Interactions of Substrates and Inhibitors with a Family of Tethered HIV-1 and HIV-2 Homo- and Heterodimeric Proteinases*

(Received for publication, August 18, 1993, and in revised form, October 29, 1993)

Jonathan T. Griffiths‡§, Lorraine A. Tomchak‡, John S. Mills, Mary C. Graves, Neil D. Cook**, Ben M. Dunat‡, and John Kay‡§§

From the ‡Department of Biochemistry, University of Wales College of Cardiff, Cardiff CF1 1ST, Wales, United Kingdom, the §Department of Biotechnology, Roche Research Center, Nutley, New Jersey 07110, the ||Department of Molecular Virology, Roche Research Center, Welwyn Garden City AL7 3AY, United Kingdom, **Amersham International plc, Forest Farm, Cardiff CF4 7YT, Wales, United Kingdom, and the §§Department of Biochemistry and Molecular Biology, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610

Genes were constructed to encode single-chain tethered human immunodeficiency virus HIV-1/HIV-1 and HIV-2/HIV-2 homodimeric proteinases and two HIV-1/ HIV-2 heterodimers which differed in the nature of the interface strands. All four constructs under the control of a heat-inducible promoter were expressed in E. coli and the resultant proteinases were purified therefrom. Kinetic parameters (K_m, k_cat, and k_cat/K_m) were derived for the interaction of the tethered homo and heterodimeric proteinases with two distinct substrates at a variety of pH values.

All four enzymes were comparably active toward one substrate. With the second substrate at pH 4.7, the k_cat/K_m value was best for the HIV-1/1 tethered homodimer, 15-fold lower for the two heterodimeric proteinases, and was reduced by an additional 6-fold for the HIV-2/2 homodimer. From the K_i values determined for the interactions of the four tethered dimer proteinases with a systematic series of synthetic inhibitors, a parallel trend was observed. Whereas several inhibitors were equipotent against all four enzymes, two were discriminatory in that they inhibited strongly the HIV-1/1 homodimer and the two heterodimeric proteinases but had little effect on the HIV-2/2 tethered homodimer (or its untethered wild-type counterpart from HIV-2). The significance of these findings for active site interaction with HIV-proteinases is considered.

In the human immunodeficiency viruses (HIV-1 and HIV-2), synthesis of viral Gag and Pol proteins as a Gag-Pol polyprotein necessitates cleavage to release the mature forms of each viral protein. The aspartic proteinase encoded therein is responsible for these cleavages, including its own catalytic release (1, 2). The generation of a fully competent aspartic proteinase requires two monomers to combine as each monomer contributes one of the two Asp residues required for catalytic activity (3).

The case of such dimerization in polypeptide precursors may well be a rate-limiting factor in viral processing and assembly. Indeed, because of the spatial juxtaposition of the N-terminal (residues 1-5) strand of one subunit to the C-terminal (residues 94-99) strand of its partner subunit, as defined in the crystal structures of retroviral proteinases, e.g. from Rous sarcoma virus and HIV (4, 5), a number of reports have described the construction and expression of single-chain enzymes formed by insertion of a short linker peptide to connect the N and C termini covalently (6-10). When inserted into proviral clones, these single-chain enzymes, often termed tethered dimers, bring about premature processing of viral polyprotein and prevent the formation of infectious particles (8, 10). One implication from this observation is that such tethered dimers may be more active than their "free" counterparts with two subunits that are not covalently linked.

The sequences of the proteinases from HIV-1 and HIV-2 are less than 50% identical overall. Their enzymic properties, however, are generally very similar (11), and it has been suggested that, in the active site residues that make contact with a bound substrate or inhibitor, only 5 residues are different between the two proteinases (12, 13). The N- and C-terminal strands that form the interface between the two domains of the dimer are considerably different, and it has been suggested that formation of heterodimers is a result of a association of a HIV-1 subunit with a subunit from HIV-2 PR generates an inactive enzyme (14). In order to examine these facets in these therapeutically important target enzymes in a quantitative manner, a family of genes was constructed to encode four single-chain proteinases consisting in two cases of homodimeric subunits and in the other two cases, of heterodimeric or chimeric enzymes. The interactions of these enzymes with substrates and a series of synthetic inhibitors under a variety of conditions are described.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Escherichia coli K-12 strain MC1061 transformed with pBR248clts was used as the host strain for all plasmid constructs. pRK248clts is a low copy number plasmid that is compatible with pBR322 derivatives and contains a gene encoding a temperature sensitive ApL repressor (15). Expression work used a derivative of pBR322, plasmid pRC23, which contains the ApL promoter, a model ribosome binding site, and the ampicillin resistance gene (15). Four expression plasmids were constructed (Fig. 1) to encode single-chain HIV PRs in which residue 99 of the first subunit (residues 1-99) was connected covalently by -Gly-Gly- (residues 100a and 100b) to residue 101 of the second subunit (residues 101-199). (a) In plasmid pBR26; residues 1-99 and 101-199 were both derived from the PR region of the HXB-3 strain of HIV-1. The resultant product was thus a tethered HIV-1 homodimer. Using PCR technology, DNA encoding residues 1-99 was directly amplified from pPTAN (2) using primers containing appropriate restriction enzyme sites to facilitate cloning; the 5' forward primer incorporated an EcoRI site and an NdeI

4787
site, which also provided the initiating Met codon. The 3′-reverse primer incorporated the two glycines (residues 100a and 100b), the second of which was encoded together with Pro-101 from the second domain in an ApaI site (GGGCCG). The resulting PCR-amplified product was gel purified using Geneclean (Bio 101) and digested with EcoRI and ApaI. These DNA fragments for residues 101-199 (followed by two stop codons) was made synthetically using 4 overlapping oligonucleotides (alternating coding and non-coding strands) and PCR amplification as described by Dillon and Rosen (16). The 4 oligonucleotides were annealed and extended using a thermal cycler for 8 cycles. An aliquot of this reaction mixture (2 μl) was used as DNA template for a second round of PCR using flanking primers encompassing ApaI and SalI at the 5′ and 3′-ends, respectively. The resultant product was gel-purified and digested with ApaI and SalI to generate a 306-bp fragment. In this way, residues 1-99 were generated to be identical to 101-199. However, the DNA sequence encoding residues 101-199 was synthesized to optimize codon usage for E. coli and to incorporate new restriction sites including a unique SmaI site. The 315-bp EcoRI/ApaI fragment of domain I and the 306-bp ApaI/SalI fragment of domain II were ligated to the 3975-bp EcoRI/SalI fragment from PLC23 (15) to create the 4596-bp expression plasmid pPR2O (Fig. 1).

(6) In plasmid pPR2O, residues 1-99 and 101-199 were both derived from the PR region of the ROD strain of HIV-2, thus producing a tethered HIV-2/2 homodimer. DNA encoding residues 1-99 was generated as described in (a) by using PCR to amplify the PR region from pPR2, a plasmid containing the HIV-2 PR gene isolated from p2PROT (17). DNA encoding residues 100a, 100b, and 101-199 of HIV-2 PR was made synthetically and amplified by PCR (15). An appropriate fragment of the two DNA fragments were ligated to the 3975-bp EcoRI/SalI fragment of PLC23 to make pPR2O (Fig. 1).

(b) In plasmid pPR2, residues 1-99 from HIV-1 PR were linked to -Gly-Gly-to residues 101-199 of HIV-2 PR to make a tethered HIV-1/2 heterodimer. The 315-bp EcoRI/ApaI fragment of pPR20 and the 306-bp ApaI/SalI fragment of pPR21 were ligated to the 3975-bp EcoRI/SalI fragment of PLC23 to create pPR2O (Fig. 1).

(c) In plasmid pPR22, residues 1-99 from HIV-1 PR were linked by (-Gly-Gly-) to residues 101-199 of HIV-2 PR to make a tethered HIV-2/2 heterodimer. The 315-bp EcoRI/ApaI fragment of pPR20 and the 306-bp ApaI/SalI fragment of pPR21 were ligated to the 3975-bp EcoRI/SalI fragment of PLC23 to create pPR2O (Fig. 1).

(d) Plasmid pPR23 encodes a tethered HIV-1/2 heterodimer in which residues 1-99 and residues 101-104 and 195-199 were all derived from HIV-1, while those for 105-194 were HIV-2 in origin. This was accomplished by using a 2-step PCR approach, in which the forward and reverse primers were designed to amplify the PR region of the two HIV-2 strains. The forward primers were designed to anneal to the 5′-end of the first domain and the reverse primers to the 3′-end of the second domain. The two DNA fragments were ligated to the 3975-bp EcoRI/SalI fragment of PLC23 to create pPR2O (Fig. 1).

Transformations were performed as described previously (18), and ampicillin-resistant colonies were screened by PCR using primers specific to each PR sequence. The sequence of DNA encoding tethered PRs was confirmed by DNA sequencing.

Cell growth—In analytical scale cultures, transformants were grown at 30 °C in supplemented M9 medium to mid-log growth phase. The temperature was then shifted to 42 °C, and aliquots were withdrawn at appropriate time intervals for analysis by SDS-PAGE and Western blotting as described previously (2) except that a Tricine buffer system was used (19). Further aliquots of harvested cells were resuspended in 10 mM Tris-HCl, pH 8.0, containing 10% sucrose and 0.4 mM phenylmethylsulfonyl fluoride and lyzed by sonication (four 30-s bursts). The supernatant obtained after centrifugation at 15,000 × g for 15 min at 4 °C was assayed for PR activity as described previously (20).

Results and discussion—The native HIV-1/2 PRs contain 22 amino acids and 2 cysteines, which are conserved in the HIV-1/2 homodimers, HIV-1/2 heterodimers, and HIV-2/2 homodimers, as well as in the HIV-1/2 heterodimer. The addition of a Gly-Gly spacer between the two domains of HIV-1/2 heterodimers results in a decrease in activity of approximately 40%. The addition of a Gly-Gly spacer between the two domains of HIV-2/2 homodimers results in a decrease in activity of approximately 40%. The addition of a Gly-Gly spacer between the two domains of HIV-2/2 homodimers results in a decrease in activity of approximately 40%.
in the insoluble fraction. In the cases of the HIV-1/2 and HIV-2/2 PRs, approximately 30% of the material was present in the supernatant fraction, however, and for the HIV-1/1 and HIV-1/2* preparations, approximately 40% remained soluble. These amounts were considered adequate for the present purposes, and, since all four soluble extracts had demonstrable activity in a peptide cleavage assay (20), the unnecessary complications arising from attempts to refold the insoluble material could thus be avoided. Operational conditions for optimal expression of each of the four tethered PR genes were established on analytical scale inductions. Large scale inductions were then performed using these individualized optimum conditions (see “Materials and Methods” section). Supernatants were prepared by wild-type HIV-2 PR. Kinetic parameters for the cleavage of the non-identical HIV-1 and HIV-2 subunits produced catalytically defective heterodimers. It was for this reason that the HIV-1/2* PR gene was constructed in such a way that the interface region between the N- and C-terminal strands of the two domains (which is crucial for dimer formation; Ref. 5) would consist solely of HIV-1 residues. No significant difference was detected between the “authentic” HIV-1/2 heterodimer and the HIV-1/2* PR encoding the modified interface region (Table I).

The peptide employed for these analyses is an excellent substrate for both HIV-1 and HIV-2 PRs and consequently does not readily permit discrimination and detection of subtle differences in activity of the various engineered enzymes. For this purpose, a second substrate was employed, which has been demonstrated previously (22) to be an excellent substrate for wild-type HIV-1 PR but to be cleaved much less efficiently by wild-type HIV-2 PR. Kinetic parameters for the cleavage of this pseudo-symmetrical peptide, Ac-Tyr-Arg-Ala-Arg-Val-Phe*-Nph-Val-Ala-Ala-Lys, by the four tethered dimer PRs were determined (Table I). These reflected the findings derived previously for the wild-type HIV-1 and HIV-2 PRs, in that the tethered HIV-1/1 homodimer PR cleaved the substrate very efficiently but the HIV-2/2 homodimer displayed a specificity constant \( k_{cat}/K_m \) that was only 1% -1% of that for the HIV-1 derived homodimer; this is so similar to the situation observed previously for wild-type HIV PRs (22) that it would appear that the covalent linkage of the two subunits of wild-type HIV-PRs to form a single-chain entity has no significant effect on intrinsic activity. Similar conclusions for the HIV-1 enzyme have been reached by others (6, 7) and for the proteases of retroviruses affecting non-primate species provided that an appropriate length of linker peptide is introduced between the subunits (10).

The \( K_m \) and \( k_{cat} \) values measured for the two heterodimeric PRs with respect to this substrate lay between those of the two homodimers. Consequently, specificity constants \( k_{cat}/K_m \) for the two tethered heterodimers were midway between those for the tethered homodimers (Table I), being 6-fold improved on...
Tethered HIV-1 and HIV-2 Homo- and Heterodimeric Proteinases

FIG. 2. Determination of the size of HIV-1 and HIV-2 tethered homo- and heterodimeric PRs by Western blotting. Samples of the purified preparations of the HIV-1/1, HIV-2/2, HIV-1/2, and HIV-1/2*, tethered dimeric PRs were analyzed by SDS-PAGE followed by Western blotting. Immunostaining was with a HIV-1 PR-specific antibody (left panel) and a HIV-2 PR-specific antiserum (right panel). Samples of wild-type HIV-1 (WT1) and HIV-2 (WT2) PRs are included for comparative purposes. Distances of migration of marker standard proteins were as indicated.

TABLE I

Kinetic parameters for the hydrolysis of two nitrophenylalanine (Nph)-containing chromogenic substrates by tethered homodimeric and heterodimeric HIV-1 and HIV-2 proteinases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PR 1/1</th>
<th>PR 1/2</th>
<th>PR 1/2*</th>
<th>PR 2/2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>km</td>
<td>km</td>
<td>Km</td>
</tr>
<tr>
<td></td>
<td>kcat</td>
<td>km</td>
<td>kcat/Km</td>
<td>kcat</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>44</td>
<td>2,200</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>23</td>
<td>1,530</td>
<td>100</td>
</tr>
</tbody>
</table>

From the data derived with the heterodimers (Table II), it appears that for those compounds (e.g. 1 and 2) that permit distinction, the heterodimers behave more like the HIV-1/1 homodimer in terms of binding. Clearly the two domains are not equivalent. This too reflects the conclusion, drawn from the comparisons of the HIV-2 and HIV-1 PR crystal structures, that differences between them are not a result of simple side chain substitutions but are complicated also by different flexibility in the main chains (27, 28).

All of the measurements described so far were determined at pH 4.7, a close to optimal pH for HIV PRs (21, 29, 30). In order to extend these characterizations further, the hydrolysis of the two chromogenic substrates described earlier was examined over a wide pH range. The turnover numbers (kcat) and the specificity constants (kcat/Km) derived for their hydrolysis by the four tethered dimers are shown in Fig. 3 (panels a and b). The Km parameter is typically a complex kinetic constant; however, for classical aspartic proteinases, it has been demonstrated to reflect the binding of substrate accurately (31). For substrate 1, the Km values were maintained (at 20–30 μM) between pH 4.0 and 5.0 but increased progressively in magnitude as the pH was elevated to higher values. Each set of data fitted well to a single titration curve yielding an apparent pKₐ of 7.2 ± 0.2, 6.3 ± 0.3, 6.6 ± 0.1, and 6.8 ± 0.1 for the tethered HIV-1/1, 1/2*, 1/2, and 2/2 PRs, respectively. A comparable result was reported previously (21) for wild-type HIV-1 PR. No such simple titration curves were observed, however, with substrate 2; Km values for its cleavage by each of the tethered heterodimers and by the HIV-2/2 tethered homodimer were maintained approximately constant (3-4-fold variation) between pH 4.7 and 8.2, whereas the value measured for the HIV-1/1 homodimer did increase steadily (from 20 to 195 μM) between pH 4.7 and 9.0.

Toward substrate 2, the specificity constants (kcat/Km) for the HIV-1/1 and HIV-1/2 tethered dimers were essentially constant over the pH range 4.7–7.5 (Fig. 3, panel b, top) but diminished at higher pH values. For the homodimeric HIV-2/2 enzyme and the 1/2* PR with the HIV-1 interface, the diminution in this parameter was already apparent at pH 7.5, implying an apparent pKₐ lying between pH 6.2 and 7.5. The corresponding pKₐ value for the HIV-1/1 and HIV-1/2 enzymes would appear to be slightly higher (at approximately 7.5). This dissociation can

the HIV-2/2 PR value but 15-fold poorer than that for HIV-1/1 PR. From these data (Table I), it would appear that the two domains of the heterodimers are not equivalent and that the nature of the substrate has considerable influence on the efficiency of cleavage by the family of tethered enzymes.

In order to characterize the tethered dimeric PRs further, inhibition constants (Kᵢ) for the interactions of all four tethered PRs with a series of synthetic inhibitors were determined (Table II) and compared to those measured for wild-type HIV-1 and HIV-2 PRs. The inhibitor Ro31-8959 (compound 6 in Table II) has been shown previously to be a potent, specific inhibitor of wild-type HIV-1 and HIV-2 PRs (23), and it is currently undergoing clinical evaluation as a potential anti-viral agent for the treatment of AIDS. This compound proved to be an effective inhibitor of the four tethered PRs also (Table II); it did not, therefore, permit distinction among the enzymes. The compound bound sufficiently strongly to all four enzymes to permit its use as an analytical tool in active site titrations of the four tethered PRs.

The Kᵢ values measured for the interactions of all six inhibitors with the tethered homodimers were comparable to those determined for wild-type HIV-1 and HIV-2 PRs, with a 3-4-fold difference at most between pairs (e.g. for that between inhibitor 2 and the tethered wild-type HIV-2 PRs). In view of the well documented motion of the PR molecule necessary for interaction with substrate/inhibitor, such relatively minor differences in any of the inhibitor/enzyme interactions will not be considered further, particularly in the light of the much larger distinctions observed with inhibitors 1 and 2 (and to a lesser extent, inhibitor 3). Compounds 1, 2, and 3 form a homologous series, based on hydroxyethylene transition state analogues of -Hydrophobic*-Hydrophobic- type cleavage junctions (26), in which compounds 1 and 2 have a more bulky hydrophobic cyclohexylalanine residue in P₇, while compound 3 has a Phe. Compounds 1 and 2 are potent against HIV-1 PR (wild-type and tethered homodimer) and much less effective against HIV-2 PR, whereas this distinction is not so marked for compound 3 (Table II). This indication that HIV-2 PR tolerates a large P₁ side chain less well than HIV-1 PR is totally consistent with the conclusion derived from the recently described three-dimensional structure of HIV-2 PR (27) that it has a better ability to hydrolyze substrates with smaller P₁ (and P₁') side chains.
Inhibition constants ($K_i$) for the interaction of six synthetic inhibitors with the four tethered dimeric proteinases and with wild-type HIV-1 and HIV-2 proteinases

Values were determined using substrate 1 as described in the legend to Table I and the estimated precision of each was always <15%. W-T 1 and W-T 2, wild-type proteinases from HIV-1 and HIV-2 respectively.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>W-T 1/1</th>
<th>1/2*</th>
<th>1/2</th>
<th>2/2</th>
<th>W-T 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.8</td>
<td>2</td>
<td>15</td>
<td>10</td>
<td>&gt;500</td>
</tr>
<tr>
<td>2.</td>
<td>0.4</td>
<td>0.3</td>
<td>3</td>
<td>0.3</td>
<td>200</td>
</tr>
<tr>
<td>3.</td>
<td>0.8</td>
<td>0.3</td>
<td>3</td>
<td>0.6</td>
<td>20</td>
</tr>
<tr>
<td>4.</td>
<td>6</td>
<td>10</td>
<td>15</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>5.</td>
<td>20</td>
<td>40</td>
<td>10</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>6.</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
</tbody>
</table>

arise from either the free substrate or the free enzyme (31, 32); however, since substrate 2 does not contain a group that would ionize between pH 6.2 and 7.5, the loss in efficacy at higher pH values may reflect ionization of an important group in the hydrophobic binding cleft of the enzyme, e.g. Asp<sup>25</sup>, Asp<sup>39</sup>, or Asp<sup>30</sup> (5, 12, 13, 28). In a separate study with wild-type HIV-1 PR and a different type of substrate, a $pK_a$ value of 6.8 was reported and attributed also to the free enzyme (32).

Substrate 1 does contain a potentially ionizable Glu residue in the crucially important $P_2'$ position (26, 28). Thus, the steady decline (with a slope of -1) from the optimum at pH 4.7 that was observed in the $k_{cat}/K_m$ parameter for this substrate with all four enzymes (Fig. 3, panel a, top) may well be a combination of the influence of the ionization of the Glu side chain in position $P_2'$ in the substrate and an ionizable side chain that is common to HIV-1 and HIV-2 subunits in the enzymes. Hydrolysis of substrate 1 then is monitored optimally for all four tethered enzymes in the region of pH 4.7 (Fig. 3, panel a, top), thus substantiating the selection of this pH for the quantitative determinations described in Tables I and II.

In a number of previous investigations with archetypal aspartic proteinases, a $pK_a$ between 6.0 and 7.5 has commonly been derived from the pH dependence of the $k_{cat}$ parameter and has been assigned to the loss of a proton from the catalytic apparatus (see, e.g., Ref. 33). A similar phenomenon was observed for all four tethered dimers (Fig. 3, panels a and b, bottom) where, for example, apparent $pK_a$ values around 7.5 were determined with substrate 1 for three of the enzymes, whereas the corresponding value for the HIV-1/2* heterodimer with the HIV-1 interface was somewhat lower at ~6.2.
the tethered dimers of the present study behave comparably to wild-type HIV-1 PR, whereas the tethered HIV-1/2* heterodimeric proteinase.

Comparative pK<sub>a</sub> values for the E-S complex were derived in a separate study on untethered HIV-1 PR (32). In the wild-type PR (32), a value of 6.9 was derived (with a different class of substrate), whereas a pK<sub>a</sub> of 6.0 was calculated for a mutant homo- and heterodimeric proteinases. The substrates used were as follows: Lys-Ala-Arg-Ile-Nle*Nph-Glu-Ala-Nle-U*Val-Ala-Lys (left panels, a) and Ac-Tyr-Arg-Ala-Val-Phe*Nph-Ala-Ala-Lys (right panels, b). The buffers used were: sodium acetate, pH 4.0, 4.7, and 5.5; MES, pH 5.2, Tris, pH 7.5, 8.2, and 9.0, containing 4% EDTA (and 5 M mercaptoethanol) at pH values 7.5-9.0, and sufficient NaCl to give a constant ionic strength of 0.3 M at 37 °C. Values up to pH 6.2 were determined spectrophotometrically, while those at the higher pH values were estimated by the less precise fast protein liquid chromatography method. □, HIV-1/1 tethered homodimer; ▽, HIV-1/2* heterodimeric proteinase; △, HIV-2/2 tethered heterodimer; O, HIV-2/2 tethered homodimeric proteinase.

The requirement for precise spatial juxtaposition of catalytic and neighboring residues in the active site of HIV PR is thus evidenced by these findings. The kinetic constants that were measured for the two heterodimers indicated that these enzymes are not simple hybrids. In catalytic aspects, the heterodimers appeared to behave more like HIV-2 PR, whereas, in terms of binding, particularly of certain inhibitors, they resembled HIV-1 PR more closely. Detailed studies with inhibitors have not been described in previous work on tethered homodimers (6-10) nor in the one short report on a tethered heterodimer (34) that appeared while the present work was nearing completion.

The perfect C<sub>2</sub> symmetry intrinsic in wild-type HIV PRs is maintained, for all practical purposes, in the tethered homodimers if the directionality introduced by the Gly-Gly linker (on the periphery) is ignored in relation to the distant active site. (On this arbitrary basis, a HIV-2/1 heterodimer would thus display essentially the same spatial, but not chemical, arrangement as the HIV-1/2 PR). Thus substrates or inhibitors can bind to the tethered homodimers, as in the case of the wild-type untethered enzymes, in the “nose-to-the-left” and “nose-to-the-right” fashion observed in several crystal structures that have been solved for inhibitor complexes (28, 35–37). This is not the case, however, for the tethered heterodimers where an asymmetric active site is presented. It would appear that ligand (substrate or inhibitor) binding is being selected preferentially to direct certain residues into the HIV-1 domain rather than that contributed by HIV-2. Since there are only at most 5 residues that differ between HIV-1 and HIV-2 PR in the active site which make contact with substrate or inhibitor, this conclusion is amenable to direct examination by a mutagenesis program.

Acknowledgments—We acknowledge the valuable contributions of Jo-Ann and colleagues, Tom Krohn, Simon Broome, Alison Ritchie, and Noel Roberts in the Chemistry, Physical Methods, and Virology Departments at Roche (Welwyn Garden City, United Kingdom), and the excellent technical assistance of Waleed Danbo, Warner McComas, and Rich Motyka in the Molecular Sciences Group and Nikhil Mehta in EPT at Roche (Nutley, NJ).

REFERENCES
Tethered HIV-1 and HIV-2 Homo- and Heterodimeric Proteinases