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# Catalytic contribution of flap–substrate hydrogen bonds in "HIV-1 protease" explored by chemical synthesis

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**ABSTRACT** **An analogue of "HIV-1 protease" was designed in which the ability to donate important water-mediated hydrogen bonds to substrate was precisely and directly deleted. Chemical ligation of unprotected peptide segments was used to synthesize this "backbone-engineered" enzyme. The functionally relevant amide -CONH- linkage between residues Gly$^{49}$-Ile$^{50}$ in each flap of the enzyme was replaced by an isosteric thioester -COS- bond. The backbone-engineered enzyme had normal substrate specificity and affinity ($K_m$). However, the catalytic activity ($k_{cat}$) was reduced ≈3000-fold compared to the native amide bond-containing enzyme. Inhibition by the reduced peptide bond substrate analogue MVT-101 was unaffected compared with native enzyme. By contrast, the normally tight-binding hydroxyethylamine inhibitor JG-365 bound to the backbone-engineered enzyme with an ≈2500-fold reduction in affinity. The reduced catalytic activity of the -Gly$^{49}$-ψ(COS)-Ile$^{50}$- backbone-engineered enzyme analogue provides direct experimental evidence to support the suggestion that backbone hydrogen bonds from the enzyme flaps to the substrate are important for the catalytic function of the HIV-1 protease.**

The mature proteins packaged within virions of the human immunodeficiency virus (HIV) are initially translated as the large precursor polyproteins gag and gag–pol. The role of the aspartic proteinase from HIV-1 ("HIV-1 protease") is to proteolytically process these translation products to give functional core proteins and the enzymes required for viral replication (1).

In contrast to the single-chain nonviral aspartic proteinases such as pepsin, the HIV-1 protease has been characterized as a homodimeric molecule (2). The monomer of the HIV-1 protease consists of 99 amino acid residues, and each monomer contributes one of the two conserved active-site aspartyl residues. X-ray crystallography has revealed the HIV-1 protease to be structurally related to the larger two-domain pepsin-like aspartic proteinases (2–4). Despite the difference in quaternary structure, the similarity between the two types of aspartic proteinases is immediately obvious upon superposition of the molecular structures (2).

An important structural feature of the aspartic proteinases is the β-hairpin region known as the "flap". The flap is a mobile surface feature, characterized by high thermal factors in the crystal structures of the free enzyme (4). The homodimeric HIV-1 protease molecule possesses two flaps, in contrast to the single flap of the pepsin-like proteinases (2). Upon binding inhibitor (and presumably substrate), the flap(s) undergoes movement to interact with and lock the substrate into the active site, simultaneously desolvating the enzyme-bound ligand (4, 5).

Potentially the most significant and intriguing difference between the HIV-1 protease and nonviral aspartic proteinases is in the mode of interaction of the tip of the flap(s) with the inhibitor. In crystal structures of both types of aspartic proteinases, hydrogen bonds are observed between the protein backbone near the tip of the flap(s) and the carbonyl oxygens flanking the pseudoscissile bond of bound, substrate-based peptide inhibitor. These flap–substrate hydrogen bonds have been implicated in an out-of-plane distortion of the scissile amide bond, thereby facilitating attack by a nucleophilic water molecule (6–9). However, while the amide -NH- of peptide bonds near the tip of the single flap in pepsin-like proteinases donate hydrogen bonds *directly* to the substrate (6, 10), the corresponding hydrogen bonds in the HIV-1 protease are contributed one from each flap and appear to be *mediated* by a specific, tetrahedrally coordinated internal water molecule, water 301 (5), poised between the flaps and the inhibitor (Fig. 1*A*). This water molecule (which is not the nucleophilic water) has been observed in all HIV-1 protease enzyme–inhibitor cocrystal structures so far reported (11). This raises the question, are the water-mediated hydrogen bonds from the flaps to substrate/inhibitor important to the enzyme activity of the HIV-1 protease?

We sought to experimentally address the role of the hydrogen bonds between enzyme flaps and the substrate in the mode of action of the HIV-1 protease. An enzyme analogue was designed in which the protein backbone would lack the ability to donate the flap hydrogen bonds observed in the x-ray crystal structure (5, 7, 8). A sulfur atom specifically substitutes for an -NH- in a single peptide bond in the backbone of each flap (Fig. 1*B*). This could be done with surgical precision using the chemical dovetailing approach (13), which creates a backbone analogue structure at the ligation site. This report describes the synthesis and enzymatic properties of a "backbone-engineered" analogue of the HIV-1 protease, in which the functionally relevant amide bond between residues Gly$^{49}$ and Ile$^{50}$ has been replaced by a thioester linkage.

## MATERIALS AND METHODS

**Bromomethylvaleric Acid.** (2*R*,3*S*)-2-Bromo-3-methylvaleric acid (BrMV) was synthesized from D-allo-isoleucine [i.e., (2*R*,3*S*)-2-amino-3-methylvaleric acid] by *in situ* formation of the diazonium salt in the presence of bromide ion (14, 15). The crude reaction product was purified by reverse-phase HPLC, and the identity of this product was confirmed by $^1$H NMR. The stereochemistry and chiral purity of this compound was verified by synthesis of its diastereomer (2*S*,3*S*)-2-bromo-3-methylvaleric acid from L-isoleucine and comparison of their $^1$H NMR spectra. Within the limits of $^1$H NMR sensitivity, BrMV was chirally pure.

**Peptide Synthesis.** HIV-1 protease (SF2 strain) segments 1–49 and 51–99 were synthesized as described elsewhere (13,

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Abbreviations: HIV, human immunodeficiency virus; BrMV, (2*R*,3*S*)-2-bromo-3-methylvaleric acid; Boc, *t*-butoxycarbonyl; Aba, L-α-amino-*n*-butyric acid.

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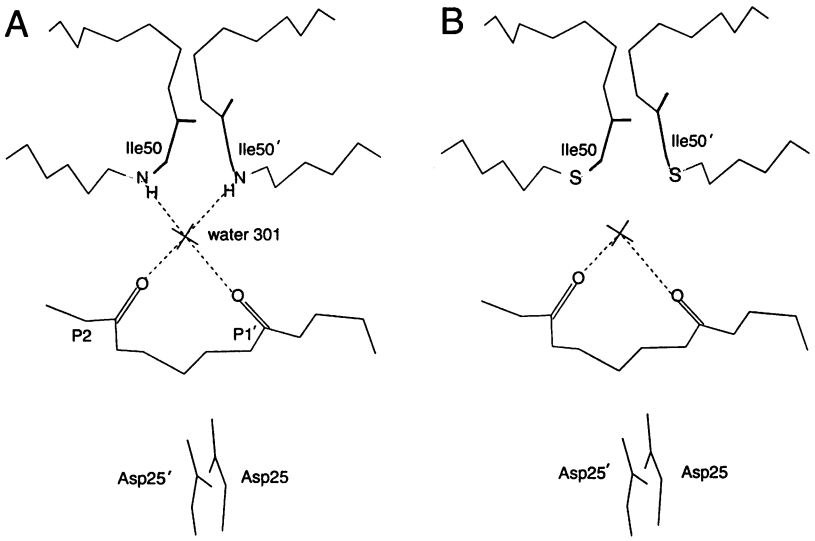



FIG. 1. Hydrogen-bonding interactions between the flap regions of the HIV-1 protease and a substrate-based inhibitor. (*A*) Hydrogen bonds from the enzyme backbone $Ile^{50}$ -NH- in each flap to the inhibitor $P_2$ and $P_{1'}$ carbonyls [notation of Schecter and Berger (12)] on either side of the isostere of the substrate scissile bond. The hydrogen bonds are mediated by a specific, tetrahedrally coordinated water (water 301) observed in all HIV-1 protease–inhibitor complexes. The data were taken from the x-ray crystal structure of synthetic HIV-1 protease bound to the reduced isostere hexapeptide inhibitor MVT-101 (5). (*B*) Design of a backbone-engineered HIV-1 protease. This shows a hypothetical structure in which the single pertinent -CONH- in each flap has been converted to a -COS-, thus removing the ability to donate a hydrogen bond. Water 301 is modeled as hydrogen bonded only to the substrate-based inhibitor. [Note that in HIV-1 protease the scissile peptide bond is attacked by a water molecule (*not* water 301) located between the $Asp^{25}$ and $Asp^{25'}$ side chains.]

16). The 51–99 segment contained L-$\alpha$-amino-*n*-butyric acid (Aba) as an isosteric replacement for cysteine at positions 67 and 95 (2). BrMV was coupled as the preformed symmetric anhydride (17) to the (51–99)HIV-1 protease segment, after first removing the His(dinitrophenyl) and $N^{\alpha}$-*t*-butoxycarbonyl (Boc) protecting groups from the resin-bound peptide (16). Removal of remaining side chain protecting groups and cleavage from the resin was effected by treatment with hydrogen fluoride (16) to yield BrMV (51–99)HIV-1 protease. The peptide segment 1–49 was synthesized on a Boc-Gly-S-CH(phenyl)phenoxyacetamidomethyl resin (18). Cleavage and deprotection to yield the desired (1–49)-$\alpha$COSH segment was similarly performed after prior removal of the $N^{\alpha}$-Boc group.

Each peptide segment was purified by reverse-phase HPLC using linear gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid. Fractions collected were combined based on analysis by isocratic HPLC and ion-spray MS. The covalent structures of the purified peptides were confirmed by MS: BrMV (51–99)HIV-1 protease, observed molecular mass = 5405.0 ± 1.0 Da (calculated: monoisotopic = 5402.9 Da; average = 5407.2 Da); (1–49)-$\alpha$COSH HIV-1 protease, observed molecular mass = 5442.4 ± 1.4 Da (calculated: monoisotopic = 5441.9 Da; average = 5445.5 Da).

**Chemical Ligation.** Attempts to ligate the (1–49)-$\alpha$COSH and BrMV(51–99) HIV-1 protease segments under the aqueous reaction conditions used by Schnölzer and Kent (13) gave no detectable product. This was attributed to reduced reactivity of the $\beta$-branched secondary alkyl halide BrMV versus the simple bromoacetyl group (19). Modest yields of the ligation product [[$NHCH_2COSCH(^{i}Bu)CO]^{49-50}$, $Aba^{67,95}$] HIV-1 protease, hereafter referred to as [$(COS)^{49-50}$]HIV-1 protease monomer, were obtained by reacting the two peptide segments in suspension in dimethylformamide containing 5% 1 M aqueous sodium phosphate (pH 3.5) at room temperature. The concentration of each peptide segment was ≈0.5 mg/ml, and although the peptides were not fully soluble in this solvent, a product yield of ≈10% was obtained after 36 hr of reaction. The desired [$(COS)^{49-50}$]HIV-1 protease monomer ligation product was purified by reverse-phase HPLC using a virgin column to eliminate possible contamination by native amide enzyme.

Guanidine hydrochloride was added to the pooled HPLC fractions, and the solution was dialyzed against decreasing concentrations of guanidine hydrochloride in dialysis buffer (25 mM Mes, pH 5.5/20% glycerol). The concentration of the folded protein was determined by absorbance at 280 nm of a dilute sample in 6 M guanidine hydrochloride using a molar extinction coefficient of 25,000 $M^{-1}\cdot cm^{-1}$ (20). Typically, 0.35 mg of folded enzyme analogue was obtained, reflecting a yield of 4.5% based on the starting peptide segments. Folded enzyme was stored in dialysis buffer at 4°C, and under these conditions, no significant autolytic or chemical degradation of the enzyme was observed after a period of 4 weeks.

The molecular mass of folded [$(COS)^{49-50}$]HIV-1 protease dimer (hereafter referred to as backbone-engineered HIV-1 PR) was investigated by gel filtration using a Pharmacia Superdex 75 column, running at 0.5 ml/min with 100 mM NaOAc/0.5 M NaCl, pH 5.3, as the mobile phase. The concentration of enzyme as loaded onto the column was 0.4 $\mu$M, and protein peaks were detected by fluorescence at 335 nm (excitation 280 nm). The observed molecular mass of backbone-engineered HIV-1 PR was determined from a plot of log(molar mass) versus retention time, calibrated by coinjection of reference proteins.

**Enzymatic Properties.** Comparison of the substrate specificity of backbone-engineered HIV-1 PR with that of "native amide" enzyme† was assessed by the cleavage of two synthetic peptides spanning the p17/p24 and p24/p15 cleavage sites in the viral gag–pol protein (22). The p17/p24 peptide (500 $\mu$M) was incubated with backbone-engineered HIV-1 PR (35 nM) at 37°C in a pH 5.5 assay buffer containing 50 mM HOAc, 50 mM Mes, 100 mM Tris, 10% glycerol, and bovine serum albumin at 0.5 mg/ml, with the ionic strength adjusted to 1.0 by the addition of NaCl. Formation of cleavage products over several days was followed by HPLC, and their identity was confirmed by MS. Cleavage of the p24/p15 peptide (250 $\mu$M) by the backbone-engineered HIV-1 PR (70 nM) was similarly assessed. The backbone-engineered HIV-1 PR itself showed no breakdown when stored in assay buffer under the conditions used in these studies.

Kinetic parameters were determined for cleavage of the fluorogenic substrate (23) Abz-Thr-Ile-Nle-Phe($NO_2$)-Gln-Arg-amide [Nle = norleucine, Abz = anthranilic acid, Phe($NO_2$) = 4-nitrophenylalanine] by backbone-engineered HIV-1 PR. Assays were performed at 37°C in the same buffer described above. Cleavage products were quantitated by HPLC with peak detection by either fluorescence or UV absorption. Initial rates were determined by single time points of reactions in which consumption of substrate was <20% of the starting concentration (60- to 90-min reaction

†References made in the text to native amide enzyme refer to synthetic [$Aba^{67,95}$]HIV-1 protease dimer (folded homodimer), which is the form of the HIV-1 protease used to generate the original correct structural data for the viral enzyme (2, 5, 7, 8). Subsequent studies with recombinant cysteine-containing enzyme yielded indistinguishable results (21).

time). Kinetic parameters were obtained by fitting data to the Michaelis–Menten equation using a nonlinear regression computer program (24).

Inhibition of backbone-engineered HIV-1 PR by Ac-Thr-Ile-Nle-$\psi$($CH_2NH$)-Nle-Gln-Arg-amide (MVT-101) and Ac-Ser-Leu-Asn-Phe-$\psi$[$CH(OH)CH_2N$]-Pro-Ile-Val-OH (desmethyl-JG-365, *S* isomer), well-characterized competitive inhibitors of the native enzyme (5, 7), was assessed in the chromatographic assay as above, at a fixed substrate concentration of 50 $\mu$M. $IC_{50}$ values were determined by least-squares analysis using Dixon plots (25), and these values were converted into inhibitor dissociation constants ($K_i$) by use of the expression $K_i = IC_{50}/(1 + [S]/K_m)$.

## RESULTS AND DISCUSSION

The basic chemical dovetailing strategy followed for the preparation of a polypeptide chain with a backbone thioester at a single defined site is that of Schnölzer and Kent (13). Two unprotected peptide segments, one containing a C-terminal $\alpha$-thiocarboxylic acid and the other an N-terminal $\alpha$-bromoacyl residue, were chemoselectively ligated via a nucleophilic substitution reaction to generate the thioester moiety (Fig. 2). Nucleophilic displacement of bromide from an N-terminal $\alpha$-bromoacyl residue proceeds via an $S_N2$ mechanism and results in the inversion of stereochemical configuration at the reaction center (15). Thus the initial stereochemistry at the $\alpha$ carbon of BrMV corresponded to that of a D-amino acid and gave the same configuration as an L-amino acid after ligation (Fig. 2). The stereochemistry of the $\beta$ carbon in the BrMV residue was unaffected by the ligation reaction and remains equivalent to that of the $\beta$ carbon in L-isoleucine. Ligation of the two peptide segments led to modest yields of the target molecule, and subsequent purification by HPLC followed by folding of the synthetic monomer gave enzymatically active homodimer.

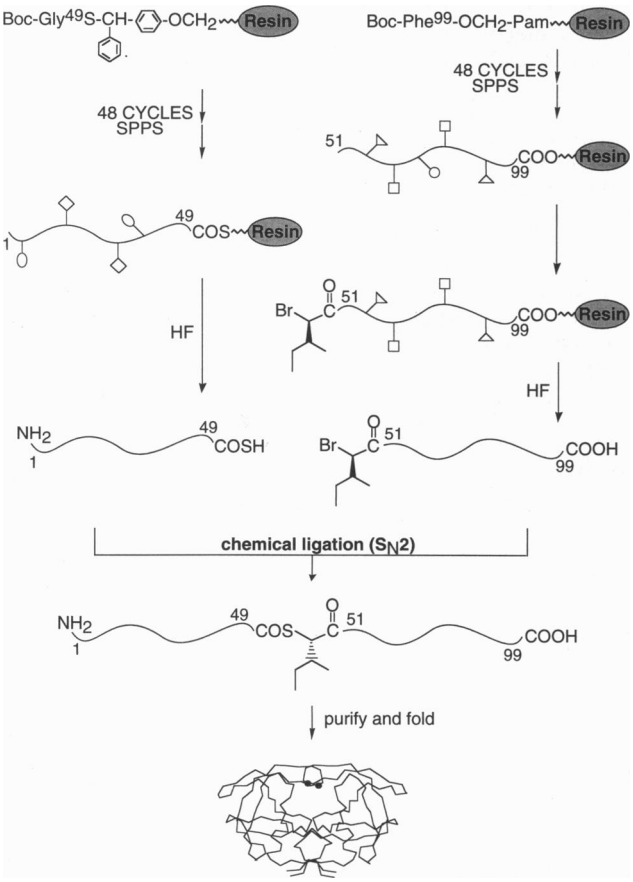



FIG. 2. Total chemical synthesis of the backbone-engineered HIV-1 PR. The unprotected HIV-1 protease peptide segments (1–49)-$\alpha$COSH and BrMV(51–99) were joined by chemical ligation, to give the [(COS)$^{49-50}$]HIV-1 protease monomer 99-residue polypeptide chain. Inversion of stereochemistry at the residue 51 $\alpha$ carbon in the $S_N2$ ligation reaction gave the desired (2*S*,3*S*) configuration. After purification by reverse-phase HPLC, the homodimeric folded enzyme analogue was generated by slow dialysis of the ligated monomer from denaturing conditions into pH 5.5 buffer. The peptide segments were prepared by Boc chemistry stepwise solid-phase synthesis (16) and purified before ligation.

The homogeneity and covalent structure of the synthetic [(COS)$^{49-50}$]HIV-1 protease monomer were confirmed by analytical HPLC and MS: observed molecular mass = 10,769 Da (calculated = 10,771 Da, average isotope composition). The molecular mass of the folded backbone-engineered HIV-1 PR, as determined by gel filtration under native conditions, was found to be consistent with that of a homodimer (observed molecular mass = 21.5 ± 0.5 kDa; calculated = 21.5 kDa). At the concentration of enzyme used (0.4 $\mu$M), there was no detectable peak corresponding to monomeric protein (detection limit ≤ 5 mol %). Injection of an identical amount of folded native amide enzyme gave a peak of the same area at the same elution time. This is strong evidence that the backbone-engineered [(COS)$^{49-50}$]HIV-1 PR has folded to a native tight homodimeric three-dimensional structure.

Enzymatic activity and substrate specificity of backbone-engineered HIV-1 PR were confirmed by cleavage of a fluorogenic substrate (23), as well as by cleavage of known synthetic peptide analogues of the p17/p24 and p24/p15 junctions in the viral gag–pol polyprotein (26). As anticipated, the p17/p24 peptide was cleaved at the Tyr-Pro site, whereas the p24/p15 peptide was cleaved at the expected Met-Met and Leu-Ala sites (Fig. 3). No additional cleavage sites were observed in any of these peptides upon prolonged incubation for several days. These cleavages showed normal characteristics of an enzyme-catalyzed reaction and were at the identical sites for native amide enzyme (26) and for the processing observed in virus maturation *in vivo* (1). The backbone-engineered protein is thus an enzyme with the same substrate specificity as native enzyme.

Calculation of the kinetic parameters for cleavage of fluorogenic substrate by the backbone-engineered HIV-1 PR (Fig. 3) revealed an ≈3000-fold reduction in the $k_{cat}$ [(8.6 ± 0.3) × $10^{-3}$ $s^{-1}$], but an essentially unchanged $K_m$ value (5.3 ± 0.4 $\mu$M), when compared to the kinetic parameters for native amide enzyme assayed under the same conditions: $k_{cat}$ = 25 $s^{-1}$, $K_m$ = 8 $\mu$M (S.B.H.K., P. Alewood, and D. Bergman, unpublished work). Furthermore, an enzyme analogue containing a thioester bond in another region of the flap had normal kinetic parameters (13), ruling out general effects from altered backbone flexibility in this sensitive region. These results show that hydrogen bonds from the backbone of the flaps, at the Gly$^{49}$-Ile$^{50}$ peptide bond, to the $P_2$ and $P_{1'}$ carbonyls of the substrate are essential for normal turnover of substrate under steady-state conditions. However, they do not contribute to the binding of substrate, as measured by the $K_m$ values.

Interestingly, the observed reduction in activity of the backbone-engineered enzyme (≈3000-fold) corresponds to an increase of ≈5 kcal/mol in the activation energy for enzyme-catalyzed substrate hydrolysis. This is consistent with the average binding energy that could be contributed by two hydrogen bonds in a protein–ligand complex (27). This analysis assumes that all other interactions between enzyme and substrate are maintained as in the native enzyme–substrate complex. Given the exquisite precision of the single atom replacement in each flap in the backbone-engineered enzyme analogue, this seems reasonable. Nonetheless, that

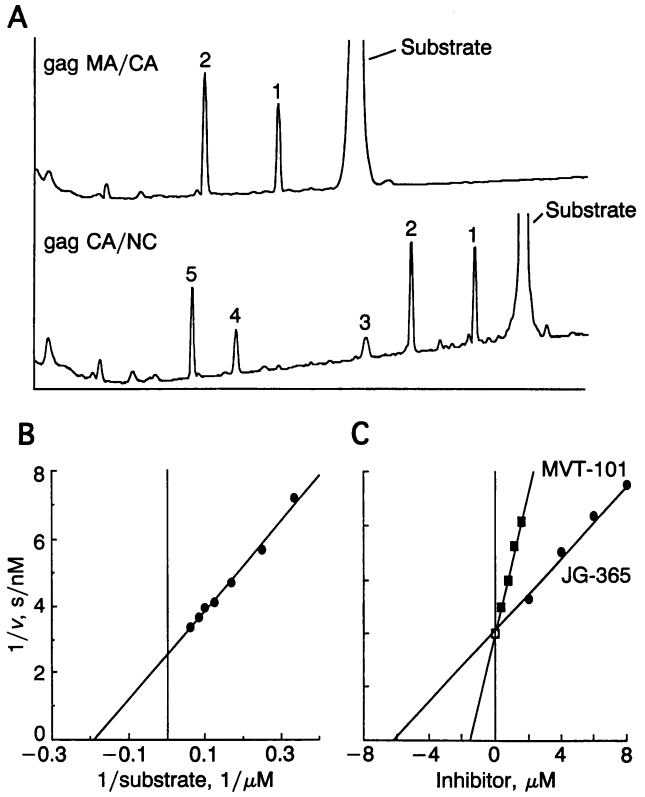
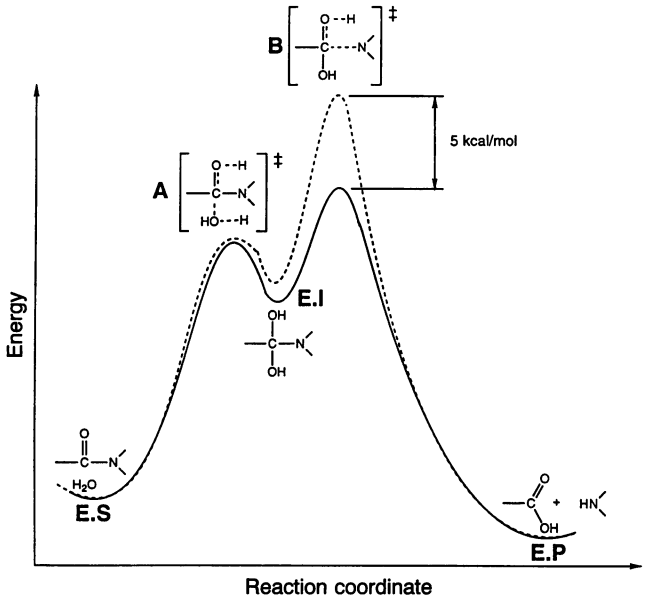



FIG. 3. Enzymatic activity of folded backbone-engineered HIV-1 PR. (*A*) Substrate specificity: analytical reverse-phase HPLC assays, with UV detection. (*Upper*) The peptide RRSNQVSQNY~PIVQNIQGRR, a synthetic analogue of the matrix/capsid (MA/CA) processing site in the HIV-1 *gag* translation product was progressively cleaved over 72 hr at pH 5.5 by the analogue enzyme to give the fragments PIVQNIQGRR (peak 1) and RRSNQVSQNY (peak 2), resulting from specific cleavage at the Tyr-Pro peptide bond. (*Lower*) Similarly, the peptide GHKARVL~AEAMSQVTNSATIM~MQRGNFRNQRK, a synthetic analogue of the HIV-1 capsid–nucleocapsid (CA/NC) processing sites, was progressively cleaved over 72 hr at pH 5.5 to yield GHKARVLAEAMSQVTNSATIM (peak 1), AEAMSQVTNSATIMMQRGNFRNQRK (peak 2), AEAMSQVTNSATIM (peak 3), GHKARVL (peak 4), and MQRGNFRNQRK (peak 5). These fragments result from specific cleavage at the Leu-Ala and Met-Met peptide bonds. For both substrates, no other cleavages were observed. All cleavages were identical to those observed with native amide HIV-1 protease and were the same as the processing events in viral maturation *in vivo*. (*B*) Substrate kinetics: determination of steady-state kinetic parameters for the action of backbone-engineered HIV-1 PR on the fluorogenic substrate Abz-Thr-Ile-Nle~Phe($NO_2$)-Gln-Arg-amide. Initial velocities were measured at different concentrations of substrate. A double-reciprocal plot yielded $k_{cat} = 8.6 \times 10^{-3}$ $sec^{-1}$ and $K_m = 5.3$ μM. (*C*) Inhibition: determination of inhibitor affinities for the backbone-engineered HIV-1 PR from steady-state kinetic measurements as a function of inhibitor concentration. $IC_{50}$ values were converted to $K_i$ values. Derived values were $K_i = 140$ nM for MVT-101 and $K_i = 590$ nM for desmethyl-JG-365.

the reduction in activity is not a consequence of some unanticipated more drastic perturbation in structure awaits verification by crystallography or other direct means.

Inhibition (Fig. 3) by the reduced amide bond peptide inhibitor MVT-101 was found to be similar for the backbone-engineered HIV-1 PR ($K_i = 140$ nM) and the native amide enzyme [$K_i = 780$ nM (5)]. By contrast, the potency of the hydroxyethylamine peptide inhibitor JG-365 against the backbone-engineered enzyme is dramatically reduced. While JG-365 is a tight-binding inhibitor of the native amide HIV-1 protease with an inhibition constant of 0.24 nM (7, 28), the $K_i$ for inhibition of backbone-engineered HIV-1 PR was found to




| Inhibitor peptide bond isostere | Transition state mimicked |
|---|---|
| Reduced amide (MVT-101) | A |
| Hydroxyethylamine (JG-365) | B |

FIG. 4. Hypothetical reaction coordinate diagram illustrating the relative energies of [enzyme·reaction intermediate] species for the native amide enzyme and for the backbone-engineered enzyme, under the assay conditions used in this study. ——, Energy profile for the native amide HIV-1 protease; - - - -, energy profile for the backbone-engineered HIV-1 PR. Deletion of the specific hydrogen bonds between the flap(s) and the substrate carbonyls on either side of the scissile peptide bond results in an ≈5 kcal/mol increase in energy of the rate-limiting step (B), the breakdown of the enzyme·tetrahedral intermediate (E.I) complex to form the enzyme·products (E.P) complex. Deletion of these flap–substrate hydrogen bonds has little effect on the energy barrier (A) to formation of the enzyme·tetrahedral intermediate complex. The reduced amide isostere inhibitor MVT-101 mimics transition state A and therefore has similar affinity for native amide and backbone-engineered enzymes. The hydroxyethylamine isostere inhibitor JG-365 mimics transition state B and therefore has its affinity for the backbone-engineered enzyme reduced by ≈5 kcal/mol (≈2500-fold). E.S, enzyme·substrate complex.

be 590 nM. Thus, JG-365 is ≈2500-fold‡ less potent against the backbone-engineered HIV-1 PR. This figure is remarkably similar to the reduction in the rate of substrate hydrolysis by the backbone-engineered HIV-1 PR (3000-fold) and again is consistent with the effect that would be expected from the loss of two hydrogen bonds.

It is not surprising that MVT-101 was found to be an equally effective inhibitor of the backbone-engineered HIV PR and the native amide enzyme, given that both enzymes have equal affinity for substrate (as indicated by the $K_m$ values). The onus then is to explain the reduced potency of JG-365. Inspection of the crystal structures of complexes of these two inhibitors with the HIV-1 protease offers one

‡The exact value will depend on $K_i$ for JG-365 under the same assay conditions used in this study. Additionally, the JG-365 used in this work lacked the C-terminal methyl ester, although this is known to have negligible effect on its potency (29).

possible explanation (5, 7, 30). At the core of MVT-101 is the reduced amide bond $\psi(CH_2NH)$ replacing the scissile peptide bond in what is otherwise a substrate. The hydroxyethylamine moiety [$CH(OH)CH_2N$] present in JG-365 is not a true peptide-bond isostere due to an extra backbone atom. In the complex between JG-365 and HIV-1 protease, the enzyme accommodates the added methylene group by pushing the $P_{1'}$ proline ring deep into the $S_{1'}$ binding pocket, resulting in a conformation of $P_{1'}$ not directly analogous to that of bound MVT-101. Hydrogen bonds from the $P_2$ and $P_{1'}$ carbonyls to water 301 would assist in this additional deformation and so their absence may prevent attainment of an optimal inhibitor conformation to maximize interactions with the enzyme.

In proteolytic enzymes, the strain mechanism theory has generally been proposed to assist in catalysis by distortion of the scissile peptide bond from planarity, resulting in a reduction of its double bond character and making the carbon atom susceptible to attack by the nucleophilic water (6–9, 31). This assumes that the slow step in catalysis is the formation of the tetrahedral intermediate. However, recent detailed kinetic analysis of the HIV-1 protease indicates that the rate-determining step in catalysis is not the formation of the tetrahedral intermediate but rather breakdown of the tetrahedral intermediate into the cleavage products (32, 33). Hydrogen bonds from the enzyme flaps to the $P_2$ and $P_{1'}$ carbonyls of the substrate would then serve to lower the activation barrier of the rate-determining step, perhaps by correctly orienting the lone-pair orbital of the leaving group nitrogen. The effect of deleting these hydrogen bonds would be to retard breakdown of the tetrahedral intermediate (Fig. 4).

This hypothesis correlates well with the observed inhibition data. Hyland *et al.* (33) proposed that because of the separation of the $P_{1'}$ nitrogen from the hydroxyl-bearing tetrahedral carbon by an extra methylene group, the hydroxyethylamine moiety of JG-365 better mimics the transition state for the (rate-limiting) breakdown of the tetrahedral intermediate. By contrast, reduced amide bond inhibitors (such as MVT-101) better mimic the (non-rate-limiting) transition state for the formation of the tetrahedral intermediate (Fig. 4). If, as transition state theory requires, the backbone-engineered enzyme analogue no longer binds the rate-limiting transition state as tightly (relative to the native enzyme), it follows that a mimic of this transition state (JG-365) would not inhibit the analogue enzyme as potently as it does the native enzyme, while inhibition by a mimic of the non-rate-limiting transition state (MVT-101) would remain unchanged (see Fig. 4).

In conclusion, we have shown by direct experimental observation that hydrogen bonds between the substrate and the backbone amide bond between $Gly^{49}$ and $Ile^{50}$ in each flap of the HIV-1 protease are important for the normal catalytic activity of this enzyme. Whether these hydrogen bonds are mediated by the crystallographically observed water 301, as opposed to direct flap–substrate interactions, remains to be determined. Finally, this study of a functionally relevant backbone-engineered enzyme analogue illustrates the power of the chemical synthesis approach (34) in expanding the scope of exploration of protein structure and function.

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