Defective variants of human immunodeficiency virus type 1 (HIV-1) protease (HIV PR) have been engineered to inhibit wild-type (wt) HIV PR activity. These variants were designed to promote the formation of heterodimers and to destabilize the formation of inactive variant homodimers of HIV-1 protease through substitutions at Asp-25, Ile-49, and Gly-50 (Babe, L. M., Rosé, J., and Craik, C. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10069–10073; McPhee, F., Good, A. C., Kuntz, I. D., and Craik, C. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11477–11481). The mechanism of action of these dominant-negative inhibitors was established using recombinantly expressed defective monomers. The defective monomers were refolded in vitro in the presence of wt HIV PR and showed dose-dependent inhibition of proteolytic activity. This inhibition was shown to result from the formation of inactive heterodimers between defective and wt HIV PR monomers. Heterodimer formation was detected by (i) isolating refolded, inactive heterodimers using histidine-tagged defective monomers and (ii) isolating heterodimers from bacteria coexpressing both wt and defective variants of HIV PR. Single-chain variants of HIV PR, in which the C terminus of the wt HIV PR monomer was covalently tethered to the N terminus of the defective monomer, were also expressed and analyzed. Thermal denaturation of these single-chain heterodimers using differential scanning calorimetry revealed a 1.5–7.2 °C greater thermal stability than single-chain wt HIV PR. The thermodynamic trend shown by these three variants mirrors their relative inhibition in provirus transfection assays. These data support the model that the effects seen both in tissue culture and in vitro arise from an increase in stability conferred on these heterodimers by interface mutations and identifies heterodimer formation as their mechanism of inhibition.

Human immunodeficiency virus type 1 (HIV-1) encodes a homodimeric, aspartyl protease (HIV PR) that is required for viral maturation and proliferation. Isolated HIV PR monomers are unstable (3), and dimerization results in a shared active site to which each monomer donates one catalytic aspartate residue. Because dimerization is required for activity, an alternative strategy to small-molecule inhibitors that target the active site of HIV PR is to develop compounds that destabilize or disrupt dimerization by binding at the dimer interface. Such a strategy has been investigated by several researchers (4–7), but all of the inhibitors developed thus far have IC50 values in the low μM range. The low Kd of HIV PR (39 pm–50 nM; Refs. 3, 4, 8) may also be exploited to develop new inhibitors of HIV PR in the form of defective HIV PR variants that form inactive heterodimers with wild-type (wt) HIV PR. A defective variant should be less sensitive than small-molecule inhibitors to the emergence of resistance mutations because of the large interacting surface area (3224 Å2) at the dimer interface (9, 10) as well as the strict requirement of dimerization for enzyme activity. In addition, the potency of the inhibitor could be improved by engineering the inactive, heterodimeric interface to be more stable than the wild type homodimeric interface. Since the Kd of HIV PR has been reported to be decreased by inhibitor and substrate binding (3), one possibility for increasing the potency of the dominant-negative inhibitor is to introduce substitutions that fill the neighboring wt HIV PR substrate binding pocket in the heterodimer. An understanding of the interactions that make a dominant-negative inhibitor effective may lead to the optimization of peptide or other small molecule inhibitors that are less sensitive to resistance.

The design of several such defective HIV PR monomers, with one or more substitutions at Asp-25, Gly-49, and/or Ile-50, has been reported. All defective monomers studied have demonstrated inhibition in cell culture (1, 2). Cells were co-transfected with wt HIV proviral DNA and varying amounts of proviral DNA encoding the defective HIV PR D25N. Coexpression of the inactive protease variant with wt HIV PR inhibited viral maturation in both transiently transfected and stably integrated cell lines in a dose-dependent manner, consistent with dominant-negative inhibition of the viral protease (1). In subsequent studies, other changes were introduced at the active site dimer interface to further promote heterodimer formation and to minimize defective monomer homodimerization (2). A variant monomer of HIV PR containing three substitutions, D25K/G49W/I50W (KWW), was the most potent dominant-negative inhibitor. A heterodimer composed of wt HIV PR and HIV PR KWW is shown in Fig. 1. The substitution of lysine for the catalytic Asp-25 may stabilize the heterodimer by both (i) hydrophobic interactions between the methylene groups of the lysine side chain and the hydrophobic substrate binding pocket and (ii) favorable charge-charge interactions with the aspartate of the wt monomer (2). The replacement of residues Gly-49 and Ile-50 with tryptophans introduces favorable hydrophobic interactions with the S1 and S2 subsites of an associated wt
monomer via a pseudo-substrate-like mechanism. These substitutions were predicted not only to promote heterodimer formation but also to destabilize homodimer formation through both steric and electrostatic repulsion. That some of these defective monomers demonstrated greater inhibition than others was predicted to be a result of their higher affinity for wild-type monomers and their decreased tendency to self-associate (2).

Although the results obtained from tissue culture experiments are consistent with our model of dominant-negative inhibition, there is no direct evidence showing the formation of inactive heterodimers. We report here the in vitro inhibition of wt HIV PR by defective variants, biochemical evidence of heterodimer formation, and the biophysical characterization of three inactive heterodimers. For these studies, we examined the following defective monomers with single, double, and triple mutations, respectively: (i) HIV PR D25K, (ii) HIV PR G49W/I50W (WW), and (iii) HIV PR KWW. The experimental results presented here demonstrate that dominant-negative inhibition is attributable to heterodimer formation and that the dimer interface of HIV PR can be engineered to stabilize these inactive heterodimer complexes relative to homodimeric wt HIV PR.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression**—All HIV PR variants described were expressed in *Escherichia coli* using the expression vector pTacTac, a derivative of the pPH65 vector (11). A synthetic gene for the 99-amino acid HIV-1 PR (12, 13) was cloned downstream of a gene encoding the highly transcribed CheY protein, obtained from the plasmid pCHEY15LOX (14), to facilitate ribosome capture. To further enhance translational efficiency, an intervening bicistronic linker was placed between the CheY and HIV PR coding regions. This linker, 5'-CTCGGAGGAGGGGCCCGGTACC-3', introduces a Shine-Dalgarno sequence and a non-transcribed ATTGGAAGGAGGGCCCGTAC-3' representation. Other residues shown on the wild-type monomer is the model of the HIV protease heterodimer with the D25K, G49W, and I50W (KWW) substitutions. The KWW monomer is shown in red, with space-filling representations of the D25K (red), G49W (light blue), and I50W (dark blue) substitutions. The wild-type monomer is shown in green, except for the active site Asp-25, which is depicted by the red ball-and-chain representation. Other residues shown on the wild-type monomer are those listed in the text as forming contacts with the substituted tryptophans from the KWW monomer (i.e., residues 28, 32, 47–49, and 81–84).

The clone pT2Ch3/wt&KWW-His6, used for coexpressing wt HIV PR along with a C-terminally His-tagged KWW variant in *E. coli*, was constructed using a three-fragment ligation. Gene fragments encoding the two protease genes were synthesized in two separate PCR reactions. Wt HIV PR was synthesized using a pT2Ch3/HIV-1 wt template with the primers 5'-AAAAATGATAGGGCGATGG-3' and 5'-GGCGATGGCAACCTTTATTTACAAACATG-3'; HIV PR KWW-His6 was synthesized using pBS/KWW-His6 as a template in a PCR reaction with the primer 5'-TTTAAAGGGGGCGATGGCAACCTTTATTTACAAACATG-3' and primer 5'-AAACTTATTTACAAACATG-3' (PhD, University of Pennsylvania). Nontethered heterodimers were coexpressed using related plasmids in which a second, histidine-tagged HIV PR gene was placed downstream of the untagged, wt HIV PR with an additional bicistronic linker sequence inserted between the two coding regions (Fig. 2C). All of the HIV PR constructs contain the mutation Q7K, which greatly reduces autoproteolysis (15).

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Plasmids encoding variant HIV PR monomers, including the C-terminal histidine-tagged variants, were made by Kunkel mutagenesis (16). Mutagenesis on the HIV PR gene in a pBluescript (pBS) based shuttle vector (Stratagene, La Jolla, CA), which contained the entire bicistronic region between unique BamHI and SalI sites (Fig. 2A), was carried out using various oligonucleotides singly or in combination. To introduce a lysine at position 25 of the protease, the oligonucleotide 5'-GAAGCTTTCCTAAAGCCGTGCTGAC-3' was used, and positive clones were identified by digestion with the restriction endonuclease AflII (underlined nucleotides). Tryptophan codons were introduced at positions 49 and 50 using the oligonucleotide 5'-AAAATGATAGGGCGATGGCGGGGGGAGGCTGTTATAC-3'; the sequences encoding Gly-(His)3 and Gly-(His)6 were introduced at the C terminus of wt HIV PR and the defective monomers using the oligonucleotides 5'-GTACCTTTAATTTTGCGTCACCACACTAGTGCCAGCTCG-3' (Gly-(His)3) and 5'-GTACCTTTAATTTTGCGTCACCACACTAGTGCCACCTCG-3' (Gly-(His)6). For all oligonucleotides, mutated sequences are indicated in bold. The BamHI-SalI fragment was then ligated into BamHI-XhoI-restricted pT2Ch3/HIV-1 to generate the expression plasmid. All constructs were sequenced through the protease gene to confirm the mutations.

Constructs expressing tethered heterodimers were made using Kunkel mutagenesis, in two parallel mutagenesis steps: (i) the stop codon at the 3' end of the HIV PR gene was replaced with a sequence encoding three glycines using the oligonucleotide 5'-GTACTTTAATTTTGCGTCACCACACTAGTGCCAGCTCG-3'; this sequence also introduces an Apal site (underlined); and (ii) the start codon at the 5' end of the His9-tagged variants was replaced with a sequence also encoding an Apal site using the oligonucleotide 5'-GAAGCTTTCCTAAAGCCGTGCTGAC-3'; this sequence also introduces an Apal site (underlined). Tryptophan codons were introduced at positions 49 and 50 using the oligonucleotide 5'-AAAATGATAGGGCGATGGCGGGGGGAGGCTGTTATAC-3'; the sequences encoding Gly-(His)3 and Gly-(His)6 were introduced at the C terminus of wt HIV PR and the defective monomers using the oligonucleotides 5'-GTACCTTTAATTTTGCGTCACCACACTAGTGCCAGCTCG-3' (Gly-(His)3) and 5'-GTACCTTTAATTTTGCGTCACCACACTAGTGCCACCTCG-3' (Gly-(His)6). For all oligonucleotides, mutated sequences are indicated in bold. The BamHI-SalI fragment was then ligated into BamHI-XhoI-restricted pT2Ch3/HIV-1 to generate the expression plasmid. All constructs were sequenced through the protease gene to confirm the mutations.
using nickel chelate chromatography. Clarified cell lysates containing (His)~t~/tagged wt HIV PR were adsorbed to a cellulose phosphate (Whatman, Fairfield, NJ) column (2.5 × 6 cm) in 100 mM Tris (pH 8.0) and eluted with a 0–1 M NaCl gradient. The fractions containing protease were pooled and applied to a Ni-nitriotropic acid-agarose (Qiagen, Santa Clarita, CA) column (150 × 5 cm) and eluted with a 0–300 mM imidazole gradient. Wt and inactive single-chain (His)~t~/tagged variants were expressed and purified in the same manner but eluted with a 0–350 mM imidazole gradient.

(His)~t~/tagged, defective HIV PR variants were expressed in insoluble inclusion bodies and purified by solubilizing the inclusion bodies in 10 ml of 8 M deionized urea. The solubilized inclusion bodies were clarified by centrifugation and then applied to a G-50 (Amersham Pharmacia Biotech) size exclusion column (2.5 × 120 cm) equilibrated in 8 M urea and eluted at a flow rate of 2 ml/min. The fractions containing protease were identified by immunoblot analysis, pooled, and applied to a Ni-nitriotropic acid affinity column (0.25 × 5 cm), and eluted with a 0–300 mM imidazole gradient in 8 M urea. Wt and inactive single-chain (His)~t~/tagged variants were expressed and purified in the same manner but eluted with a 0–350 mM imidazole gradient.

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**RESULTS**

**HIV Protease Expression and Purification**—For all protease variants, a bicistronic expression system was used in which the first 293 nucleotides of the efficiently translated CheY gene were inserted upstream of the HIV PR gene to improve translation levels (14). This system does not rely on the use of a fusion protein to overcome the poor translational efficiency of the protease gene and thus eliminates the requirement for post-translational processing. In general, synthesis of the protease diminished ~2 h after induction, concurrent with a dramatic decrease in cell viability. Protease yields were typically between 1 and 3 mg/l of growth media. Postinduction times were lengthened for expression of defective variants of HIV PR and the inactive tethered heterodimers. Cells expressing these inactive HIV PR constructs did not show a similar, rapid decrease in cell viability (data not shown). The introduction of a second, defective HIV PR gene downstream of the wt HIV PR gene resulted in the co-expression of approximately equal amounts of wt and defective variants as determined by immunoblot analysis (data not shown).

Strategies used to purify the D25K, WW, and KWW variants under nondenaturing conditions, with or without a C-terminal (His)~t~/tag, were unsuccessful. All three of these variants were relatively insoluble and precipitated after bacterial cell lysis. The majority of expressed protein was found in insoluble inclusion bodies. The binding of poly-L-histidine to nickel was unaffected by denaturants, inclusion bodies containing the His~t~/tagged variant monomers were resolubilized in 8 M urea and purified to homogeneity by nickel chelate chromatography under denaturing conditions. To ensure that the histidine tag was
not cleaved during expression or purification, the purified Gly-
(His)₃-tagged wt HIV PR was analyzed by mass spectrometry. An HIV PR monomer containing a Gly-(His)₃ C-terminal extension showed the expected molecular mass (measured molecular mass, 11,257.4 ± 1.1 Da; expected molecular mass, 11,257 Da), indicating that the histidine tag was not cleaved.

Wt HIV PR was assayed with all the aforementioned modifications as well as in all relevant buffer systems to ensure that the protein being studied was catalytically competent. Neither poly-l-histidine tags nor a three-glycine tether linking two wt monomers reduced the solubility or activity of wt HIV PR in HIV PR assay buffer. All wt HIV PR variants were active in MES Buffer A. Under the same conditions, the single-chain wt PR retained proteolytic activity in both MES Buffers A and B, suggesting that the protease is well folded under all conditions studied here.

Repeated attempts were made to crystallize tethered wt/ KWW heterodimers using crystal conditions reported previously (22, 23), as well as using the Hampton screening kit (Hampton Research, Laguna Niguel, CA). None of the conditions yielded crystals, most likely because the heterodimers could not be concentrated at >1 mg/ml protein.

**In Vitro Inhibition of HIV PR—Varying amounts of (His)₃-tagged D25K, WW, and KWW variants were refolded in the presence of a fixed amount of untagged wt HIV PR.** Inhibition profiles of all three variants demonstrated a similar dose dependence (Fig. 3). At equimolar ratios of wt and defective variant, wt HIV PR activity was reduced by ~50%. When the molar ratio of variant monomer to wt was >3:1, proteolytic activity became undetectable.

**Isolation of Refolded and Coexpressed Heterodimers—**Heterodimers were isolated after either refolding from urea or coexpression in E. coli. After refolding, histidine-tagged heterodimers were isolated using nickel-chelate affinity chromatography. Heterodimer formation was confirmed by the appearance of two bands on an SDS-polyacrylamide gel that corresponded to the molecular masses of an unmodified HIV PR monomer (wt) and a (His)₃-tagged monomer (defective variant). These refolding experiments were performed under conditions in which either the wt HIV PR or the (His)₃-tagged variant HIV PR was in excess. In both cases the two monomer bands on SDS-polyacrylamide gels were of equal intensity, indicating that equimolar amounts of wt HIV PR and defective variant were bound to the nickel resin (Fig. 4A). No proteolytic activity was detected in purified heterodimer samples. Controls with untagged wt HIV PR were applied to the Ni-affinity resin; however, nonspecific binding was not detected.

Clariﬁed lysates of E. coli coexpressing wt and variant HIV PR contained approximately equal amounts of each species based on immunoblot analysis (data not shown). All species bound to and eluted from cellulose phosphate in a manner similar to wt HIV PR. Material eluted from cellulose phosphate was mixed with pepstatin affinity resin. In every case, wt HIV PR was present and bound to the affinity resin. No histidine-tagged variants were observed to bind to the pepstatin affinity resin. The fraction of protein that did not bind to pepstatin was further puriﬁed by nickel chelate afﬁnity chromatography. Immunoblot analysis showed that the two species elute from the Ni resin in an 1:1 ratio for all analyzed heterodimers (Fig. 4B). Wt HIV PR, when expressed alone, does not signiﬁcantly bind to the Ni afﬁnity resin.

**Thermal Denaturation of Tethered Homo- and Heterodimeric Variants of HIV PR—**One measure of protein stability is the temperature at which it is half-denatured (Tₘ). All calorimetry studies (except those otherwise marked) were conducted using tethered dimers of HIV PR. Tethered dimers were studied to ensure homogeneity within each sample as well as to eliminate the concentration dependence of thermal stability for dimers (24). The Tₘ values of un tethered and tethered wt HIV PR in MES Buffer A were determined to compare the effect of the three-glycine tether covalently linking the two monomers. In MES Buffer A, untethered wt HIV PR melts with a single transition at 52.5 °C, compared with 64.5 °C for tethered wt HIV PR (Fig. 5). The shapes of these two denaturation curves both indicate a single transition. The shape of the tethered wt denaturation curve is sharper than untethered wt, which is typical for proteins with higher Tₘ values and higher enthalpies of unfolding. In Buffer A, the tethered heterodimers were soluble only to a concentration of ~0.5 mg/ml, which is below the detection level of the instrument. Attaining sufficient con-
The tethered heterodimers are apparent by the formation of inactive HIV PR heterodimers. We have biophysically characterized a series of inactive heterodimers of HIV PR. The results indicate that it is possible to engineer the dimer interface of HIV PR to form heterodimers that are more thermostable than wt homodimers.

To express defective HIV PR variants for biochemical studies, we used a bicistronic expression system that takes advantage of the high expression level of the endogenous E. coli CheY gene. The bicistronic vector facilitates ribosome capture to enhance translation of the HIV PR gene. Because this system does not require autoproteolytic processing, it can be used to express wt HIV PR as well as inactive variants. To coexpress wt and variant monomers, a tricistronic expression system was developed in which the two protease genes are separated by an additional Shine-Dalgarno sequence followed by three spacer nucleotides. The second, less-soluble His-tagged defective variant appears to be expressed at the same levels as wt HIV PR, suggesting that ribosomes remain associated with the mRNA transcript past the first HIV PR gene. The bicistrionic and tricistrionic systems have proved useful for the production of multiple, diverse variant proteases.

Cells harboring bicistronic constructs encoding wt HIV PR under the control of two Tac promoters suffered from the effects of protease toxicity even in the absence of induction, suggesting that repression of the promoters in pTacTac was incomplete. It was therefore necessary to use a high lacI background strain of E. coli (X90) for all cloning manipulations. In contrast, cells harboring vectors that encoded inactive protease were less affected by toxicity, and induction resulted in less drastic declines in growth rates. Cells coexpressing wt and defective HIV PR variants had growth rates more similar to those expressing defective variants alone than to those expressing wt HIV PR alone. These results, showing that either inhibition or inactivation of proteolytic activity reduces toxicity, suggested that the soluble expression of HIV PR is toxic to E. coli because of unregulated proteolytic activity.

Solubility was a limiting factor in defective HIV PR expression. When expressed alone the (His)₃-tagged defective monomers D25K, WW, and KWW were predominantly found in the insoluble fraction of cell lysates. Any D25K, WW, or KWW protease not in inclusion bodies precipitated after cell lysis despite many attempts at purification using a variety of conditions and cosolvents. The formation of inclusion bodies is consistent with an overall observed decrease in solubility of these variants compared with wt. The variant homodimers are likely destabilized by steric repulsion between the four tryptophans in the flap region, as well as by both steric and charge repulsion between the two lysines at the active site. These destabilizing interactions may result in dimer dissociation. The insolubility of defective HIV PR monomers compared with wt HIV PR suggests that defective monomers do not form homodimers and that they aggregate in solution.

Conversely, both tethered and untethered inactive heterodimers were soluble after expression and could be purified under the same buffer conditions used for wt HIV PR. Likewise, previously insoluble, defective monomers were found in the soluble fraction when refolded with wt monomers. Changes at Asp-25, Gly-49, and Ile-50 are thus destabilizing in the context of a variant homodimer but stabilizing in the presence of wt HIV PR. This strongly suggests that wt HIV PR enables the dimerization of defective HIV PR variants, mostly likely through the formation of heterodimers with the wt monomers.

Heterodimer formation and subunit exchange in vitro between wt HIV PR and defective HIV PR variants, as well as between nonidentical subunits of HIV PR, have been reported (26, 27). In these cases, heterodimer formation leads to HIV PR inactivation. However, monitoring wt activity as a function of monomer exchange under conditions used in these previous studies was not possible in the present study because of the insolubility of D25K, WW, and KWW. Therefore, we examined the activity of wt HIV PR when refolded from urea in the presence of increasing concentrations of variant monomers.

FIG. 4. Formation of HIV PR heterodimers was demonstrated using denaturing SDS-polyacrylamide gel analysis. A, mixtures of wt and variant HIV PR monomers were refolded, and this material was repurified using nickel affinity chromatography. Lanes were loaded as follows: 1, wt/D25K; 2, wt/WW; 3, wt/KWW; 4, untagged HIV PR; and 5, His₃-tagged HIV PR. B, heterodimers were coexpressed and purified as described under “Experimental Procedures.” Lanes were loaded in the same order as in panel A.
Because purified heterodimers have no proteolytic activity, this assay measures the amount of wt HIV PR that is present after refolding. Inhibition by the three variants was dose-dependent, suggesting the formation of a complex between wt and defective variants. Although previous cell culture assays had demonstrated differences in the relative inhibition of the three variants (2, 28), these in vitro refolding experiments revealed no significant difference in the dose dependence of inhibition by different defective variants. Refolding was not conducted under equilibrium conditions; thus the results likely reflect a mix between equilibrium heterodimer formation and a purely stochastic process, in which relative levels of each species are a function only of initial monomer concentrations. Although heterodimer formation by refolding in vitro does not completely model heterodimer formation in vivo, the dose dependence in both the transfection and refolding and inhibition assays strongly suggests that in both systems the mechanism of the dominant-negative inhibition is through the formation of inactive complexes between wt and defective HIV PR.

Complex formation between wt and variant HIV PR during refolding was demonstrated by isolating heterodimeric HIV PR that contained C-terminally histidine-tagged defective monomers. Histidine-tagged dimers were separated from untagged wt HIV PR by nickel chelate chromatography and analyzed by electrophoresis. Upon immunoblot analysis, bands corresponding to tagged and untagged HIV PR were of equal intensity. This indicates that the histidine-tagged, defective monomer and the untagged, wt monomer bound to the resin in a 1:1 ratio. When considered in combination with previous results that demonstrated the formation of a complex between wt and defective HIV PR, these results suggest that heterodimer formation can occur after refolding in vitro, and that it results in inhibition of wt HIV PR activity.

Refolding in vitro demonstrates that heterodimers form between wt and the engineered inhibitors. We wished to know whether these heterodimers could also form in vivo; however, the concentration of HIV PR is below detection levels both in virus core particles and in eukaryotic cultured cells. We therefore resorted to the pTacTac-based bacterial overexpression system to test whether heterodimers could form in E. coli. For all three dominant-negative inhibitors (D25K, WW, and KWW), the untagged, wt monomer co-purified on an affinity column with the tagged monomer, indicating heterodimer formation in E. coli. Furthermore, the equal levels of expression of both wt and defective variants from the same plasmid suggest that dimerization and folding take place soon after translation. If the participation of adjacent gag-pol sequences, cellular chaperones, or other cofactors in either bacterial cells or tissue culture cells is excluded, heterodimer formation in bacteria should model potential heterodimer formation in transfected COS cells demonstrated in previous studies (2, 28).

According to the model that inhibition is caused by heterodimer formation, an increase in inhibitor potency should correspond to a more stable heterodimer between the inhibitor and wt HIV PR. We chose to characterize tethered heterodimers to ensure that the sample in any given experiment contained one single species: the heterodimer of interest. As expected, the inclusion of the three-glycine tether stabilized the wt HIV PR by several degrees, presumably by eliminating the concentration dependence of dimer stability. Heterodimers between wt HIV PR and D25K, WW, or KWW variants were all more stable than the wt tethered homodimer. In fact, the order of increasing strength is reflective of the order of increasing potency in the co-transfection cell culture assay. This evidence further supports the model that inhibition in vitro as well as in cell culture is attributable to the formation of inactive heterodimers between defective variants and wt HIV PR. Although we have not obtained diffraction quality crystals of the wt/KWW heterodimer for direct structural analysis, we can make certain structural predictions based on computer modeling. We propose that the increased stability of the tethered heterodimers is attributable to favorable interactions between the substituted lysine and tryptophan residues and residues in the binding pocket of the opposing wt HIV PR monomer. In cell culture, any combination of the substitutions results in a better inhibitor than D25N HIV PR (2), demonstrating that the substitutions can improve the potency of the inhibitor. In addition, the fact that KWW is the strongest inhibitor of all variants tested suggests that these three substitutions strengthen the heterodimer in an additive fashion. An Asp-25 to lysine substitution likely stabilizes the heterodimer through hydrophobic interactions between its methylene groups and neighboring hydrophobic residues, as well as through a potential charged interaction between the lysine amine and the carboxylate group of the wild-type catalytic aspartic acid. The two tryptophan substitutions almost certainly interact with substrate binding subsites in the wild-type monomer. Modeling indicates that Trp-49 interacts with residues 81–84 of the wild-type monomer, shown to make contacts to bound inhibitors in the S1/S1′ subsite (29). Similarly, Trp-50 makes contacts with residues 28, 32, and 47–49, associated with inhibitor contacts in the S2/S2′ subsite. It is likely that in filling subsites of the binding pocket, the tryptophan substitutions stabilize the flap region of both dimers, further strengthening dimer formation. The amino acids in the flap region are predicted to have a low contribution to protein stability compared with amino acids elsewhere in the active site (30). The flaps have a high degree of flexibility because of the presence of 8–12 flexible residues in
the flap region. This flexibility is necessary for the entry of substrate into the active site. The flaps of wt HIV PR are in an open, unstructured conformation in the absence of substrate. Substrate binding causes the flaps to close and form stabilizing contacts between the substrate and the enzyme. Thermal and chemical denaturation studies on wt HIV PR with inhibitors bound in the active site have greater stability than PR without substrate (3). It is likely that the two tryptophan mutations at Gly-49 and Ile-50 stabilize heterodimer formation by closing the flaps and making contacts in the active site analogous to the stabilization of HIV PR by small-molecule inhibitors.

In treating HIV infection, the effectiveness of small-molecule inhibitors that bind in the active site is limited, because they inevitably give rise to resistant viral strains. Defective HIV PR monomers that preferentially associate with wt HIV PR should be less sensitive to the emergence of resistance mutations than small-molecule inhibitors, because they interact with wt HIV PR through extensive structural features that are necessary for homodimerization and, thus, activity. We have demonstrated here that the mechanism of action for dominant-negative inhibition is through the formation of inactive heterodimers. We have also demonstrated that the potency of inhibition can be increased through the rational design of a more stable heterodimeric interface. Further understanding of the interactions involved in dominant-negative inhibition of HIV PR may help develop a class of inhibitors that interact with regions involved in dimerization and thus delay the selection of resistant viral strains.

Acknowledgments—We gratefully acknowledge Hoang-Anh Le and Nancy Douglas for protein preparation and Zhonghua Yu for mass spectrometric analyses.

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