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Mutagenesis of the dimer interface residues of tethered and untethered HIV-1 protease result in differential activity and suggest multiple mechanisms of compensation

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Abstract

As is the case for all retroviruses, the protease of HIV-1 is only functional as a homodimer; dimerization of two protease monomers results in the formation of the enzyme active site. This dimer structure is supported primarily by interactions between the first four amino-terminal and the last four carboxy-terminal amino acids. These eight amino acids form a β-sheet in which hydrophobic residues are oriented towards the core of the molecule and polar residues are directed towards the solvent. Although the structure of the dimer interface has been determined, the forces that support dimerization have not been fully characterized. Here, we describe a tethered construct in which two protease monomers are joined by a 5 amino acid linker. We evaluate the relative role of each dimer interface residue in functional homo- and heterodimers. Our studies indicate that the hydrophobic residues of the dimer interface are particularly important in maintaining enzyme activity and that enzyme activity is more sensitive to substitutions of the C-terminal amino acids. Further, we demonstrate that the presence of the tether is able to compensate for mutations within the dimer interface that inactivate the enzyme.
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Introduction

Dimerization of the HIV-1 protease is an essential step in viral replication. Enzyme activity requires the formation of protease homodimers (Wlodawer et al., 1989). Mutations that block dimerization interfere with protease function; viral variants encoding non-functional enzymes are aberrantly assembled and are non-infectious (Kaplan et al., 1993; McPhee et al., 1996; Schramm et al., 1993; Zhang et al., 1991).

Crystallographic studies demonstrate that the protease monomers are joined by a total of 34 hydrogen bonds and 4 salt bridges and half of these interactions occur in a region known as the dimer interface (Wlodawer et al., 1989). The dimer interface is comprised of eight interdigitating N- and C-terminal residues on each of the two monomers (residues 1–4 and residues 96–99) in a four-stranded β-sheet arrangement that orients the polar side chains of Q2, T4, T96 and N98 towards solvent. The side chains of P1, I3, L97 and F99 are oriented toward the body of the enzyme and are involved in hydrophobic interactions (Fig. 1). C-terminal residues 96–99 are predicted to be particularly important in dimer formation as they contribute roughly 50% of the inter-subunit ionic and H-bond interactions and 45% of the buried surface area during dimer formation (Weber, 1990). Dimerization results in the formation of an active site pocket. The two catalytic aspartic acids are located in the floor of the pocket and the roof is formed by two flexible flaps that close over the active site (Navia et al., 1989; Wlodawer et al., 1989).

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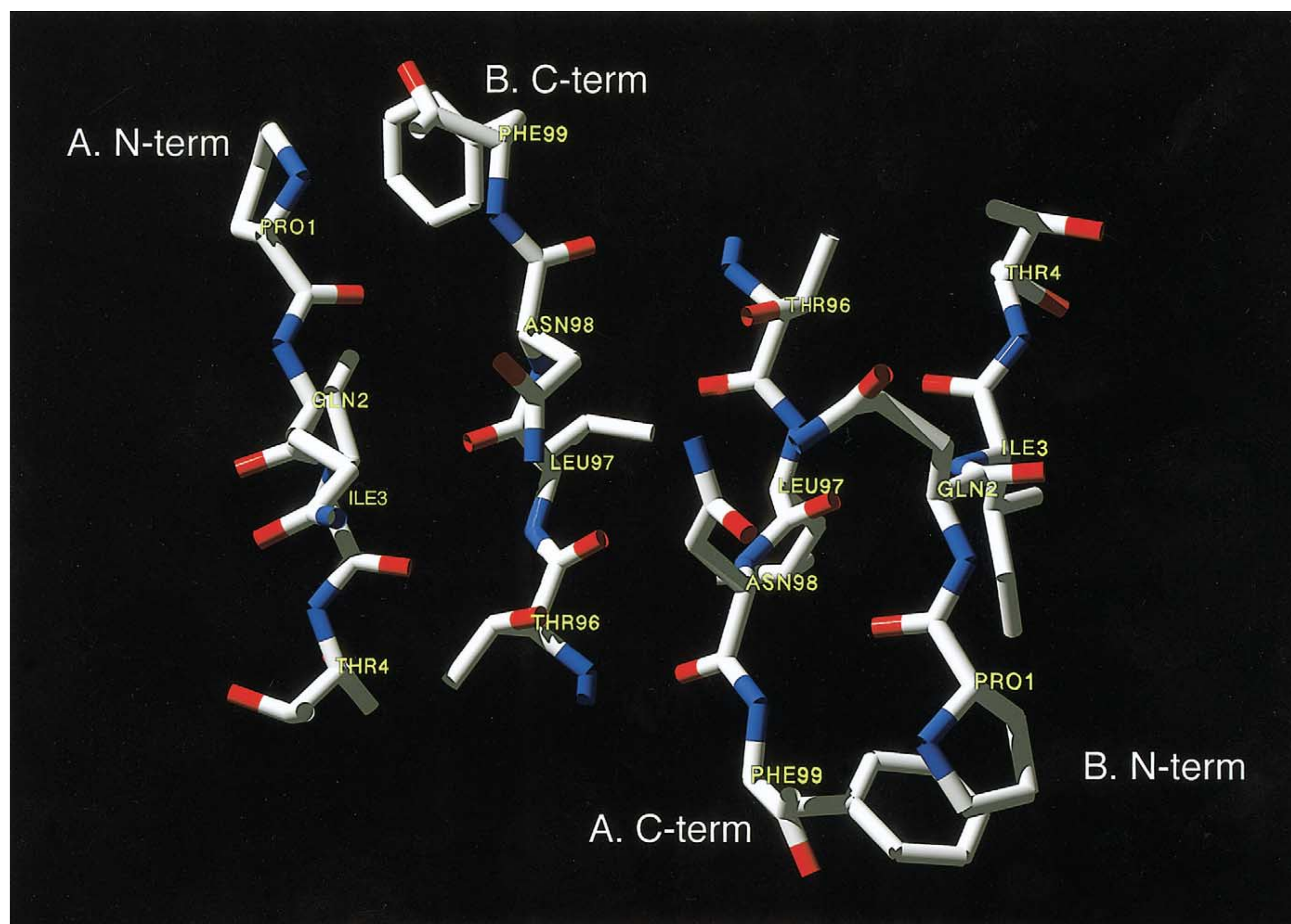

Fig. 1. Stick model of the antiparallel β-sheet arrangement of the dimer interface of HIV-1 protease. Based on the crystal structure of Erickson et al. (1990). Nitrogens are shown as blue; oxygens as red. The side chains of polar residues Q2, T4, T96, and N98 extend into solution; hydrophobic residues P1, I3, L97, and F99 extend away from the viewer towards the body of the enzyme.

Little is known about the individual contributions of dimer interface residues in maintaining enzyme activity. Residues 1–4 and 96–99 are conserved between different HIV-1 isolates. However, there is considerable variation in the sequence of the dimer interface between divergent retroviruses (Blundell et al., 1991; Weber, 1989) despite conservation of the 4 stranded β-sheet structure (Gustchina et al., 1996; Rose et al., 1993b; Vondrasek et al., 1997; Weber, 1990). This observation suggests that a large degree of sequence variation is allowed without disrupting overall structure and activity.

Previous mutational analyses of the HIV-1 dimer interface have been limited in selection and have been primarily restricted to extended protease forms that use protease activation and self processing as a measure of activity. Loeb et al. (1989) found that dimer interface mutations evaluated in the context of a Pol precursor were relatively insensitive to non-conservative substitution with the exception of Q2, L97 and F99 position.

Since the functional enzyme is a homodimer, any substitution introduced into the protease coding domain will be present in both monomers symmetrically. A number of groups have produced “tethered” dimers of the HIV-1 protease in which two monomers are joined C-terminus to N-terminus by a flexible linker of 2–5 amino acids (Babe et al., 1992; Bhat et al., 1995; Cheng et al., 1990; Dilanni et al., 1990; Krausslich, 1991; Krausslich, 1992; Panchal and Hosur, 2000; Tozser et al., 1997). This “head-to-tail” arrangement is based on crystal structures of the native, two-chain dimers which demonstrate that the N- and C-termini of different subunits are located within a few Å of each other. It has been shown that tethered dimers have a wild-type substrate specificity (Bhat et al., 1994; Cheng et al., 1990; Griffiths et al., 1994; Phylip et al., 1995). Crystallographic studies demonstrate that the structure of the single chain dimers is nearly identical to the native, two-chain protease (Bhat et al., 1994; Bhat et al., 1995).

The evaluation of these tethered constructs has furthered our understanding of the relative contribution of each half of the functional enzyme to enzyme structure and function. However, the biochemical assays required to assay enzyme activity are relatively cumbersome and not particularly well

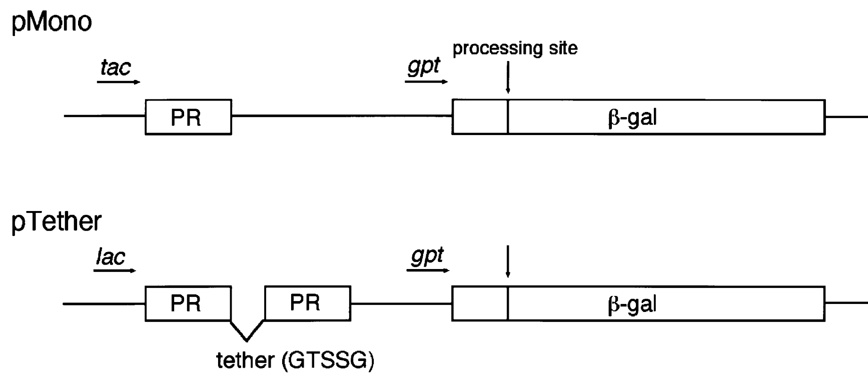

Fig. 2. Schematic of pMono and pTether expression constructs. In both constructs, β-galactosidase containing an inserted HIV-1 protease cleavage site is expressed from the constitutive *gpt* promoter (Baum et al., 1990). Monomeric protease in pMono is expressed from the *tac* promoter. In pTether, the two protease subunits are expressed as a single chain linked by a 5 residue tether (GTSSG) from the *lac* promoter.

suited to the rapid assessment of large numbers of mutants. As part of our evaluation of protease dimer formation, we have modified an expression system that produces a monomeric or single-chain tethered dimers of the protease together with a heterologous β-galactosidase substrate altered by the insertion of an HIV-1 protease cleavage site (Baum et al., 1990). This system provides us with a color screen with which we can rapidly evaluate the phenotype of mutations introduced asymmetrically into one or the other subunit of the dimer. Further, using a monoclonal antibody specific for β-galactosidase, we can estimate the relative activity of these mutations by quantitative Western blot. Our data identify the contribution of individual residues within the dimer interface important for maintaining enzyme activity and suggest important roles for hydrophobic and C-terminal residues of the dimer interface. In addition, our results show that the deleterious effects of mutations within the dimer interface can be compensated by multiple mechanisms. These mechanisms include the presence of the tether, the presence of a wild type monomer, and by second-site phenotypic reversion.

Results

Alanine substitution of each dimer interface residue results in a differental effect on enzyme activity

Baum et al. (1990) have described a system in which monomers of the HIV-1 protease are co-expressed in *E. coli* with β-galactosidase modified by the addition of an HIV-1 protease cleavage site (Fig. 2). These studies demonstrate that expressed monomeric protease undergoes dimerization to cleave a heterologous β-galactosidase substrate modified by the inclusion of an HIV-1 processing site (Baum et al., 1990). The β-galactosidase substrate provides a convenient color screen and a quantitative assay that allows us to evaluate the phenotype of a mutated enzyme. We began our characterization of the role of interface residues in enzyme activity by alanine scanning mutagenesis of the untethered homodimer. As expected, expression of wild-type HIV-1 protease in *E. coli* produced white colonies and fully cleaved β-galactosidase (107 kDa product), whereas blue colonies and full-length β-galactosidase (123 kDa product) resulted from the expression of a protease containing an active site D25N substitution (Fig. 3). Using independent expressions of wild-type or mutant protease, we determined that values derived by quantitative Western blot was reproducible with a standard deviation of no more than 5% cleavage and that the amount of error was independent of the extent of β-galactosidase cleavage (data not shown).

Next we replaced each of the dimer interface residues individually with alanine and assayed the activity of each of the mutants in *E. coli.* The transition from blue to white colonies corresponded to roughly 40–50% cleavage of

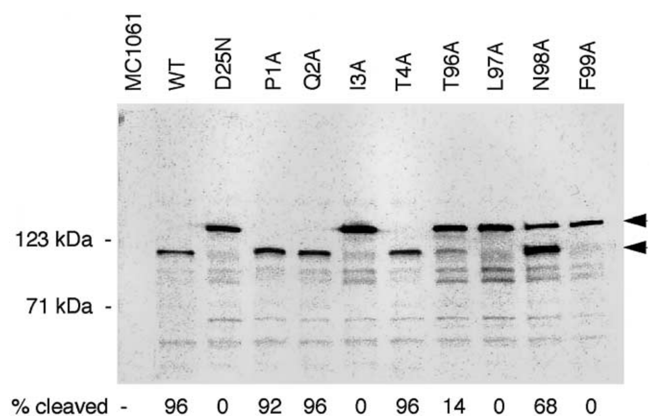

Fig. 3. The effect of single alanine substitutions in the dimer interface of untethered homodimeric protease (pMono) on the cleavage of the β-galactosidase substrate. Lysates from *E. coli* 1061 expressing wild-type (WT) or mutated protease were probed with anti-β-galactosidase antibody as described in Materials and methods. Expression of protease carrying an inactivating D25N substitution of the catalytic aspartate resulted in full length uncleaved 123 kDa β-galactosidase (upper band). Expression of fully active wild-type (WT) protease produced 107 kDa cleaved β-galactosidase (lower band). The percent cleavage of β-galactosidase substrate estimated by quantitative Western blot is shown below each lane.

Table 1
Effect of the tether on the cleavage of β-galactosidase and color screen analysis in homodimeric protease

Mutation	Monomeric			Tethered		
	% Cleavage*	Std dev	Color screen	% Cleavage*	Std dev	Color screen
WT/WT	99%	2.0	white	95%	2.6	white
D25N/D25N	0%	0.0	blue	0%	0.0	blue
1A/1A	98%	4.0	white	91%	3.9	white
2A/2A	99%	2.0	white	90%	3.0	white
3A/3A	0%	0.0	blue	65%	4.8	white
4A/4A	99%	2.0	white	92%	1.4	white
96A/96A	14%	1.3	blue	85%	3.8	white
97A/97A	0%	0.0	blue	10%	7.2	blue
98A/98A	60%	7.9	white	87%	4.6	white
98D/98D	12%	7.0	blue	91%	5.1	white
99A/99A	0%	0.0	blue	37%	10.8	blue
2A3A/2A3A	0%	0.0	blue	45%	8.0	b/w
3A4A/3A4A	0%	0.0	blue	22%	7.0	blue
96A97A/96A97A	0%	0.0	blue	11%	8.6	blue
97A98A/97A98A	0%	0.0	blue	12%	8.1	blue

* Mean of 4–5 replicates.

β-galactosidase as determined by Western blot (Table 1). Alanine substitution of P1, Q2, and T4 resulted in activity near wild-type levels (Fig. 3). However, the substitution of alanine for I3, T96, L97, and F99 resulted in limited cleavage of β-galactosidase as demonstrated by blue bacterial colonies and the persistence of uncleaved β-galactosidase (Table 1 and Fig. 3). Substitutions for N98 were intermediate in phenotype; alanine and aspartic acid substitutions resulted in 68% and 12% cleavage respectively (Table 1). This result indicates that substitutions of individual side chains in the dimer interface can have a dramatic detrimental effect on enzyme activity. The effect of alanine substitution on activity was position dependent; many positions tolerated substitution with little loss of activity. Generally, residues on the amino terminal strand, with the exception of I3, were less sensitive to substitution than residues on the C-terminal strand.

We generated double alanine substitutions to examine the relative importance of the N- and C-terminal strands of the dimer. These substitutions placed an alanine on both the hydrophobic and polar side of the interface (Fig. 1). Paired alanine substitution of 2 and 3, 3 and 4, 96 and 97, and 97 and 98 resulted in profound effects on enzyme function. For each of these strand-specific double substitutions, no detectable cleavage of β-galactosidase was observed (Table 1). This result suggests that while the amino terminal strand is tolerant of single alanine substitutions in 3 of the 4 positions, both N- terminal and C-terminal strands are necessary for protease activity.

Compensation of detrimental interface mutations in the tethered enzyme

We extended our evaluation of dimer interface mutations to the single chain tethered dimer. The plasmid pTether expresses protease with the two subunits linked by a 5 amino acid tether (Fig. 2). To assess the activity of the tethered dimer, we expressed the wild-type tethered construct (wt/wt) together with the modified β-galactosidase. White colonies and near complete cleavage of β-galactosidase (107 kDa cleavage product) was observed by Western analysis, thus demonstrating enzymatic activity of the single chain tethered form of protease (Fig. 4A).

We next compared the effect of the alanine substitution on enzyme activity when expressed as monomeric protease or as a single chain tethered protease. As shown in Table 1, many of the substitutions showed increased activity when expressed in the tethered dimer. The amount of increase in activity was position dependent, with I3A and N98D showing the largest amount of increase. In general, interface substitutions that had the greatest effect on activity when expressed as untethered protease maintained a larger effect when expressed as tethered. Several of the substitutions that displayed no activity when expressed in monomeric form had recovered measurable activity when expressed as a single chain form (e.g., L97A, F99A, and all 4 double substitutions, Table 1 and Fig. 4A). The presence of the tether did not significantly reduce activity in any of the mutations tested. These data indicate that a tether alone can compensate in a position dependent manner for the detrimental effect of dimer interface mutations. In contrast, substitution of the active site catalytic aspartate (D25N/D25N) showed no compensating effect from the tether (Table 1).

Finally, to assess whether the length of the tether effects the amount of compensation we evaluated several of the mutations in a construct in which the tether was lengthened from 5 to 25 amino acids. Our results showed that the longer tether is still able to compensate the effects of substitutions in the dimer to the same degree as the shorter tether (data not shown).

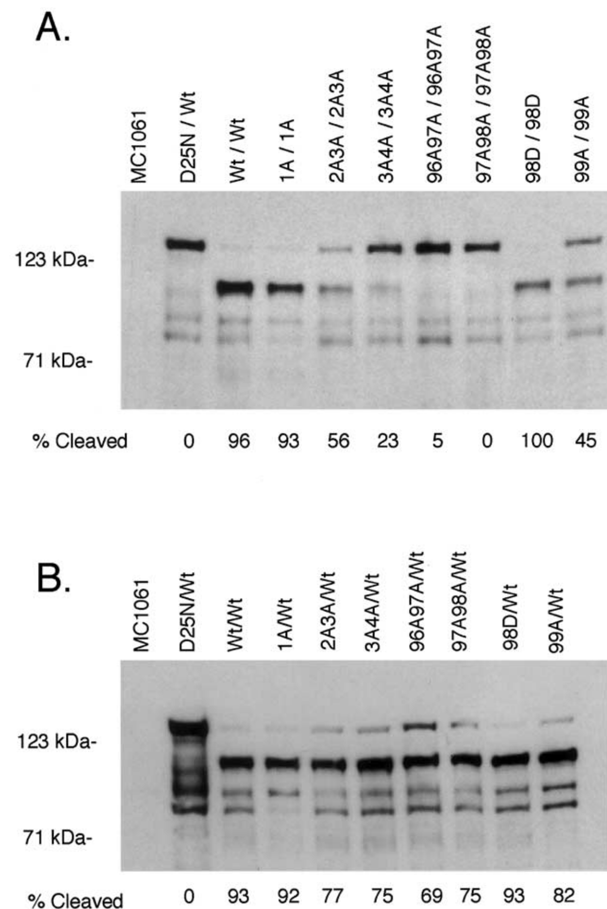

Fig. 4. The effect of interface mutations placed doubly in both subunits or singularly in the 2nd subunit on the activity of tethered protease. Tethered protease was expressed with the β-galactosidase substrate in *E. coli* MC1061 and probed by Western blot with an anti-β-galactosidase antibody. A wild-type subunit is designated Wt. The D25N substitution is a mutant of the catalytic aspartate residue. The percentage of cleaved β-galactosidase substrate is shown below. A. The effect of expressing mutations as a tethered homodimer. B. The effect of the same mutations as a tethered heterodimer with a wild-type (Wt) second monomer.

A wild type subunit in one half of the tethered protease can compensate for mutations in the dimer interface

Previous mutational analyses of the HIV protease dimer interface residues have been limited to an evaluation of the homodimeric enzyme. Although these studies have demonstrated that these residues are important for enzyme function, the phenotype of each mutant enzyme reflected the presence of a double, identical substitution in each subunit. Therefore, to characterize the contribution of individual dimer interface residues to enzyme activity, we introduced substitutions in only one subunit of the tethered dimer.

We found that the presence of a wild-type monomer compensated to some degree for every interface mutation tested (Table 2, compare Fig. 4A to Fig. 4B). However, the amount of compensation by the wild-type subunit varied with position. Single alanine substitutions for L97 and F99 showed the largest degree of compensation (10% to 80%, 34% to 88% cleavage respectively) (Table 2). In general all of the dimer interface mutations tested were able to recover activity to at least 70% cleavage of β-galactosidase when linked to a wild-type monomer. Thus, the compensation of detrimental interface mutation by a wild-type subunit was additive to the compensating effect of the tether. The most dramatic compensation was seen in the L97A and F99A mutations which were inactive when expressed in monomer form, but recovered activity to 80% and 88% cleavage of β-galactosidase when expressed alone in the tethered enzyme (compare Tables 1 and 2). Although we observed compensation of all dimer interface mutations we tested by a wild-type subunit, we observed no compensation of the catalytic D25N mutation. This observation is in agreement with previous studies of enzyme catalysis that two catalytic aspartates are required for enzymatic activity (Dilanni et al., 1990; Jaskolski et al., 1991)

We also examined whether there was position dependence to the placement of the wild-type subunit within the single chain protease. We reversed the order of mutated and wild-type subunits within the tethered protease in the 2A3A, 3A4A, 96A97A, and 97A98A mutations and compared activity (data not shown). We found that the degree of compensation was similar whether the mutation was place first or second subunit. This result indicated that the compensating effect of the wild-type subunit is independent of the position of the subunit.

Second site substitutions within the same subunit can compensate for deleterious interface mutations

These data suggest that a tethered dimer can retain function if the dimer interface of just one of the subunits is intact, even if the other subunit contains destabilizing substitutions in dimer interface residues. Although the presence of wild-type dimer interface residues on one subunit may rescue enzyme function, the mechanism by which this is accomplished is unclear. For example, it is uncertain whether specific side chain interactions between the two monomers are required to maintain enzyme activity or if any properly folded monomer in one position will compensate for an inactivating mutation in the opposite dimer interface.

To characterize the interaction between the dimer interface residues on the two subunits further, we attempted to identify compensatory substitutions that are capable of restoring enzyme activity. Previously, we had determined that a substitution of Gln to Pro at position 2 partially restores the enzyme activity that is lost when the Asn at position 98 is replaced by an Asp (data not shown). A tethered dimer containing the N98D substitution on one subunit and the 96A97A substitution on the other subunit decreased the percentage cleavage of β-galactosidase from 94% to 34% (Fig. 5). However, when the second-site Q2P substitution was added to the subunit containing the N98D mutation, much of the activity of the heterodimer was restored (Fig. 5,

Table 2
Rescue of inactivating mutations by a WT monomer in the tethered protease

Homodimer				Heterodimer			
Mutation	% Cleavage*	Std dev	Color screen	Mutation	% Cleavage	Std dev	Color screen
WT/WT	95%	2.6	white	WT/WT	95%	2.6	white
D25N/D25N	0%	0.0	blue	D25N/WT	0%	0.0	blue
1A/1A	91%	3.9	white	1A/WT	93%	2.6	white
2A/2A	90%	3.0	white	2A/WT	92%	2.9	white
3A/3A	65%	4.8	white	3A/WT	77%	3.0	white
4A/4A	92%	1.4	white	4A/WT	95%	4.4	white
96A/96A	85%	3.8	white	96A/WT	92%	2.5	white
97A/97A	10%	7.2	blue	97A/WT	80%	4.1	white
98A/98A	87%	4.6	white	98A/WT	95%	4.1	white
98D/98D	91%	5.1	white	98D/WT	93%	5.6	white
99A/99A	37%	10.8	blue	99A/WT	86%	3.1	white
2A3A/2A3A	45%	8.0	b/w	2A3A/WT	83%	5.1	white
3A4A/3A4A	22%	7.0	blue	3A4A/WT	75%	4.0	white
96A97A/96A97A	11%	8.6	blue	96A97A/WT	64%	6.9	white
97A98A/97A98A	12%	8.1	blue	97A98A/WT	82%	5.3	white

* Mean of 4–5 replicates.

compare lanes 5 and 6). Thus, the Q2P N98D combination in one tethered protease subunit was able to increase activity of the N98D/ 96A 97A mutant.

Discussion

Several structural analyses have suggested that the residues of the dimer interface play a critical role in maintaining the HIV protease dimer structure. We have extended these studies by comparing the activity of untethered dimers containing symmetric mutations and tethered dimers containing symmetric and asymmetric dimer interface substitutions. Further, we have adapted a bacterial expression system that allows us to screen large numbers of substituted enzymes rapidly and then assess the relative ability of these mutants to cleave an indicator protein. This approach provides new insights into the mechanism and control of protease dimerization.

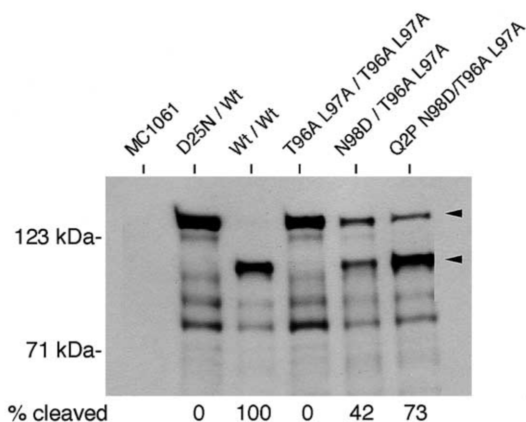

Fig. 5. Increase in activity in tethered N98D/T96A L97A protease by a second site Q2P substitution. Western blot analysis with anti-β-galactosidase antibody is shown. The percent cleavage of the β-galactosidase substrate is shown below.

Our alanine substitution studies of the monomeric protease suggest that allowed side chain substitutions are position dependent within the 8 residues of the dimer interface. Generally, we found that substitution of the hydrophobic residues (I3, L97, F99) produced a greater decrease in activity than the polar residues (Q2, T4, N98). This supports previous suggestions that hydrophobic packing forces play a major role in protease dimerization (Weber, 1990). The polar residues Q2 and N98 have been proposed by structural studies to form interstrand hydrogen bond pairs in the dimer interface (Weber, 1990; Wlodawer et al., 1989). Our results suggest that the hydrogen bonding of polar residues is a minor contribution to dimerization. The position dependent effects of single alanine substitutions reported here correspond well to the energetic contribution of the single residues previously determined by Todd et al. (1998). In particular, Todd et al. indicated that Q2 and T4 provide less Gibbs free energy towards dimerization than other residues of the dimer interface.

We also observed that the C-terminal residues were generally less tolerant of substitution than the N- terminal residues. This finding is in agreement with Weber et al. (1990), who suggested that the C-terminal strand contributes more to dimerization than the amino terminal strand. However, we also find that particular mutations of both strands are able to inhibit enzyme activity completely. Others have found that the amino terminal strand is necessary for enzyme activity. In these studies, deletion of residues 1–5 (Babe et al., 1991) or removal of the same by autolysis (Mildner et al., 1994; Rose et al., 1993a) resulted in an enzyme that was inactive, yet still capable of exerting a trans-dominant negative effect. The conclusion that a func-

tional amino terminal strand is necessary for enzyme function conflicts with the intramolecular processing model proposed by others for the cleavage of the TF/PR site (Co et al., 1994; Louis et al., 1999; Louis et al., 1994; Navia et al., 1989). Our results suggest that dissociation of the amino terminal strand to act as a cis-acting substrate would produce an inactive enzyme. Nevertheless, our observations support previous structural and biochemical analyses of the wild-type tethered protease dimer and suggest that the relative significance of each of the dimer interface residues is maintained, although to a less degree, when the two subunits are held together by the tether.

It seems likely that the tether would promote the formation of a stable dimer by increasing the local concentration of each of the subunits. Our observation that the presence of a tether can compensate for mutations in the enzyme dimer interface supports this reasoning. The compensating effect appears specific to dimer interface mutations since tethered enzymes containing mutations that inactivate one of the active site aspartic acids or interfere with the motion of the enzyme flap on one of the subunits (Tozser et al., 1997) remain inactive in tethered form. In the virus, protease is translated as part of the GagPol precursor (Jacks et al., 1988); thus it is possible that the multimerization of GagPol molecules during virion assembly function to promote dimerization by a mechanism analogous to that of the tether. Others have noted previously that the presence of the tether can promote enzyme stability and dimerization (Cheng et al., 1990; Dilanni et al., 1990), although these studies were performed only on wild-type protease.

One limitation of our evaluation of the effect of dimerization mutants is that our assessment of phenotypes is limited to enzyme activity measurement rather than the actual dissociation of the enzyme. The observation that all detrimental dimer interface mutations tested showed a measurable increase in activity when expressed in tethered form, while the catalytic D25N substitution showed no increase, suggests that dimer interface mutations tested affect dimerization. However, we can not rule out that particular mutations affect catalytic activity as well. One possible example is found in L97 that makes a hydrophobic interaction with T26 neighboring the catalytic aspartate, D25.

Thus, disruption of activity observed with the L97A mutation and others may have a catalytic component as well.

The observations reported here suggest several conclusions. First, in all cases tested, tethered enzymes in which a single subunit contains inactivating mutations in dimer interface residues are functional. Therefore, it seems unlikely that these dimer interface substitutions produce unintended major perturbations in the enzyme active site. Second, the ability of the tether to compensate for mutations that disrupt the dimer interface also indicates that whether the enzyme active site is formed as a result of interactions at the dimer interface or through the action of the tether, the protease retains its function. Finally, our data also demonstrate compensation of deleterious dimer interface mutations can occur by multiple, additive mechanisms.

Materials and methods

Plasmid construction and mutagenesis

Plasmid pMono, which expresses mutant or wild-type monomeric protease under control of the *tac* promoter, was constructed from the plasmid PI + IQ (Baum et al., 1990) as follows: The protease coding domain downstream of the *tac* promoter was deleted by inverse PCR. A linker consisting of 5′ XhoI-XbaI-SalI-PstI 3′ was inserted to produce p1CVx. Mutant or wild type protease sequences were obtained by PCR of pET-PR by primers 5′Xho (AATATA-XhoI-GAAGGAGATATACAT and 3′Xba (ATAAAT-XbaI-CTTGGGCTGCAGGG) and inserted into p1CVx to produce pMono. The plasmid pET-PR contains the 99 residue coding domain of monomeric protease HXB2 isolate (Ratner et al., 1987) inserted behind an initiating methionine into the NdeI-BamHI sites of pET24a (Novagen).

Plasmid pTether was constructed by insertion of a complimentary oligonucleotide linker encoding 5′ XhoI-Gly-Thr-Ser-Ser-Ser-XbaI 3′ into the XhoI-XbaI site of p1CVx to create p1CVspe. The linker contained a unique SpeI site overlapping the codons for Gly-Thr for insertion of the 1st and 2nd protease monomers. Wild-type or mutant protease sequences encoding the first protease monomer were obtained from pET-PR by PCR with primers 5′Xho and 3′Spe (5′AATATA-SpeI-ACCAAAAATTAAAGTGCAACC). Insertion of the 1st monomer sequences into p1CVspe created pPR1spe. Insertion of the 2nd protease domain into pPR1spe was performed by PCR of pET-PR with primers 5′Spe (TATTAT-SpeI-TCTGGGCCTCAGATCACTCTT-TGG) and 3′Xba.

To limit cell toxicity, the *tac* promoter (de Boer et al., 1983) in pTether was mutated to a wild-type *lac* promoter by substitution of positions within the −35 and −10 regions as follows: −35: TTGACA to TTTACA, −10: TATAATG to TATGTTG. Mutagenesis was performed on the p1CVx parental plasmid by the QuikChange mutagenesis protocol according to the directions of the manufacturer (Stratagene). pTether25 was constructed by insertion of complimentary oligonucleotides encoding four additional translationally-optimized (CUTG-Codon Usage Tabulated from Genbank) repeats of Gly-Thr-Ser-Ser-Gly into the SpeI site of pTether.

Mutagenesis of the protease coding domain in pET-PR was performed as previously described using uracil-substituted single-stranded templates (Bebenek and Kunkel, 1989; Kunkel, et al., 1991). Mutations were confirmed by DNA sequencing prior to transfer of the protease sequences in pMono or pTether constructs. For construction of a library of possible 2nd site mutations in the pTether 98D/96A97A construct, site-directed mutagenesis was per-

formed as described above on pETPR 98D with random oligonucleotides degenerate for either the Q2, I3, or T4 codons prior to insertion of the first monomer into pTether WT/96A97A. Possible phenotypic revertants were screened for the presence of light blue or white color on X-gal indicator plates as described below. Following screening, the presence of a 2nd site mutation was confirmed by sequencing prior to Western blot determination of the percent of β-galactosidase cleaved (described below).

Colony color-screen analysis for β-galactosidase activity

The plasmid pMono or pTether was transformed into *E. coli* MC1061 cells and plated on LB with 100 μg/ml ampicillin. Colonies were replica plated onto LB supplemented with 100 μg/ml ampicillin, 100 μg/ml X-gal, and either 100 μg/ml isopropyl β-D-thiogalactopyranoside (IPTG) (pMono) or 30 μg/ml IPTG (pTether). Replica plates were incubated at 37°C for 2–3 h until color developed.

Western blot determination of the percent of β-galactosidase cleavage

Samples for Western blot analysis were collected from uninduced mid-log (OD_{600} 0.5) cultures of E. coli strain MC1061 carrying either pMono or pTether grown in YT media. Cells were pelleted and resuspended in 400 μl X (OD_{600}) of SDS–PAGE sample buffer (Pettit et al., 1991) heated to 95°C for 4 min, and clarified by centrifugation. SDS polyacrylaminde gel electrophoresis (SDS–PAGE) was performed on standard 7.5% Laemmli gels (Laemmli, 1970). Separated proteins were transferred to nitrocellulose by electroblot, blocked for 1 h with 3% bovine serum albumin in Tris buffered saline–0.1% Tween 20 (TBS-tw) (10 mM Tris pH 8.0, 8% w/v NaCL) and probed by anti-β-galactosidase monoclonal antibody (Boehringer) at a 1:5000 dilution. Following subsequent washes in TBS-tw, β-galactosidase was detected with the ECL Plus system followed by autoradiography according to the instructions of the manufacturer (Amersham Pharmacia).

Densitometric analysis of the cleavage of β-galactosidase was performed by placing blots directly on a Molecular Dynamics Storm model 800 phosphoimager. For scanning, the phosphoimager was set in blue fluorescence/chemifluorescence mode at a PMT voltage of 700 V. Determination of the final value for the percent cleavage of the β-galactosidase substrate was determined by taking the average value of 2 or more independent inductions. We found that the value of the percentage of β-galactosidase cleavage varied by 5% cleavage or less between duplicate inductions in the Western blot and that the amount of variation was independent of the extent of β-galactosidase cleavage (data not shown).

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