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Systematic mutational analysis of the active-site threonine of HIV-1 proteinase: Rethinking the "fireman's grip" hypothesis

KVIDO STRISOVSKY,[1] UWE TESSMER,[2] JOSMAR LANGNER,[3] JAN KONVALINKA,[1] AND HANS-GEORG KRÄUSSLICH[2]

[1]Department of Biochemistry, Institute of Organic Chemistry and Biochemistry, Czech Academy of Science, Flemingovo n.2, 166 10 Praha 6, Czech Republic
[2]Heinrich-Pette-Institut für experimentelle Virologie und Immunologie, Martinistr. 52, D-20251 Hamburg, Germany
[3]Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

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Abstract

Aspartic proteinases share a conserved network of hydrogen bonds (termed "fireman's grip"), which involves the hydroxyl groups of two threonine residues in the active site Asp-Thr-Gly triplets (Thr26 in the case of human immunodeficiency virus type 1 (HIV-1) PR). In the case of retroviral proteinases (PRs), which are active as symmetrical homodimers, these interactions occur at the dimer interface. For a systematic analysis of the "fireman's grip," Thr26 of HIV-1 PR was changed to either Ser, Cys, or Ala. The variant enzymes were tested for cleavage of HIV-1 derived peptide and polyprotein substrates. PR(T26S) and PR(T26C) showed similar or slightly reduced activity compared to wild-type HIV-1 PR, indicating that the sulfhydryl group of cysteine can substitute for the hydroxyl of the conserved threonine in this position. PR(T26A), which lacks the "fireman's grip" interaction, was virtually inactive and was monomeric in solution at conditions where wild-type PR exhibited a monomer–dimer equilibrium. All three mutations had little effect when introduced into only one chain of a linked dimer of HIV-1 PR. In this case, even changing both Thr residues to Ala yielded residual activity suggesting that the "fireman's grip" is not essential for activity but contributes significantly to dimer formation. Taken together, these results indicate that the "fireman's grip" is crucial for stabilization of the retroviral PR dimer and for overall stability of the enzyme.

Keywords: aspartic proteinase; dimerization; fireman's grip; HIV; mutagenesis

Formation of infectious retrovirus particles depends on the activity of the viral proteinase (PR) that cleaves viral precursor polyproteins (Gag and Gag-Pol) into individual functional proteins (Vogt & Eisenman, 1973; Vogt et al., 1975). Cleavage is followed by a dramatic morphological rearrangement of the internal virion structure, termed maturation. PR itself is made as part of the Gag-Pol polyprotein and is activated in the process of maturation. Inactivation of PR by either mutation or chemical inhibition leads to formation of only immature viruses and consequently to loss of infectivity (Kohl et al., 1988). Therefore, inhibitors targeted against the active site of human immunodeficiency virus type 1 (HIV-1) PR have recently led to a major breakthrough in the treatment of AIDS (von der Helm, 1996).

Retroviral PRs belong to the family of aspartic proteinases that includes proteinases of eukaryotic cells, such as pepsin, chymosin, cathepsins D and E, and renin. All aspartic proteinases form bilobal structures with a deep, extended cleft containing the active site, which is formed quasi-symmetrically from both domains, each conferring one catalytic aspartate. Retroviral PRs are equivalent to one domain of the eukaryotic enzymes and are only active as homodimers, where each molecule in the dimer contributes one half of the active site. The two monomers are related by a twofold axis of symmetry with the dimer interface being formed by a four-stranded antiparallel β-sheet containing two strands from each molecule (Weber, 1990). The close spatial arrangement of the N- and C-termini of the two monomers allowed the two chains to be artificially linked together by a short peptide sequence (Cheng et al., 1990; DiIanni et al., 1990; Bizub et al., 1991; Kräusslich, 1991). Such covalently linked dimers of retroviral PRs have been shown to cause premature processing of viral polyproteins and consequent loss of particle formation (Burstein et al., 1991; Kräusslich, 1991), supporting the hypothesis that dimerization is an important regulatory factor governing PR activation. In addition, the availability of single chain PR dimers permitted the analysis of asymmetric mutations in an otherwise symmetrical molecule (Griffiths et al., 1994; Bagossi et al., 1996; Tözsér et al., 1997).

Reprint requests to: Hans-Georg Kräusslich, Department of Virology, University of Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany; e-mail: Hans-Georg_Kraeusslich@med.uni-heidelberg.de.

Retroviral and other aspartic proteinases have the same general fold of the polypeptide chain, even in pairs with considerable divergence in primary sequence and length (Rao et al., 1991). In particular, the three-dimensional (3D) structures of the active sites are nearly identical in all crystal structures and the catalytic Asp residues are within hydrogen bond distance of each other and essentially in the same spatial relationship in all structures determined to date. These aspartate residues are part of the conserved amino acid triplet Asp-Thr-Gly (DTG). A particular feature of the catalytic center is the interaction between the side chain of the active site Thr (Thr26 in the case of HIV-1 PR) with the main-chain amide of the active site Thr (Thr26′) of the other molecule in the dimer. Similar interactions take place between the side chain of this Thr (Thr26) and the main-chain carbonyl of the preceding Leu (Leu24′ in the case of HIV-1 PR) of the other molecule in the dimer. Both hydrogen bonds are strong and are dependent on the side-chain hydroxyl of the Thr residues. An X-ray crystallographic model of the 3D structure of HIV-1 PR and a detailed view of the active site region depicting the described hydrogen bond network is shown in Figure 1. The two loops holding the active site Asp residues in the correct orientation are effectively stabilized by four hydrogen bonds and this elaborate hydrogen bond network has been given the name "fireman's grip" (Fig. 1) (Pearl & Blundell, 1984; Pearl & Taylor, 1987). The position of the active site Thr residue and the resultant hydrogen bonds are conserved in virtually all aspartic proteinase structures, and it is believed that this feature plays an essential role in stabilizing the active site geometry.

The only sequence divergence within the catalytic triplet of retroviral aspartic proteinases is a conservative Thr → Ser substi-

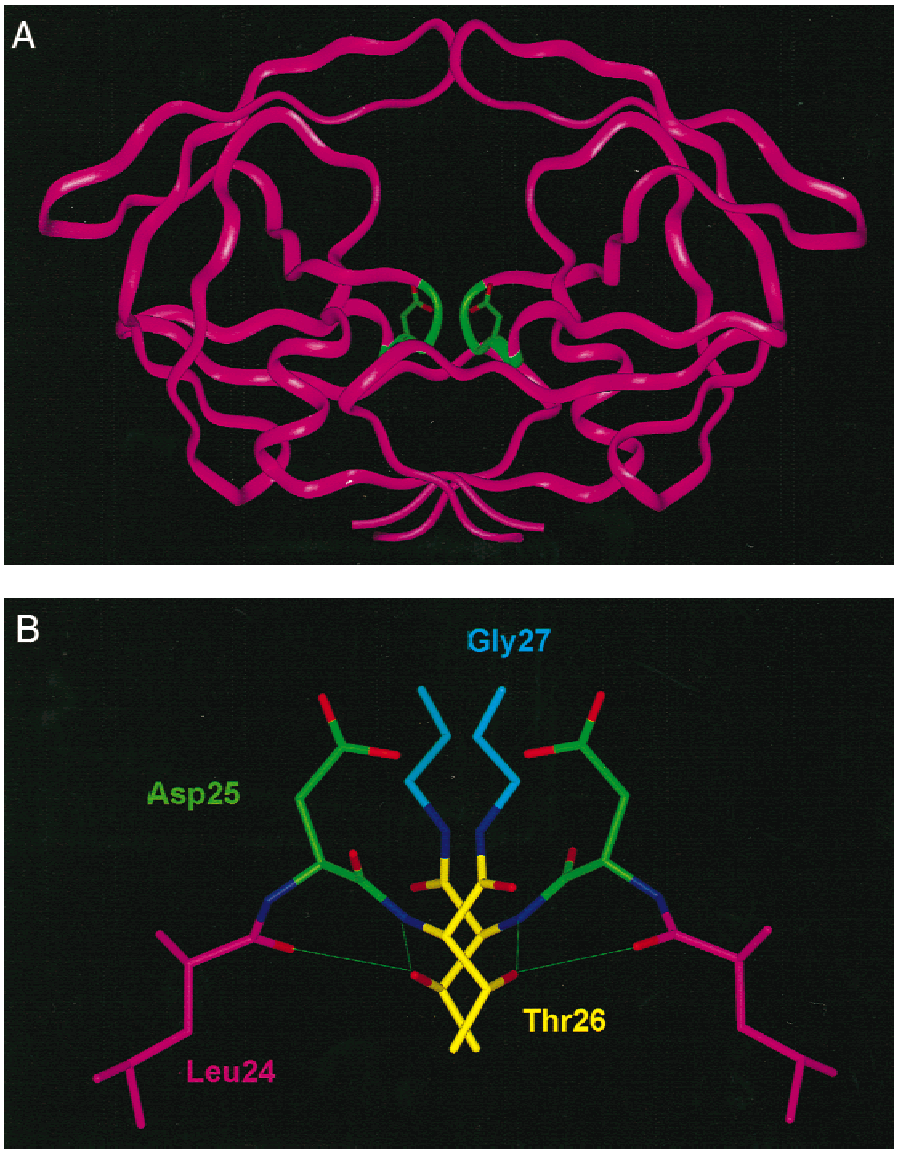

Fig. 1. Three-dimensional structure of HIV-1 PR. **A:** The structure of HIV-1 PR as determined by X-ray crystallography (PDB entry 1hhp). The active-site region Leu24–Gly27 comprising the "fireman's grip" is highlighted in green. Side chains of the two catalytic aspartic acid residues are also shown. **B:** A detailed view of the active site region (Leu24–Gly27) of HIV-1 PR forming the "fireman's grip." Notice the network of hydrogen bonds between Thr26, Thr26′ (in yellow) and Leu24, Leu24′ (in purple).

tution (leading to a DSG triplet) in the PRs of avian retroviruses, foamy viruses, and some yeast retrotransposons (Barrett et al., 1998). Serine, with a side-chain hydroxyl similar to that of threonine, is the only other small amino acid that is able to form the described specific interactions of the "fireman's grip." Interestingly, avian retroviruses encode their PR as part of the Gag polyprotein leading to equimolar amounts of PR and structural proteins in the virion (Bennett et al., 1991). Most other retroviruses encode PR as part of the Gag-Pol polyprotein, which is expressed by translational frameshifting or read-through of a termination codon at a 10- to 20-fold lower ratio compared to the Gag polyprotein (Hatfield et al., 1992). Consistent with the higher expression levels, avian retroviral PRs have been shown to be less active than the corresponding PRs of mammalian retroviruses (e.g., HIV-1 PR) (Konvalinka et al., 1992). Taken together, these observations suggest that the Thr → Ser exchange, while maintaining the essential hydrogen bond network of the "fireman's grip," may play an important role in regulating PR activity. This hypothesis is supported by studies concerning a variant of HIV-1 PR with a Thr26 → Ser substitution (Konvalinka et al., 1995b; Rose et al., 1995). Kinetic analysis of this enzyme showed an eightfold reduction in activity at neutral or slightly acidic pH when compared to wild-type PR. This effect was largely due to an increase in K_m, while the turnover number and substrate specificity were unaltered. In the context of an infectious clone of HIV-1, on the other hand, normal polyprotein processing and viral infectivity was observed. This phenotype is probably due to the very high concentrations of both enzyme and substrate in the nascent virion (~0.1 and 4 mM, respectively), which are well above the K_m value of the PR(T26S) variant. In the cytoplasm of infected cells, on the other hand, the PR concentration is much lower leading to reduced processing of intracytoplasmic (cellular) substrates by PR(T26S) (Konvalinka et al., 1995b). A reciprocal increase in PR activity was observed when the DSG triplet of an avian retroviral PR was changed to a DTG triplet, while this mutation reduced the number of infectious virus particles (Arad et al., 1995).

Surprisingly, the concept of the "fireman's grip" and the role of the critical Thr residue has not been subjected to systematic experimental studies. In a comprehensive mutational analysis of HIV-1 PR, mutating Thr26 to either Ala, Ile, Lys, or Arg led to inactivation of the enzyme (Loeb et al., 1989). However, in this study, only cis-cleavage of a bacterially expressed artificial polyprotein was analyzed. Analysis of heterodimers containing a substitution of Thr26 for either Ser or Asn in just one or both chains of a linked dimer has also been reported (Bagossi et al., 1996). In this case, no change in activity was observed for the Ser-containing enzymes, while the Asn-containing variants were virtually inactive. To systematically examine the role of the active site threonine of HIV-1 PR, we changed this residue to either Ser, Cys, or Ala and analyzed the variant enzymes regarding their activities against peptide and polyprotein substrates. In addition, the same mutations were introduced into one chain of a linked HIV-1 PR dimer, thereby creating asymmetric heterodimers. Here, we report that preventing formation of the hydrogen bond network of the "fireman's grip" virtually abolished PR activity, while removing a single Thr residue in the context of a linked dimer had only a minor effect. Surprisingly, even exchanging both Thr residues to Ala did not abolish activity of the linked dimer, suggesting that the "fireman's grip" is not absolutely required for enzyme activity and contributes significantly to dimer stability. This hypothesis was confirmed by analytical ultracentrifugation experiments.

Results

Expression and purification of HIV-1 PR variants

To analyze the role of the "fireman's grip" for the stability and catalytic activity of HIV-1 PR, we exchanged Thr26 of HIV-1 PR to either a Ser, Cys, or Ala residue. In the case of serine, the hydroxyl involved in the hydrogen bond network is preserved. In the case of cysteine, this hydroxyl group is exchanged for a sulfhydryl group that should, in principle, be capable to donate and accept the same hydrogen bonds without causing significant steric hindrance. An alanine in this position, on the other hand, should completely abolish the hydrogen bond network of the "fireman's grip" (see Fig. 1B for a detailed view of this region of the HIV-1 PR structure). The mutations were introduced into the bacterial expression vector pET11-PR, which contains the coding region for the 99 amino acids of HIV-1 PR preceded by a methionine initiation codon (Konvalinka et al., 1995b). N-terminal amino acid sequencing showed that all expression products were effectively demethionylated in *Escherichia coli*. The yield of recombinant proteins was inversely correlated with their respective activities, giving the highest yields for the least active enzymes. The variant proteins were purified from inclusion bodies by denaturation and refolding. A critical step in the purification procedure (modified from Cheng et al., 1990; Konvalinka et al., 1995a) was the renaturation of denatured inclusion bodies by dissolving them in a chaotropic agent and dialysis against an appropriate buffer to yield active, refolded enzyme. Refolded proteins were subsequently purified by cation exchange chromatography and active-site titrated using a tight binding inhibitor of HIV PR. Purity was >90% in all cases (data not shown).

Catalytic activities and substrate specificities of variant PRs

Activity of the variant enzymes was determined using the synthetic, chromogenic octapeptide substrate KARVNleF(NO_2)EANle (Richards et al., 1990). Since we had previously shown that the T26S mutation altered the pH profile of this enzyme compared to wild-type PR (Konvalinka et al., 1995b), kinetic properties of all PRs were analyzed at two pH values. These results are summarized in Table 1. At pH 4.7, differences between PR(WT), (T26S), and (T26C) were mainly in K_m values, while the k_{cat} values of these three enzymes were comparable. Specific activities, expressed as k_{cat}/K_m, were approximately threefold lower for the T26S variant and 10-fold lower for the T26C variant compared to PR(WT). PR(T26A) exhibited only traces of peptidolytic activity and could not be titrated. At the higher pH (6.8), the differences between wild-type and variant PRs became more pronounced. Taken together, the peptide cleavage data showed that substituting Thr26 of HIV-1 PR by Ala resulted in a virtual loss of activity, while introduction of either Ser or Cys into the same position yielded active enzymes with reduced specific activities and altered pH profiles.

The kinetics of hydrolysis of peptide substrates and the specific activities of different enzymes do not always directly correspond to their relative activities on polyprotein substrates (e.g., Klabe et al., 1998). We therefore analyzed the activities and specificities of the T26X PR variants against the HIV-1 $Pr55^{gag}$ Gag polyprotein, its native substrate (Fig. 2). During virion maturation, $Pr55^{gag}$ is cleaved into the functional proteins matrix (MA; 17 kDa), capsid (CA; 24 kDa), nucleocapsid (NC; 7 kDa), p6, and several smaller peptides. For in vitro cleavage, $Pr55^{gag}$ was obtained from recombi-

Table 1. *Comparison of pH-dependent peptidolytic activity of T26 mutants of HIV PR*[a]

	pH 4.7, 0.3 M NaCl			pH 6.8, 1.0 M NaCl		
Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
26T(wt)	7.8 ± 0.8	19.7 ± 0.7	2.5 ± 0.3	93 ± 8	20 ± 1	0.22 ± 0.03
T26S	15 ± 2	14.9 ± 0.4	1.0 ± 0.1	190 ± 50	12 ± 2	0.063 ± 0.030
T26C	52 ± 9	11.5 ± 0.7	0.22 ± 0.04	>250[b]	n.d.[b,c]	n.d.[c]
T26A	60 ± 13	0.1 ± 0.01[d]	0.0016 ± 0.0006[d]	n.d.	n.d.	n.d.

[a] Synthetic peptide substrate KARVNleF(NO_2)EANle was cleaved by PR variants at 37 °C in 100 mM buffer (potassium acetate pH 4.7 or MES pH 6.8), 1 mM EDTA and 0.3 M NaCl (pH 4.7) or 1.0 M NaCl (pH 6.8). Enzyme concentration in assays ranged from 3–80 nM. Initial reaction rates were determined spectrophotometrically.

[b] Inhibition by substrate precludes determination.

[c] n.d., not determined

[d] Active site concentration of the PR (T26A), used for computation of k_{cat}, was estimated from total protein concentration and comparison with the active site titrated wild-type HIV-1 PR.

nant virus-like particles and incubated for 2 h with different concentrations of purified, active-site titrated PR variants. Cleavage products were determined by immunoblotting using an antiserum against MA. Incubation with wild-type HIV-1 PR (Fig. 2A) at a concentration of 17 nM led to almost complete processing of $Pr55^{gag}$ to MA with few intermediate cleavage products remaining, both at pH 4.7 and 6.8. In contrast, no cleavage was observed upon incubation with PR(T26A), even at the highest concentration possible (~1.5 μM, Fig. 2D). Incubation with PR(T26S) (Fig. 2B) and (T26C) (Fig. 2C), on the other hand, led to efficient cleavage of the Gag polyprotein with an apparently unaltered specificity (as judged from the relative mobilities of the cleavage products). At the acidic pH, PR(T26S) was approximately threefold less active compared to PR(WT), while PR(T26C) was at least 10-fold less active. These differences are in good agreement with the specific activities of the respective enzymes determined on peptide substrates (Table 1). At neutral pH, the difference between PR(WT) and the T26S and T26C variants became much more pronounced. Incubation at pH 6.8 showed an approximately 10-fold lower activity for PR(T26S) compared to PR(WT). The activity of PR(T26C) was reduced significantly more and no complete processing to the MA product was observed even at the highest concentration analyzed (450 nM; Fig. 2C, Lane 6). Similar results were obtained using an HIV-1 Gag-derived substrate containing only a single cleavage site for HIV-1 PR (Gross et al., 1998; data not shown).

Polyprotein cleavage by T26X PR variants in viral maturation

Besides cleaving $Pr55^{gag}$ *in trans*, it was of interest to determine whether the variant PRs can cleave viral polyproteins in the process of virion maturation. Therefore, the three mutations were introduced into the subgenomic HIV-1 expression vector pK-HIV that contains the entire coding region of HIV-1 except for the *nef* gene under control of the strong eukaryotic promoter/ enhancer element of human cytomegalovirus (Konvalinka et al., 1997). Transient transfection of tissue culture cells with pK-HIV containing a wild-type PR gene leads to release of HIV-like particles that are morphologically indistinguishable from HIV-1 and undergo normal polyprotein processing and maturation.

Figure 3 shows immunoblot analysis of virus-like particles collected after transfection of COS-7 cells with pK-HIV (Lane 2) or T26X variants as indicated above each lane. PR(WT) + inhibitor (Lane 3) corresponds to a transfection cultured in the presence of the HIV PR-specific inhibitor saquinavir (Roberts et al., 1990). As described previously (Konvalinka et al., 1995b), particles produced from pK-HIV (WT) or -(T26S) transfected cells exhibited virtually complete processing of polyproteins, while particles produced in the presence of saquinavir contained only uncleaved precursor polyproteins (Fig. 3, Lanes 2–4). Particles produced from pK-HIV(T26A) transfected cells also did not show any processing of the Gag polyprotein (Fig. 3, Lane 6), while weak processing occurred in the case of the T26C variant, reproducibly showing partially cleaved intermediate products (Fig. 3, Lane 5). These data are in good agreement with the results of the in vitro cleavage reactions at neutral pH, where only limited activity of the T26C variant was observed.

Proteolytic activity of tethered dimers of HIV PR

From the described experiments, we conclude that the critical Thr residue of the "fireman's grip" of HIV-1 PR can be functionally replaced by the two other small amino acids with the potential to serve as acceptor/donor of the hydrogen bond (Ser, Cys), but not by an amino acid lacking a side-chain hydroxyl or sulfhydryl (Ala). The "fireman's grip" consists of two pairs of symmetrical hydrogen bonds and it was of interest to determine whether abolishing the capacity to form the hydrogen bond in one subunit of the dimer while retaining it in the other subunit would suffice for enzyme activity. We therefore introduced the described T26X mutations into the first chain of a tethered dimer of HIV PR, where the two subunits of the enzyme are covalently linked by a heptapeptide hinge (Kräusslich, 1991). These constructs (schematically depicted in the bottom part of Fig. 4) were designated pET-2PR(X/T) and the resulting proteins were designated 2PR(X/T), where X can be Thr (WT), Ser, Cys, or Ala. The various heterodimers were purified from inclusion bodies as described above. The purity was higher than 90% in all cases (Fig. 4 and data not shown) and there was virtually no sign of degradation to proteins of monomeric size as judged by immunoblotting using enhanced chemiluminescence for detection (Fig. 4, Lanes 4, 5). Prior to kinetic measurements, all PRs were active-site titrated.

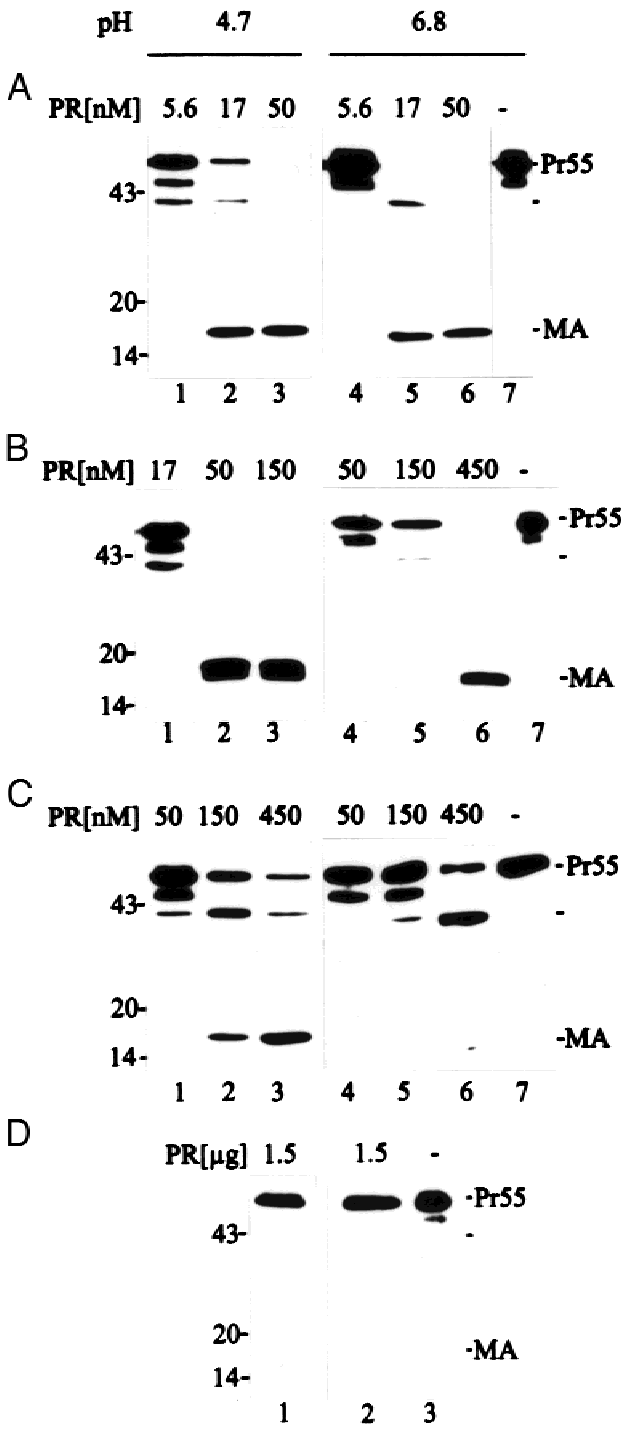

Fig. 2. Analysis of pH-dependent cleavage of HIV-1 Gag polyproteins by HIV-1 PR and T26 variants. $Pr55^{gag}$ obtained by centrifugation of virus-like particles was used at a concentration of 1 μM and cleavage reactions were performed for 2 h at 37 °C and pH 4.7 or 6.8 as indicated on top. Enzyme concentrations (determined by active-site titration) for **(A)** wild-type PR, **(B)** PR(T26S), and **(C)** PR(T26C) are indicated above each lane. In the case of **(D)** PR(T26A), no active site concentration could be determined and 1.5 μM of purified PR was used in this case. Control incubations performed in the absence of PR are indicated as (–). All samples were analyzed by immunoblotting using polyclonal antiserum against the HIV-1 matrix protein and detected by enhanced chemiluminescence. Molecular mass standards are shown on the left; HIV-specific proteins are indicated on the right.

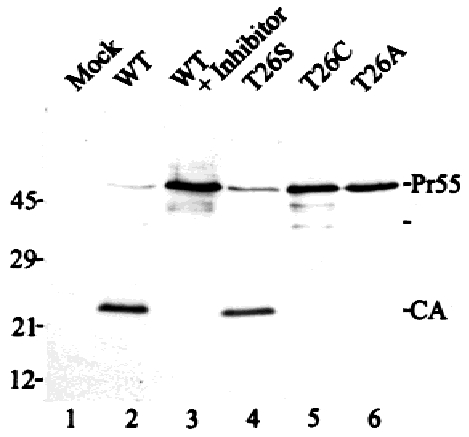

Fig. 3. Immunoblot analysis of HIV-specific proteins following transient transfection. COS-7 cells were transfected with pK-HIV or PR(T26)-derivatives as indicated on top of each lane and lysates of virus-like particles collected by centrifugation were resolved by SDS-polyacrylamide gel electrophoresis. Western blots were reacted with polyclonal antiserum against HIV-1 capsid protein. Mock refers to transfection without specific DNA, WT + inhibitor refers to cultivation of pK-HIV transfected cells in the presence of 10 μM of the PR-specific inhibitor saquinavir.

The proteolytic activity of the variant PR heterodimers was analyzed at pH 4.7 and 6.8, using the described chromogenic peptide substrate (Table 2). At pH 4.7, there was virtually no difference between wild-type HIV-1 PR (Table 1), the linked 2PR homodimer, and the three different covalently linked dimers (Table 2). The observed specific activities (k_{cat}/K_m) ranged from 2.5 $s^{-1}\mu M^{-1}$ for the wild-type enzyme to 1.7 $s^{-1}\mu M^{-1}$ for 2PR(A/T). Very similar activities for the linked wild-type homodimer and the three heterodimers were also observed at neutral pH with specific activities ranging from 0.49 $s^{-1}\mu M^{-1}$ for 2PR(S/T) to 0.21 $s^{-1}\mu M^{-1}$ for 2PR(A/T) (Table 2). All linked dimers were two- to threefold more active than the unlinked wild-type HIV-1 PR at this pH (compare Table 1). Surprisingly, the specific activity of 2PR(A/T) was only less than twofold reduced compared to the wild-type linked dimer at both pH values. This result clearly indicates that the presence of only one hydroxyl in the hydrogen bond network of the "fireman's grip" is sufficient for a functional aspartic proteinase.

To exclude that formation of mixed PR multimers of higher order might compensate for loss of the "fireman's grip" in one domain, we also prepared a tethered PR dimer with a D25A mutation (inactivating the catalytic aspartate) in the first domain. This variant was inactive in all assays. To determine whether the requirement for Thr26 may be less stringent in the context of a covalently linked PR dimer, we also constructed an expression vector for the 2PR(A/A) homodimer and purified the corresponding protein from inclusion bodies. This mutant enzyme, which completely lacks the side chains required for forming the "fireman's grip," is still an active proteinase. The large decrease in specific activity can be attributed mostly to the dramatic increase in K_m. Furthermore, specific inhibitors of HIV PR showed approximately 100-fold weaker binding to 2PR(A/A) than to WT proteinase (data not shown), which impairs active site titration of 2PR(A/A). Taken together, these observations suggest but do not prove that 2PR(A/A) may have an altered substrate specificity. The active site concentration of 2PR A/A was estimated from the

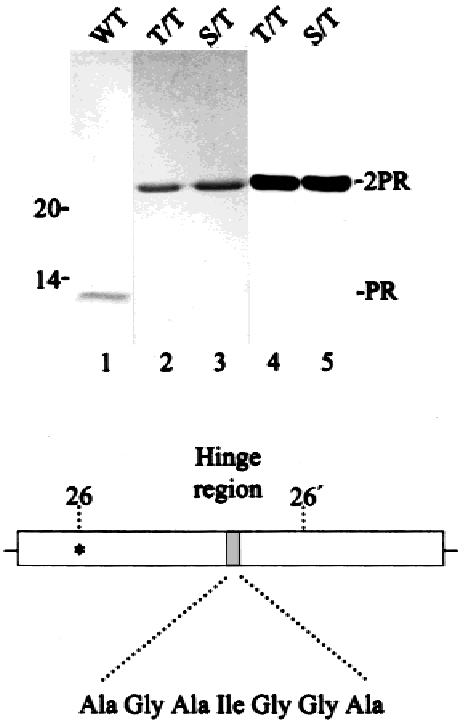

Fig. 4. Analysis of linked dimers of HIV-1 PR. A schematic representation of the linked dimers is shown at the bottom. Monomeric HIV-1 PR (WT; Lane 1) and the linked dimers containing wild-type sequences in both chains (T/T, Lanes 2, 4) or serine in the first chain (S/T, Lanes 3, 5) were analyzed on Coomassie Blue-stained SDS-polyacrylamide gels (Lanes 1–3) or by immunoblotting using polyclonal antiserum against HIV PR followed by enhanced chemiluminescence detection (Lanes 4, 5).

total protein concentration assuming a similar ratio of active PR vs. total protein in the preparation as determined for the linked heterodimers. When directly compared in one experiment, 2PR(A/A) is approximately 50-fold more active than PR(T26A) (data not shown; compare Tables 1 and 2). This experiment conclusively shows that the absence of the "fireman's grip" interactions in a single chain aspartic proteinase does not necessarily abolish its enzymatic activity.

We next tested the proteolytic activities of the tethered dimers on $Pr55^{gag}$ obtained from virus-like particles. Cleavage reactions for the wild-type linked homodimer (T/T) and the three linked heterodimers were performed at pH 6.8 at enzyme concentrations of 25 or 250 nM and a substrate concentration of 1 μM and were analyzed by immunoblotting using an antiserum against HIV-1 CA (Fig. 5A). Cleavage by 25 nM wild-type 2PR was complete after 30 min with few intermediate products remaining after incubation for 10 min (Fig. 5A, Lanes 2, 3). A very similar pattern was observed for the S/T heterodimer, which was virtually indistinguishable from the wild-type linked dimer in several different assays (Fig. 5A, Lanes 4–6 and data not shown). The Cys- and Ala-containing heterodimers, on the other hand, exhibited significantly lower activities against the polyprotein substrate with only minor amounts of completely cleaved CA protein after 30 min incubation at an enzyme concentration of 25 nM (Fig. 5A, Lanes 9, 12). Complete cleavage was observed on incubation with 2PR(C/T) or (A/T) at a concentration of 250 nM for 30 and 120 min, respectively (Fig. 5A, Lanes 10, 14). Comparative experiments indicated that these variant heterodimers were approximately five-fold (C/T) or 10- to 20-fold (A/T) less active than the wild-type linked dimer (data not shown). Very weak activity was observed for 2PR(A/A) under these conditions and the experiment was therefore repeated at pH 4.7, yielding partial cleavage of the Gag polyprotein upon incubation with 250 nM 2PR(A/A) (Fig. 5B, Lanes 2, 3). However, no complete cleavage to the CA protein was observed in this case, indicating that the Ala-containing homodimer may be significantly less active or may have an altered substrate specificity.

Analysis of the dimerization of variant PR molecules

The described experiments indicate that the requirement for the "firemen's grip" is less stringent in a single chain dimer of HIV-1

Table 2. *Comparison of pH-dependent peptidolytic activity of T26 mutants of tethered dimers of HIV PR*[a]

	pH 4.7, 0.3 M NaCl			pH 6.8, 1,0 M NaCl		
Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
T/T	9.2 ± 0.6	20.2 ± 0.4	2.2 ± 0.2	69 ± 5	27.6 ± 0.8	0.40 ± 0.03
S/T	7.4 ± 0.9	18.5 ± 0.6	2.5 ± 0.3	67 ± 3	32.7 ± 0.5	0.49 ± 0.02
C/T	4.2 ± 0.4	13.4 ± 0.3	3.2 ± 0.3	31 ± 4	12.6 ± 0.5	0.41 ± 0.06
A/T	5.7 ± 0.6	9.4 ± 0.3	1.7 ± 0.2	68 ± 6	14.4 ± 0.4	0.21 ± 0.02
A/A	67 ± 6	3.5 ± 0.1[b]	0.052 ± 0.005[b]	n.d.[c]	n.d.	n.d.

[a] Synthetic peptide substrate KARVNleF(NO_2)EANle was cleaved by mutant tethered dimers at 37 °C in 100 mM buffer (potassium acetate pH 4.7 or MES pH 6.8), 1 mM EDTA and 0.3 M NaCl (pH 4.7), or 1.0 M NaCl (pH 6.8). Enzyme concentration in assays ranged from 3–16 nM. Initial reaction rates were determined spectrophotometrically.

[b] Active site concentration of tethered homodimer 2PR(A/A), used for computation of k_{cat}, was estimated from the total protein concentration and comparison with the active site titrated wild-type tethered dimer (T/T).

[c] n.d., not determined

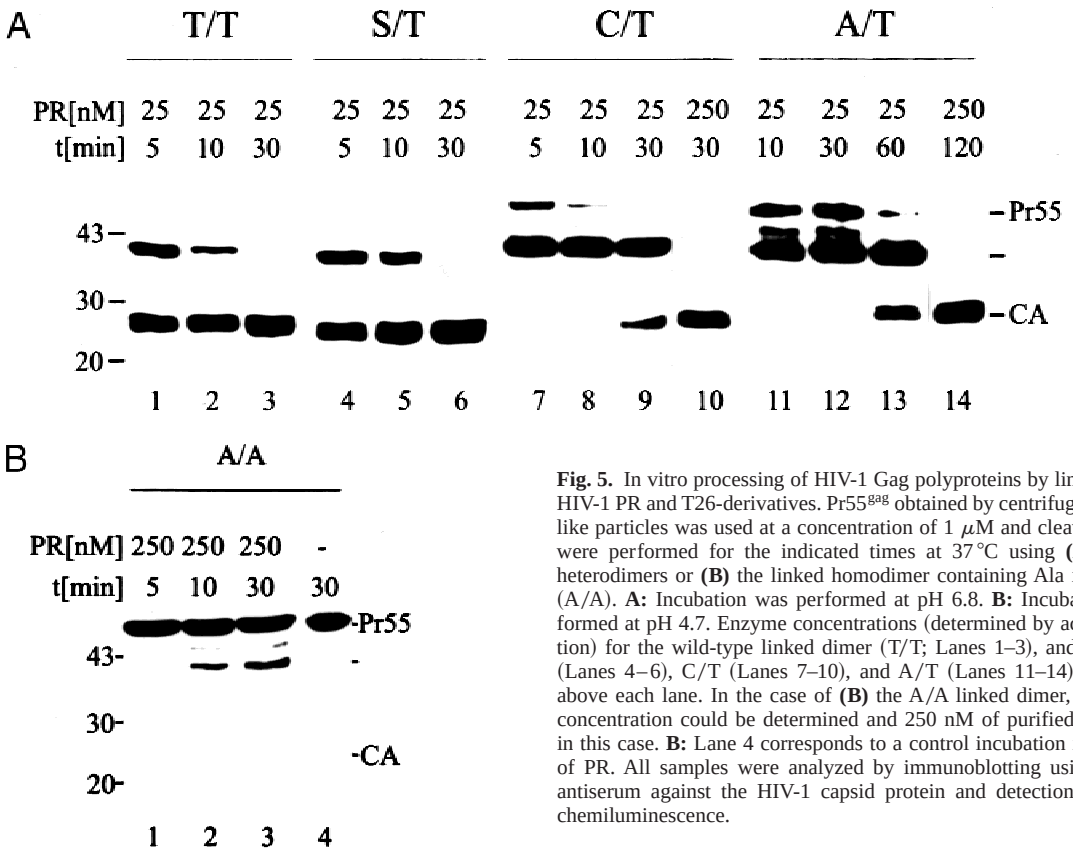

Fig. 5. In vitro processing of HIV-1 Gag polyproteins by linked dimers of HIV-1 PR and T26-derivatives. $Pr55^{gag}$ obtained by centrifugation of virus-like particles was used at a concentration of 1 μM and cleavage reactions were performed for the indicated times at 37 °C using **(A)** linked PR heterodimers or **(B)** the linked homodimer containing Ala in both chains (A/A). **A:** Incubation was performed at pH 6.8. **B:** Incubation was performed at pH 4.7. Enzyme concentrations (determined by active-site titration) for the wild-type linked dimer (T/T; Lanes 1–3), and variants S/T (Lanes 4–6), C/T (Lanes 7–10), and A/T (Lanes 11–14) are indicated above each lane. In the case of **(B)** the A/A linked dimer, no active site concentration could be determined and 250 nM of purified PR was used in this case. **B:** Lane 4 corresponds to a control incubation in the absence of PR. All samples were analyzed by immunoblotting using polyclonal antiserum against the HIV-1 capsid protein and detection by enhanced chemiluminescence.

PR, suggesting that the active site threonine may contribute to dimer stability of retroviral PRs. The lack of activity of the PR(T26A) variant could then be interpreted as a dimerization defect, which is partly overcome in the case of the 2PR(A/A) enzyme by covalent linkage of the two subunits. We therefore performed equilibrium ultracentrifugation experiments to analyze the aggregation state of the wild-type HIV-1 PR and the unlinked PR(T26A) (Fig. 6). Initial measurements indicated that the presence of low molecular weight contaminants (presumably peptides, peptidoglycans, or fragments of nucleic acids) obscured the result (data not shown), and the purification procedure was therefore modified. Using ion exchange chromatography under denaturing conditions, we were able to remove virtually all UV-absorbing low-molecular weight contaminants. A final ion exchange chromatography step was required after refolding to remove autodegradation products.

Equilibrium ultracentrifugation of the different PRs showed that wild-type HIV-1 PR (Fig. 6A), as well as PR(T26S) and -(T26C) (data not shown) were not monodisperse exhibiting an average molecular weight of 13–16 kDa. This suggests that these proteins are forming a monomer/dimer equilibrium in solution. The data obtained for PR(WT) fitted well into a model for ideal monomer–dimer association, yielding a K_D value of 77 μM (Fig. 6B). No degradation products could be observed for wild-type PR after 8 h of ultracentrifugation at 20 °C and the enzyme maintained 95% of its initial activity. In contrast to wild-type PR and the other variants, PR(T26A) was monodisperse with an average molecular weight of 10 kDa (Fig. 6B). The data obtained in this case could not be fitted into the monomer–dimer association model (hypothetical K_D is in the range of 100 M), strongly arguing for a dimerisation defect of the PR(T26A) variant.

Discussion

Alignment of all aspartic proteinases identified so far (Barrett et al., 1998) shows that the amino acid triplet Asp-Thr(Ser)-Gly is completely conserved. This "signature sequence" is such a generally recognized trademark that Hill and Phylip recently used it to search for yet unknown aspartic proteinases in the genomes of *E. coli* and *Haemophilus influenzae*, and identified novel proteins with residual proteolytic activity (Hill & Phylip, 1997). The hydroxyl group of the Thr (or Ser) residue within the catalytic core is presumed to stabilize the geometry of the active site by forming the hydrogen bond network of the "fireman's grip." Here, we show that the sulfhydryl group of cysteine can functionally substitute for this hydroxyl. Interestingly, Risk et al. (1999) recently identified *gag* and *pol* like open reading frames in retrotransposons of the Ty3/gypsy family of *Magnaporthe grisea*, which encode an Asp-Cys-Gly sequence in the position corresponding to the active site of aspartic proteinases. While the catalytic competence of the corresponding proteins has not been analyzed, our results suggest that active aspartic proteinases with a Cys residue in the active site may exist. In fact, the Cys-containing variant of HIV-1 PR exhibited only an ~10-fold lower specific activity compared to the wild-type enzyme when analyzed against a peptide substrate at pH 4.7. Given that such an activity could be sufficient to support polyprotein processing and replication (Rose et al., 1995), one may ask why such sequences have not been found in naturally occurring viruses.

Part of the answer may be the remarkable pH dependence of Gag polyprotein cleavage by PR(T26C). We had previously shown that a Ser-containing variant of HIV-1 PR was pH-sensitive with reduced activity at neutral compared to acidic pH (Konvalinka

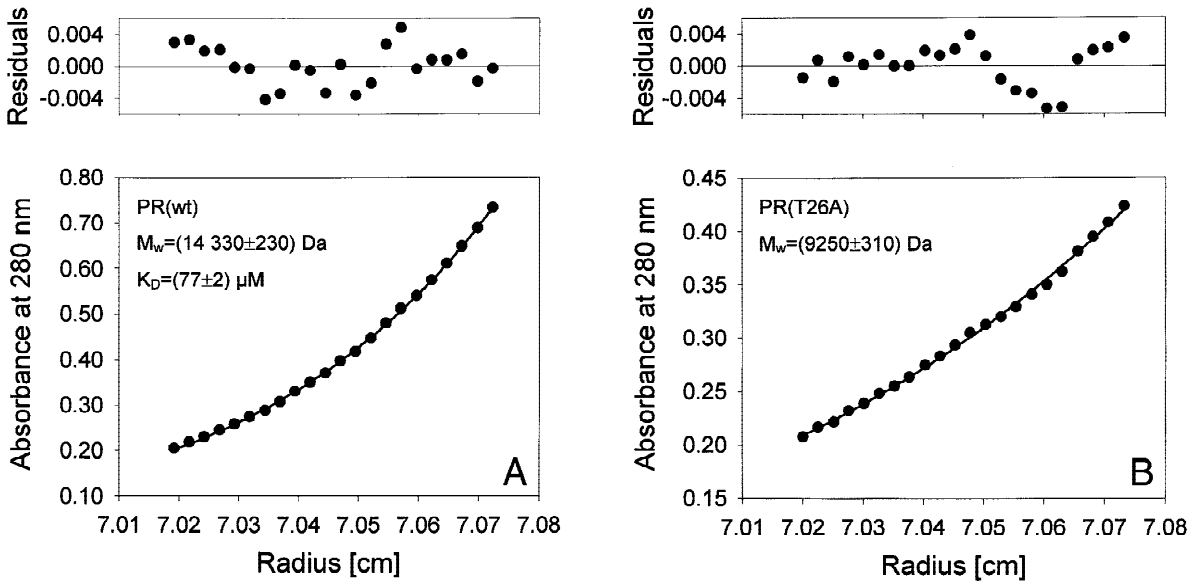

Fig. 6. Analytical ultracentrifugation. The experimental absorbance data (filled circles) plotted as a function of centrifugation radius are shown for **(A)** PR(WT) and **(B)** PR(T26A). The solid curves were obtained by fitting the experimental data to model equations describing single ideal sedimenting species (T26A) or ideal monomer–dimer equilibrium (PR(WT)). The resulting average molecular weight of the sedimenting species and in the case of PR(WT) also the dimer dissociation constant K_D are shown in insets. Data for PR(T26A) do not conform to monomer–dimer equilibrium and hence no K_D value can be computed. The residual differences of observed and fitted data are shown in the top sections of each panel.

et al., 1995b). This result was reproduced in the current study, but a much more significant difference was observed for the Cys-containing variant, which was approximately 10-fold less active at pH 6.8 compared to pH 4.7. Together with the lower specific activity compared to wild-type HIV-1 PR at pH 4.7, this results in approximately 1–2% of wild-type PR activity for PR(T26C) at neutral pH, and no complete cleavage of Gag polyproteins was observed, even at the highest concentration tested. Introduction of this mutation into an HIV-1 expression vector led to virtually complete loss of polyprotein cleavage, while particles containing wild-type PR or the T26S variant exhibited efficient processing to the mature viral proteins. The lower specific activity of the Cys-containing PR can only partially explain this result since the PR concentration in the virion is ~100 μM, while 0.45 μM PR(T26C) yielded partial Gag cleavage in vitro. Conceivably, the activity difference may be even more pronounced at physiological pH or there may be a defect in autocatalytic release of the PR variant from the polyprotein.

Our results indicate that the specific activities of wild-type HIV-1 PR and its covalently linked counterpart are very similar. Comparable results have been reported for the PR of Rous sarcoma virus and its covalently-linked dimers (Bizub et al., 1991). In contrast, a dramatic increase in intracellular polyprotein cleavage and complete loss of particle formation was observed when covalently linked PR dimers were produced as part of the viral Gag-Pol polyproteins (Burstein et al., 1991; Kräusslich, 1991). Most likely, this reflects the requirement for dimerization to produce an active PR: normally, this is only achieved when polyproteins are concentrated within the nascent virion, while a covalently linked dimer on the polyprotein can be active immediately after translation.

Using linked asymmetric PR dimers, we further show that a single hydroxyl group is sufficient for activity and even the 2PR(A/A) variant, which completely lacks the hydroxyl groups forming the "fireman's grip," retained some activity. It is noteworthy that several aspartic proteinases have a combination of DTG and DSG triplets in their two domains (e.g., the proteinases from *Candida albicans*, *Saccharomyces cerevisiae*, *Plasmodium falciparum* (Francis et al., 1994) or the recently identified protease processing the β-amyloid precursor protein, memapsin-2 (Lin et al., 2000; for review see Barrett et al., 1998)), similar to our artificially constructed enzymes. Linked dimers of HIV-1 PR with mutations T26S and T26N in the first PR domain have been reported (Bagossi et al., 1996). Similar to our results, they reported that the T26S-containing heterodimer was almost as active as the wild-type enzyme. In contrast, the T26N-heterodimer exhibited a dramatic loss in activity and the T26N-homodimer was completely inactive. Conceivably, the larger volume of the asparagine side chain and the presence of an additional hydrogen bond donor/acceptor leads to different packing of this side chain in the interior of the molecule and to distortion of the active site structure. It is interesting to note that the specific activities of the wild-type linked dimer and the three linked heterodimers in our study are virtually identical with each other, both at pH 4.7 and at pH 6.8. The specific activity was approximately 5–10-fold lower at the higher pH for all four linked dimers, but there was at most a twofold difference between any two enzymes at either pH. A much more pronounced difference was observed for polyprotein cleavage by the various heterodimers with 2PR(C/T) and 2PR(A/T) being at least 10-fold less active than the wild-type or Ser-containing linked dimer. This difference in cleavage of peptides and polyproteins may be explained by the observation that the rate limiting step in catalysis by HIV-1 PR can differ depending on the substrate. In the case of a substrate containing Glu in the P2′ position (nomenclature according to Schechter and Berger (1967)), a chemical step coupled with proton transfer appears to be rate limiting. When the substrate contained Gln in this position, on the other hand, the rate limiting step was

a conformational change upon substrate binding (Polgar et al., 1994). Most cleavage sites on the polyprotein do not contain Glu in the P2′ position, in contrast to the peptide substrate used in this study, and this difference in substrate properties may be important for cleavage by the various enzymes.

A dramatic difference was observed when the activity of the PR (T26A) variant was compared to its covalently linked counterpart 2PR(A/A). The-50 fold increase in specific activity upon covalent linkage of the two subunits indicates that PR(T26A) has a dimerization defect. Providing a covalent linkage between the C-terminus of the first and N-terminus of the second PR chain apparently increases the probability of "productive" interactions between the two subunits to yield an active enzyme. Based on reversible denaturation (Grant et al., 1992; Szeltner & Polgar, 1996) and structure-based thermodynamic analysis (Todd et al., 1998), it has been suggested previously that HIV-1 PR folding and dimer formation are concomitant processes, i.e., that folded monomers are unstable and stability largely comes from interactions at the dimer interface. Regions of the molecule that substantially contribute to its structural stability have been mapped to residues 22–32 and 84–91 (Todd et al., 1998; Wallqvist et al., 1998). Todd et al. further include the N- and C-termini (1–6, 94–99) and part of the flap region (49–51). NMR ^{1}H and ^{15}N transverse relaxation measurements, on the other hand, demonstrate unexpectedly large fluctuations of the intermonomer β-sheets that are formed by the N- and C-termini and suggest that these are a structural requirement for maturation of PR in vivo (Ishima et al., 1999). Our ultracentrifugation experiments showed that PR(T26A) is virtually completely monomeric, although traces of dimer must be present to explain its residual peptidolytic activity. The residual activity may be due to stabilization of the dimer through active site ligands (substrates, inhibitors). Assuming that some dimers are present in the concentrated preparation, these may be lost upon dilution, but may be stabilized in part by the presence of peptide substrates in kinetic assays. Ultracentrifugation experiments, on the other hand, were performed in the absence of ligand.

These results suggest that the "fireman's grip" interaction is far more important for stabilization of the dimer than would be implicated from the X-ray structure (Wlodawer et al., 1989) and subsequent theoretical thermodynamic analysis (Todd et al., 1998; Wallqvist et al., 1998). The fact that a point mutation along the PR sequence shifts the monomer–dimer equilibrium virtually completely toward monomers implies that this is a dimerization hot spot. Other, nonactive site point mutations (e.g., V82F) can increase the K_d as much as 20-fold (Xie et al., 1999), but the effect of the T26A mutation on K_d is several orders of magnitude larger, corresponding to practically pure monomer. Since folding and dimerisation of retroviral PRs are interdependent (Grant et al., 1992; Szeltner & Polgar, 1996; Todd et al., 1998), it may be speculated that the "fireman's grip" region serves as a folding core of the enzyme.

In conclusion, we provide evidence that the "fireman's grip" structure in retroviral PRs is necessary for efficient dimerization (and thus enzyme activity). We also demonstrate that the hydrogen bond network itself is not indispensable for a single chain aspartic proteinase. We can only speculate why it is still retained in bilobal aspartic proteinases, where no intermolecular dimerization occurs. All aspartic proteinases are suggested to be derived from monomeric chains requiring dimerization and these may have evolved structural features ensuring coordinated folding and dimerization. Once selected for, this feature was retained in all members of the family, irrespective of whether they are dimeric or single-chain, bilobal enzymes.

Materials and methods

Expression plasmids

All mutations were made in a subclone of the full length infectious provirus pNL4-3 (Adachi et al., 1986), containing the *Apa* I to *Pst* I fragment (nt. 2006–2839 of pNL4-3) in a pBluescript vector (Stratagene, La Jolla, California). Site-directed mutagenesis was performed on a single stranded DNA template (Kunkel, 1985). The ACA codon (nt. 2328–2330) was altered to a TCA (Ser), GCC (Ala), or TGT (Cys) codon. In addition, mutant PR (T26A) contained a noncoding nucleotide exchange (nt. 2333 A to C) introducing a new *NgoM* I restriction site. The entire HIV-specific region was sequenced and the respective *Apa* I to *Pst* I fragments were cloned into a subgenomic HIV-1 expression vector (pK-HIV; (Konvalinka et al., 1997)) to give plasmids pK-HIV(T26S), -(T26C), and -(T26A), respectively.

To generate expression vectors for linked dimers containing the T26X mutation in the first chain, the *Bcl* I-fragment of pT7-2PR (Kräusslich, 1991), containing part of the first and second PR chains as well as the linker region, was introduced into the respective pBluescript derivatives. These plasmids were used as templates for PCR reactions using a forward primer specific to the 5′-end of the PR coding sequence with a flanking *Nde* I site and a T7 terminator-specific oligonucleotide as reverse primer. Polymerase chain reaction (PCR) products were cleaved with *Nde* I and *EcoR* I and cloned into the bacterial expression vector pET11c, giving the expression plasmids pET-2PR(X/T), where X corresponds to either Thr (wild-type), Ser, Cys, or Ala. The authenticity of all clones was confirmed by sequencing. Bacterial expression vectors for monomeric PR variants were constructed from the pET-2PR(X/T) plasmids by excising the 318 bp *Bcl* I fragment and religation. These plasmids were designated pET-PR(T26X).

For construction of the tethered homodimer PR(T26A/T26′A), the coding sequence from pET-PR(T26A) was PCR amplified using a 3′ T7 terminator-specific primer and a 5′ primer specific to the first 18 nucleotides of the PR coding region which introduced unique flanking *BamH* I and *Apa* I sites at the 5′ end. The newly generated *Apa* I site upstream of the PR sequence corresponded to the 3′ end of the linker region in pET-2PR. The resulting PCR product was cloned into pUC19 via the *BamH* I and *EcoR* I sites and sequenced. Therefrom, the 302 bp *Apa* I–*EcoR* I fragment corresponding to the second PR chain of a linked dimer was isolated and ligated together with the 1353 bp *Apa* I–*Apa* I and 4,388 bp *Apa* I–*EcoR* I fragments from pET-2PR(A/T). The resulting recombinant clone with the right orientation of the *Apa* I–*Apa* I fragment was designated pET-2PR(A/A).

Bacterial expression and PR purification

All recombinant PRs were overexpressed in the *E. coli* BL21(DE3) strain using the LacI controlled T7 promoter/T7 RNA polymerase system (Studier et al., 1990). Protein expression and isolation of inclusion bodies were carried out using a modification of our published protocol (Konvalinka et al., 1995b). Inclusion bodies were solubilized in 67% (v/v) acetic acid/water at ~5 mg/mL and refolded by dilution into 30-fold excess of water with 0.1% (v/v)

2-mercaptoethanol, overnight dialysis at 4 °C against water and then against 50 mM MES pH 6.7, 10% (v/v) glycerol, 1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol (Cheng et al., 1990). The final protein concentration after refolding was typically about 0.1 mg/mL as determined by Coomassie Blue binding assay (Spector, 1978). The tethered dimers were sufficiently pure for activity studies at this stage. The other PR variants were further purified by cation exchange chromatography using either MonoS FPLC (Pharmacia, Uppsala, Sweden) or SP-Sepharose Fast Flow (Pharmacia). The purity of enzymes used for activity studies was more than 90% as judged from SDS electrophoresis (Laemmli, 1970; Schägger & von Jagow, 1987) and western blotting. The purified enzymes were stored at −20 °C.

Determination of enzyme kinetics

The chromogenic peptide substrate KARVNle*F(NO_2)EANle-NH_2 was synthesized by the solid-phase method and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) (Richards et al., 1990). Peptide cleavage was monitored either online as a decrease in absorbance at 305 nm or offline by analysis of the composition of the reaction mixture by C_{18} RP-HPLC. Measurements requiring substrate concentrations higher than 200 μM were evaluated only by HPLC. The assay conditions were 37 °C, 100 mM buffer (potassium acetate for pH 4.7 and MES for pH 6.8), 1 mM EDTA and 0.3 M NaCl (pH 4.7) or 1.0 M NaCl (pH 6.8). Concentrations of active enzymes were determined by active site titration using the tight-binding inhibitor BocF[CH(OH)CH_2]FQF-NH_2 (compound IIId in Konvalinka et al., 1997). Enzyme concentrations in assays ranged from ~3 to ~80 nM. The initial velocity data were treated by nonlinear regression using the program Enzfitter (Elsevier-Biosoft, UK, 1987).

In vitro cleavage of protein substrates

Cleavage of particle-derived $Pr55^{gag}$ was performed essentially as described previously (Konvalinka et al., 1995a). Immature HIV-like particles released from Sf21 insect cells that had been infected with the recombinant baculovirus BV12myr (Royer et al., 1991) were collected by ultracentrifugation of cleared media through a cushion of 20% (w/w) sucrose and resuspended in phosphate-buffered saline (PBS). Prior to the cleavage reaction, the substrate was diluted 10-fold into 100 mM buffer (sodium acetate pH 4.7 or sodium phosphate pH 6.8), containing 0.3 M NaCl, 2 mM DTT, 1 mM EDTA, and 1% Triton X-100 to remove the viral membrane. Incubations were performed in a total volume of 30 μL at 37 °C for the indicated times and were terminated by adding SDS-gel loading buffer followed by immunoblot analysis.

Cells and transfections

COS 7 cells were maintained in Dulbecco's modified minimal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. For transfections, cells were seeded into 10 cm culture dishes and transfected with 10 μg plasmid DNA using the calcium phosphate method. Cells and culture media were harvested 72 h after transfection. Culture media were cleared by low-speed centrifugation; virus-like particles were harvested by centrifugation at 20,000 × g for 2 h at 4 °C and analyzed by immunoblotting.

Analytical ultracentrifugation

Equilibrium sedimentation experiments were carried out as described by Yphantis (1964) using a Beckman model E analytical ultracentrifuge with spectrophotometric detection. Since the enzyme preparations purified by the above described method showed presence of small molecular weight contaminants, PRs used for analytical ultracentrifugation were purified in denaturing conditions prior to refolding by a modification of a previously published method (Todd et al., 1998). After refolding, proteins were further purified using SP-Sepharose chromatography.

Analytical ultracentrifugation was performed at 20 °C in 20 mM MES buffer pH 6.0, 0.2 M NaCl, 10% glycerol, 1 mM EDTA, and 0.05% (v/v) 2-mercaptoethanol. Initial protein concentration was 0.1–0.2 mg/mL. Rotor speed was 44,000 rpm for PR(T26A) and 48,000 rpm for PR(WT). Data sets were collected after 8 h. Molecular weights of monomers were calculated from the amino acid sequence to be 10,819 for PR(WT) and 10,788 for PR(T26A). Partial specific volume of the solute was calculated as a sum of partial specific volumes of the constituent amino acid residues (Zamyatnin, 1972) yielding a value of 0.748 mL/g. The calculated absorption coefficient at 280 nm was 12,900 $M^{-1}\,cm^{-1}$ for the monomer. Solvent density was estimated to be 1.028 g/mL. Sedimentation equilibrium data were analyzed by nonlinear regression fitting into model equations for ideal single sedimenting species and ideal monomer–dimer association as described in (Holladay & Sophianopoulos, 1972; Shire et al., 1991). The equilibrium constant for dimer dissociation is defined as $K_D = [M]^2/[D]$, where $[M]$ and $[D]$ are molar concentrations of monomers and dimers, respectively, at thermodynamic equilibrium.

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