation in the Flap+. Unfavorable \( \Delta H \) of Flap+(I54) and Flap+(I54V), compared to pWT, upon interaction with the peptide-analogue inhibitor could be consistent with the lower catalytic activity of these proteins.

In contrast to the cases of Flap+(I54) and Flap+(I54V), the assay clearly showed that the V54A change that occurs as the virus evolves from Flap+(I54V) to Flap+(I54A) greatly improves the catalytic activity of PR (Figure 5c). Given the lower dimer stability of Flap+(I54A) than pWT, the actual activity of the folded Flap+(I54A) dimer may be higher than that of pWT. Two possible mechanisms could account for this: an increase in the catalytic rate or an increase in the Michaelis constant. The latter is more likely, based on the following two points. First, because the active-site region (residues 25–30) is not altered in Flap+(I54A), the single mutation from Flap+(I54V) to

**FIGURE 6** Overlay of \(^{1}H-{^{15}}N\) HSQC spectra of DRV-bound (a) Flap+(I54) and (b) Flap+(I54A) in the deuterated buffer (red) and protonated buffer (black), (c) highlight of the HD-exchange region (red) and unexchanged region (blue), and (d) the rotated view at residues 54 and 80. In panel (a), identified backbone amides that were exchanged to deuterons are marked by black labels, amides that were not exchanged to deuterons are marked by blue labels, and the resonances of T80, two resonances due to inhibitor asymmetry of the dimer, are marked by green labels.
Flap+(I54A) is unlikely to change this region, as residue 54 is far from the active site. Second, the thermodynamic parameters of Flap+(I54A) interaction with a peptide-analogue inhibitor is similar to that of pWT, compared to Flap+(I54) and Flap+(I54V) (Table 1a), suggesting that the substrate interaction of Flap+(I54A) is more similar to that of pWT than other Flap+ mutants.

Although other factors may affect mutation evolution in the viral life cycle, we envision our in vitro observation as follows. Our data indicate that V54A mutation in Flap+ restores substrate interaction to be similar to that of pWT, and at the same time allows escape from the DRV pressure. The drawback however is lower protein stability (Figure 5a, b). Significant dynamics at the T80 site in pressure. The drawback however is lower protein stability (Figure 5a, b). Significant dynamics at the T80 site in Flap+(I54A), observed by HD-exchange, could affect substrate interaction and stability of Flap+(I54V). T80 residues in both subunits do not directly interact with DRV but are located at ~5 Å distance from DRV (Figure 6d). The side chains of the two T80 residues in the homodimer are buried to the protein core. Thus, based on this location of T80, flexibility of this region may allow conformational adaptation of flexible substrates, but weaker binding to a hydrophobic inhibitor, the latter with unfavorable entropy and favorable enthalpy.

4 | CONCLUSION

To understand the mechanism of entropy-enthalpy compensation in drug interaction with HIV-1 PR, we performed NMR, fluorescence spectroscopy, enzyme assays, and ITC experiments for four PRs. These data consistently indicate that the activity and thermodynamic balance of Flap+(I54A) are closer to pWT while those of Flap+(I54) and Flap+(I54V) are similar to each other. Based on these observations, we conclude that Flap+(I54V) to Flap+(I54A) evolution partially recovers the enzymatic activity, resulting in a thermodynamic balance similar to that of pWT, while Flap+(I54) is disadvantaged in terms of catalytic activity. Flap+(I54A) exhibits significantly faster HD-exchange at T80, which interacts with residue 54 side chain, possibly explaining the relative differences in the binding thermodynamics of Flap+(I54A) compared to Flap+(I54) and Flap+(I54V).

5 | MATERIALS AND METHODS

5.1 | Protease expression and purification

HIV-1 PR with the following amino acid sequence, PQTILWKRPL VTIRIGGLK EALDTGADD TVIEMNLPG KWKPMIGGI GGFVKVRQYD QIPIEIAGHK AIGTVLGPVT PVNiGNLL TQIGATLN, was used in this study. Note, the construct contains an L63P polymorphism and four mutations (Q7K, L33I, C67A, C95A) to reduce autoproteolysis and disulfide-bridge formation. This sequence is called pWT in this study, to distinguish it from WT. Flap+(I54) contains mutations L10I/G48V/V82A on the pWT construct (DNA2.0, Newark, CA). Flap+(I54A) and Flap+(I54V) include the same mutations as Flap+(I54A) plus a single amino acid substitution at position 54, I54A and I54V, respectively. The clones were confirmed by DNA sequencing. We expressed 15N- or 15N/13C-labeled proteins and purified these using previously published protocols. Proteins were folded with 10 mM acetate at pH 6.0, buffer exchanged to 20 mM sodium phosphate at pH 5.8. Additional details of the folding is described in each experiment below. The molecular masses of the proteins used for NMR experiments were checked by Bruker QqTOF mass spectrometer. DRV was obtained from Celia Schiffer's group. All protein concentrations in the manuscript are described by assuming the dimer unless otherwise stated.

5.2 | NMR spectroscopy

All NMR experiments were performed at 20°C using proteins in 20 mM phosphate buffer at pH 5.8; spectra were recorded on Bruker Avance spectrometers at 800 MHz or 900 MHz and processed by nmrPipe and ccpNMR. 15N/13C isotope labeled Flap+(I54A), and Flap+(I54) were folded in the presence of four-fold excess of DRV in 10 mM phosphate at pH 6.0, buffer exchanged to 20 mM phosphate buffer using a pre-equilibrated dialysis cassette (Thermo Fisher Sci., Waltham, MA), and concentrated to ~0.1 mM using Amicon (Thermo Fisher Sci., Waltham, MA). 1H-15N HSQC and HNCA spectra of DRV-bound 15N/13C-labeled Flap+(I54A) and Flap+(I54) were recorded and used to assign backbone resonances. The orientation of the two subunits relative to each other and to DRV was determined by comparing the spectra to previously assigned Flap+(I54V) resonances. Assigned resonances were deposited to Biological Magnetic Resonance Bank with the accession numbers, 27,731 and 27,727, for DRV-bound forms of Flap+(I54A) and Flap+(I54V), respectively. To confirm protein folding of Flap+ mutants, without any autoproteolysis effect, 1H-15N HSQC spectra were
recorded for $^{15}$N-labeled pWT, Flap+$^{(I54A)}$, Flap+$^{(I54)}$ and Flap+$^{(I54V)}$ at 3 μM protein concentration, in the absence and presence of 10-fold excess of DRV. In the latter, proteins were folded first and inhibitor was added after that. To confirm protein folding in the competitive ITC experiments, two $^1$H-$^{15}$N HSQC spectra using a single 16 μM Flap+$^{(I54A)}$ sample were recorded: the first was recorded after the protein was folded in the presence of four-fold excess pepstatin followed by 3 hr buffer exchange and spin down to remove the excess amount of pepstatin, and then a second was recorded after addition of excess DRV. Each NMR experiment took 3–12 hrs.

HD-exchange NMR experiments were performed for Flap+$^{(I54A)}$ and Flap+$^{(I54)}$ in the following conditions. Proteins at ~25 μM concentration in 4 mL were folded by three-fold dilution to 10 mM acetate at pH 6.0 in the presence of two times excess pepstatin, followed by dialysis to 20 mM phosphate buffer at pH 3.0, concentrated, and pH adjusted to 3.0–3.5 by using 10 mM phosphate at pH 3.0. The final protein concentration was 50–80 μM and the final phosphate concentration approximately 12.5 mM and pH 3.5. Proteins were aliquoted and lyophilized. Prior to the NMR experiments, >99% D$_2$O was added to the lyophilized sample, and three times excess DRV was immediately added. $^1$H-$^{15}$N HSQC spectra of these proteins in D$_2$O-based phosphate, at pH 3.0–3.5, were recorded at 20°C. After the experiments, the protein samples were five-fold diluted to H$_2$O-based 12.5 mM phosphate at pH 3.4, incubated 30 min at room temperature, and concentrated back to the NMR sample volume (~380 μL). $^1$H-$^{15}$N HSQC spectra of the protonated samples were also recorded.

Prior to the start of the ITC experiments, 200 μM pepstatin was added, which makes the final pepstatin concentration, 216 μM. As described above, the folding of buffer-exchanged Flap+$^{(I54A)}$ was confirmed by NMR. For comparison purposes, we used 216 μM pepstatin for all the competitive experiments and injected 250 μM DRV, that was diluted using dialysis buffer from the 50 mM DRV stock solution. For Flap+$^{(I54A)}$, direct DRV titration was also performed to examine the heat flow. Flap+$^{(I54A)}$ for this experiment was prepared using a protocol similar to that used for pepstatin titration.

For all the ITC experiments, data were acquired twice to ensure data reproducibility. After normalizing the constant control heat to zero, the raw ITC data and the integrated heat per moles of injected inhibitors, assuming a 1:1 binding model, were plotted. The thermodynamic parameters of PR (dimer) - inhibitor interaction were determined using the Analysis software (Malvern, Westborough, MA). Uncertainties of $K_D$ and $\Delta H$, $\sigma_{K_D}$ and $\sigma_{\Delta H}$, were directly obtained by the fit while uncertainties of $\Delta G$ and $-T\Delta S$, $\sigma_{\Delta G}$ and $\sigma_{-T\Delta S}$, were calculated based on the error propagation equations$^{53}$:

$$\sigma_{\Delta G} = RT \left(\frac{\sigma_{K_D}}{K_D}\right).$$

(1)

$$\sigma_{-T\Delta S} = \left(\sigma_{\Delta H}^2 + \sigma_{\Delta G}^2\right)^{0.5}$$

(2)

### 5.4 Fluorescence spectroscopy

The PR concentration dependence of WT, Flap+$^{(I54A)}$, Flap+$^{(I54)}$ and Flap+$^{(I54V)}$ was examined by recording intrinsic tryptophan fluorescence emission on a FluoroMax-4 spectrofluorometer (Horiba Scientific, Edison, NJ). Proteins, taken from a −80°C frozen stock, were diluted, firstly to 4 μM and then step-wise; fluorescence emission spectra were recorded at room temperature with an excitation wavelength of 280 nm. Emission intensity changes at 350 nm per molar concentration were plotted to compare the structural changes at different protein concentrations. Chemical denaturation of 1 μM pWT, Flap+$^{(I54A)}$, Flap+$^{(I54)}$ and Flap+$^{(I54V)}$ was monitored by recording the Trp emission at 350 nm at different urea (Sigma Aldrich, St. Louis, MO) concentrations in 20 mM phosphate buffer, pH 5.8 at room temperature. Fluorescence data of the proteins were compared by normalizing the maximum emission at zero urea concentration among all four PRs to 1.0. Note, since the proteases are enzymatically active, each set of experiments was done within 1 hr.

### 5.5 HIV-1 protease assay

Protease activity of pWT, Flap+$^{(I54A)}$, Flap+$^{(I54)}$ and Flap+$^{(I54V)}$ was characterized with HIV protease substrate
1 (Sigma Aldrich, St. Louis, MO) in a FluoroMax-4 spectrofluorometer (Horiba Scientific, Edison, NJ) using kinetic mode with excitation at 340 nm and emission at 490 nm. Protease activity assays were performed for pWT, Flap+(I54A), Flap+(I54) and Flap+(I54V) at final protein concentrations of 50 nM, 0.1 μM and 0.5 μM with 10 μM substrate 1 (Sigma Aldrich, St. Louis, MO) at ~22°C, which is similar to the temperature at which ITC and NMR were performed, 20°C. Each reaction was started by mixing 100 μL of PR solution with 100 μL of 10 μM substrate 1, both in 20 mM sodium acetate buffer at pH 5.5 and containing 100 mM NaCl and 2% DMSO.

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AUTHOR CONTRIBUTIONS

Shahid Khan: Conceptualization; formal analysis; investigation. John Persons: Data curation; formal analysis; investigation. Michel Guerrero: Formal analysis; validation. Tatiana Ilina: Conceptualization; formal analysis. Masayuki Oda: Conceptualization; supervision; writing-review and editing. Rieko Ishima: Conceptualization; supervision; writing-review and editing.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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