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Structural engineering of the HIV-1 protease molecule with a β-turn mimic of fixed geometry

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Abstract

An important goal in the de novo design of enzymes is the control of molecular geometry. To this end, an analog of the protease from human immunodeficiency virus 1 (HIV-1 protease) was prepared by total chemical synthesis, containing a constrained, nonpeptidic type II′ β-turn mimic of predetermined three-dimensional structure. The mimic β-turn replaced residues $Gly^{16,17}$ in each subunit of the homodimeric molecule. These residues constitute the central amino acids of two symmetry-related type I′ β-turns in the native, unliganded enzyme. The β-turn mimic-containing enzyme analog was fully active, possessed the same substrate specificity as the $Gly^{16,17}$-containing enzyme, and showed enhanced resistance to thermal inactivation. These results indicate that the precise geometry of the β-turn at residues 15–18 in each subunit is not critical for activity, and that replacement of the native sequence with a rigid β-turn mimic can lead to enhanced protein stability. Finally, the successful incorporation of a fixed element of secondary structure illustrates the potential of a "molecular kit set" approach to protein design and synthesis.

Keywords: β-turn mimic; chemical synthesis; HIV-1 protease; protein design; thermal stability

The virus-encoded proteinase of human immunodeficiency virus 1 (HIV-1 protease) is responsible for the processing of the viral *gag-pol* polyprotein into functional core proteins and replication enzymes (Debouck et al., 1987). The enzyme is a member of the aspartyl protease family and in its active form exists as a homodimer of identical polypeptide chains, each 99 amino acid residues in length; each subunit contributes one of the catalytically essential aspartic acid residues, these being juxtaposed at the dimer interface as part of the enzyme active site (Pearl & Taylor, 1987; Wlodawer et al., 1989). Current interest in the HIV-1 protease has been centered largely on the design of inhibitors, which may eventually lead to therapeutic drugs effective against the AIDS virus (Huff, 1991).

From a protein engineering perspective, the HIV-1 protease represents an ideal enzyme to study because it can be prepared by total chemical synthesis (Schneider & Kent, 1988). The synthetic enzyme has been prepared in crystalline form and was used to solve the three-dimensional structure of both the native molecule (Wlodawer et al., 1989) and the enzyme complexed with several different substrate-derived inhibitors (Miller et al., 1989; Swain et al., 1990; Jaskólski et al., 1991). The chemical synthesis approach brings the entire world of organic chemistry into the realm of proteins. The range of chemical alterations that can be made to a native protein is limited only by the imagination and by any peculiarities of the particular chemical methodology being employed. In addition to the synthetic accessibility of the HIV-1 protease, knowledge of its three-dimensional structure (Miller et al., 1989; Wlodawer et al., 1989; Erickson et al., 1990; Swain et al., 1990; Bone et al., 1991; Jaskólski et al., 1991) is invaluable in designing experiments to probe the interrelationship of enzyme structure with catalytic function.

The HIV-1 protease molecule is a symmetric homodimer and in its unliganded crystalline form possesses a twofold axis of symmetry. One intriguing aspect of the molecular function of HIV-1 protease is loss of C_2 symmetry upon inhibitor (and presumably substrate) binding (Miller et al., 1989; Erickson et al., 1990; Swain et al., 1990; Bone et al., 1991; Jaskólski et al., 1991). Even binding of C_2 pseudosymmetric inhibitors (Erickson et al., 1990; Bone et al., 1991) apparently induces asymmetry in the enzyme. This suggests that the observed asymmetry

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of the protease in enzyme-inhibitor complexes may not be a consequence of the binding of an asymmetric inhibitor, but may be dictated by crystal packing interactions (Bone et al., 1991). The crystallographic structure of the native (i.e., uncomplexed) enzyme shows the β-turn at residues 15–18 in both subunits to have $\psi_2 = 72°$ and $\phi_3 = 89°$, and thus to be of type I′ geometry (Richardson, 1981). However, in the numerous protease-inhibitor complexes that have been solved, the geometry of this turn varies and is not necessarily identical in both subunits. For example, in the complex with JG-365, the geometries are type I′ and II′ (Swain et al., 1990); with U85548e, type I′ and II (Jaskólski et al., 1991); and with MVT-101, type I′ and IV (Miller et al., 1989). In all cases, the residues of these turns possess high thermal factors, indicating high mobility (Jaskólski et al., 1991).

To investigate whether the exact geometry and conformational mobility of the β-turn at residues 15–18 in both subunits is important for enzymatic activity, an analog of the HIV-1 protease was prepared incorporating a rigid, bicyclic type II′ β-turn mimic replacing the native Gly[16]-Gly[17] sequence in each monomer. Both the enzymatic activity and stability of the modified enzyme were determined and compared with the enzyme of native sequence.

Results

Synthesis and characterization of the [BTD[16–17], Aba[67,95]]HIV-1 protease monomer polypeptide chain

The structure of the geometrically constrained β-turn analog (BTD, for bicyclic turn dipeptide [Nagai & Sato, 1985]) is shown in Figure 1. This β-turn mimic was incorporated into the HIV-1 protease 99-amino acid monomer covalent structure in the course of stepwise solid-phase assembly of the protected polypeptide chain, replacing the residues Gly[16] and Gly[17]. A control synthesis of the Gly[16]-Gly[17] HIV-1 protease molecule was performed simultaneously (Fig. 2). Both molecules contained L-α-amino-*n*-butyric acid (Aba) in place of the native cysteine residues (Wlodawer et al., 1989). The chemically synthesized peptide chains were partially purified as described in Materials and methods.

Synthetic [BTD[16–17], Aba[67,95]]HIV-1 protease monomer was characterized by ion-spray mass spectrometry; the observed molecular weight was 10,837 Da (calculated, 10,839 Da [average isotope composition]). The native sequence synthetic enzyme had an observed molecular weight of 10,753 Da (calculated, 10,755 Da [average isotope composition]). Thus, within experimental uncertainty, the two synthetic monomers had the correct covalent molecular weights.

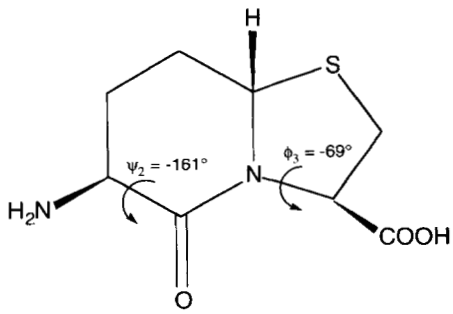

Fig. 1. Chemical structure of the rigid, bicyclic β-turn analog (3S,6S,9R)-2-oxo-3-*t*-butyloxycarboxylamino-7-thia-1-aza-bicyclo[4.3.0]nonane-9-carboxylic acid (BTD; Nagai & Sato, 1985). The angles given are those mimicking ψ_2 and ϕ_3 in a β-turn and are known from the crystal structure of BTD (Osano et al., 1989).

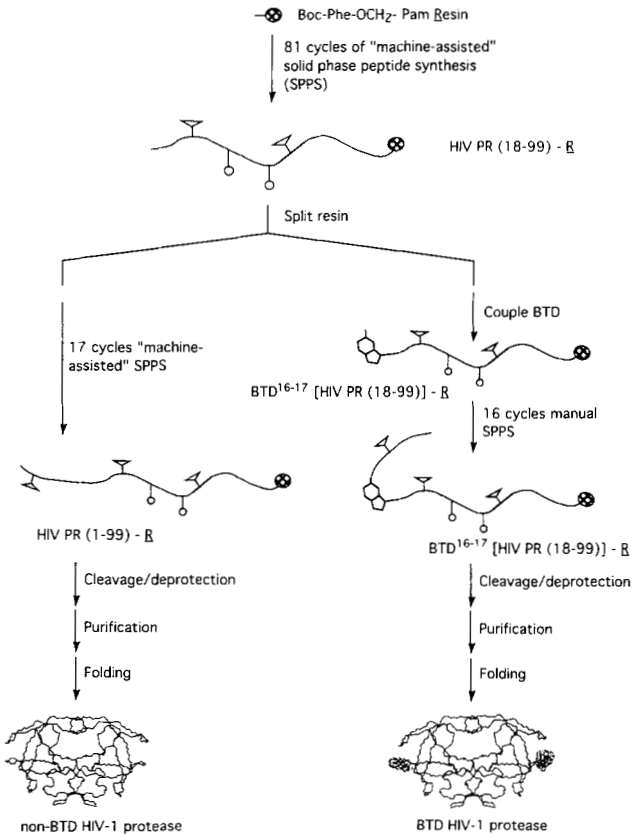

Fig. 2. Total chemical synthesis of non-BTD and BTD-containing HIV protease. The synthesis was performed in a divergent fashion, beginning with Boc-Phe-OCH_2-phenylacetamidomethyl- (Boc-Phe-OCH_2-Pam-) resin. The protected polypeptide chain from the C-terminal up to residue 18 was prepared by machine-assisted stepwise assembly on an ABI-430A peptide synthesizer. The resin was split into two portions, with one half of the resin being used to complete the native Gly[16,17] sequence. To the other half, BTD was coupled followed by the native 15 N-terminal residues. Subsequent cleavage/deprotection, purification, and folding were as described in Materials and methods.

[BTD$^{16-17,116-117}$, Aba^{67,95,167,195}]HIV-1 protease: Substrate specificity, kinetic parameters, and thermal stability

After folding by dilution of a 6 M guanidine hydrochloride (GnHCl) solution of the synthetic enzyme into assay buffer, high performance liquid chromatography and fluorogenic assays confirmed the enzymatic activity of [BTD$^{16-17,116-117}$,Aba^{67,95,167,195}]HIV-1 protease (residue numbering: first subunit, 1-99; second subunit, 101-199; hereafter referred to as BTD HIV-1 protease). Desmethyl JG-365 (Alewood et al., 1992) was shown to be a tight binding inhibitor (as for the native enzyme) by virtue of its stoichiometric titration of enzymatic activity. This enabled the concentration of active enzyme to be determined by active site titration and established the content of active protein to be 6% by weight of the lyophilized solid (uncorrected for salts and water content).

Treatment with BTD HIV-1 protease of two peptides spanning the known p17/p24 and p24/p15 sites in the HIV-1 *gag-pol* protein resulted in cleavage at the expected Tyr-Pro and Met-Met, Leu-Ala sites (Fig. 3), confirming the same substrate specificity as the native-sequence enzyme (Schneider & Kent, 1988). The identity of the cleavage products was confirmed by ion-spray mass spectrometry (data not shown). As was reported by Schneider and Kent (1988) for the native-sequence enzyme, in the p24/p15 peptide the Met-Met site is initially cleaved, followed by slower cleavage at the Leu-Ala site. No further processing of the peptide products had occurred after 24 h of digestion.

The enzymatic activity and kinetic parameters of BTD HIV-1 protease were compared with those of [Aba^{67,95,167,195}]HIV-1 protease (non-BTD HIV-1 protease). The two enzymes were assayed at a concentration of 20 nM (as determined by active site titration) in the standard fluorometric assay (see Materials and methods). The substrate concentration was varied between 20 and 100 μM. Double reciprocal plots of the kinetic data (Fig. 4) gave values of $K_m = 22 \pm 2$ μM and $V_{max} = 4.7 \pm 0.1$ (arbitrary units) for BTD HIV-1 protease and $K_m = 13 \pm 2$ μM and $V_{max} = 4.9 \pm 0.2$ (same arbitrary units) for non-BTD HIV-1 protease. These data indicate that incorporation of the rigid β-turn mimic into the HIV-1 protease did not alter the catalytic activity of the enzyme in any significant way. The maximal velocities of the two enzymes are essentially the same, although there is a minor difference (less than twofold) in the dependence of rate on substrate concentration, as indicated by the respective K_m values. This may reflect a lower affinity of the BTD-containing enzyme for substrate as a result of the increased backbone rigidity imparted by the constrained β-turn.

Measurement of the relative stabilities of the BTD and non-BTD HIV-1 protease was assessed by susceptibility to thermal inactivation (see Materials and methods). The results shown in Figure 5 indicate that the BTD-containing enzyme was more stable than the Gly^{16,17} native-sequence enzyme. Fifty percent of the activity of the non-BTD HIV-1 protease was lost after heating a solution of the enzyme for 10 min at 50 °C. By comparison, the temperature required for the same reduction in activity for the BTD HIV-1 protease was 53 °C. It is clear then that the rigid BTD moiety has stabilized the folded structure of the HIV-1 protease, without compromising the catalytic function of the enzyme.

Discussion

Many examples exist in the literature reporting the synthesis and biological properties of bioactive peptides constrained by β-turn mimics (for a review, see Ball & Alewood [1990]), and we have extended the use of such conformational constraints to the synthesis of protein analogs. β-Turns are a common element of protein secondary structure and constitute about one-quarter of the residues in typical globular proteins (Wilmot & Thornton, 1988). The in-

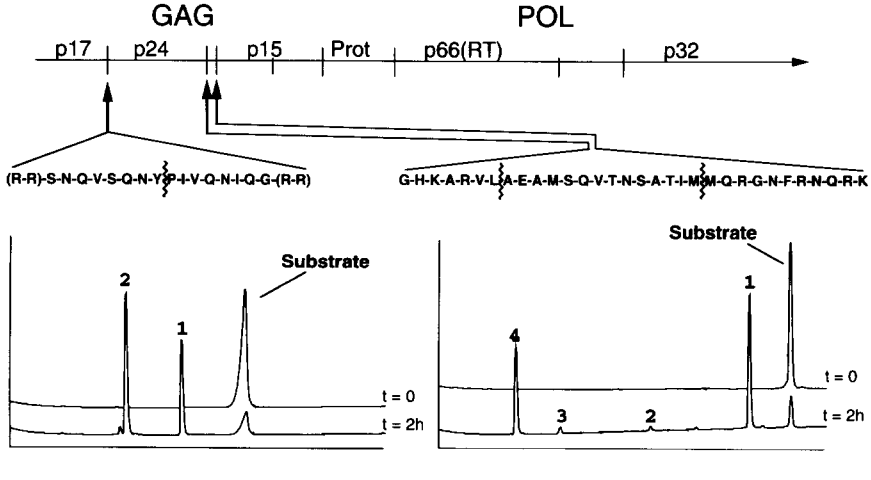

Fig. 3. Cleavage of *gag-pol* derived substrate peptides by BTD HIV-1 protease as monitored by HPLC. Traces are shown both prior to ($t = 0$) and after 2 h of proteolysis ($t = 2$ h). The identities of the proteolysis products were established by ion-spray mass spectrometry. Left-hand trace: Product 1, PIVQNIQGRR; Product 2, RRSNQVSQNY. Right-hand trace: Product 1, GHKARVLAEAMSQVTNSATIM; Product 2, AEAMSQVTNSATIM; Product 3, GHKARVL; Product 4, MQRGNFRNQRK. Traces $t = 0$ and $t = 2$ h are not plotted to the same vertical scale.

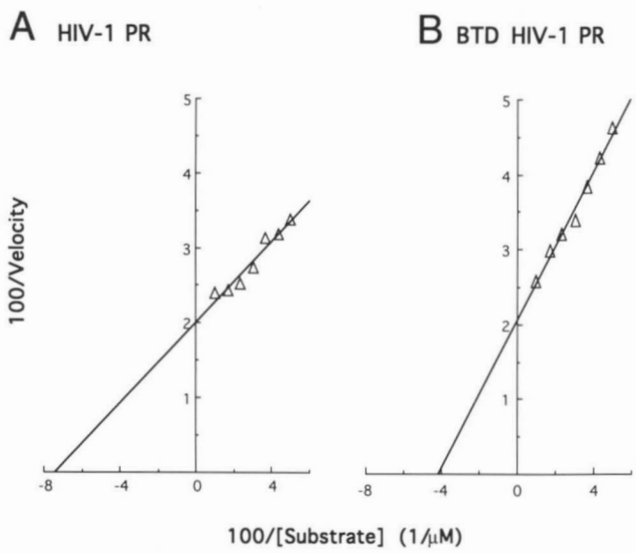

Fig. 4. Double reciprocal plots of kinetic data for the non-BTD HIV-1 protease (**A**) and BTD HIV-1 protease (**B**). The similar line slopes and intercepts show that the BTD-containing analog has comparable kinetic parameters to the non-BTD native enzyme. The respective kinetic parameters were calculated as: non-BTD: $K_m = 13 \pm 2\ \mu M$, $V_{max} = 4.9 \pm 0.2$ (arbitrary units); and BTD: $K_m = 22 \pm 2\ \mu M$, $V_{max} = 4.7 \pm 0.1$ (same arbitrary units). The V_{max} values are given in arbitrary units; the assay was not calibrated to correlate fluorescence with concentration of cleavage products.

herent polar nature of most β-turns is such that they are generally situated on the surface of proteins. The surface exposure of β-turns and their preference for residues bearing polar side chains such as Asn, Asp, Ser, Thr, and Arg (Rose et al., 1985) has led to speculation about their role as sites for molecular recognition, and this has been supported by considerable experimental evidence (for a review, see Rose et al. [1985]). In cases where β-turns do constitute a site for molecular recognition, it is likely that the precise nature of the side chain functionalities and their spatial orientation will be of critical importance. However, where the β-turn serves primarily to reverse the direction of the polypeptide backbone as required for folding into the correct protein three-dimensional structure, the relative positions and chemical composition of the side chains will be less critical.

The β-turn at residues 15–18 in each subunit of the HIV-1 protease molecule shows considerable variability in its geometry among the many enzyme–inhibitor cocrystal structures. This has been reconciled with both the surface location of this turn (Fig. 6; Kinemage 1) and its high thermal motion (Jaskólski et al., 1991), attributes common to β-turns found in globular proteins (Rose et al., 1985). In a molecular dynamics simulation of HIV-1 protease, Harte et al. (1990) found that the β-sheet comprising residues 9–24 in each subunit formed the "fulcrum" of a molecular "cantilever," whereby movements in the enzyme flaps (residues 43–58) correlated with movements in the β-sheet between residues 59 and 75. This correlated mobility was thought "likely to be involved in enzyme function at the molecular level."

The full activity of the BTD HIV-1 protease would indicate that neither the torsional mobility nor the specific geometry of the fulcrum β-turn is important for enzyme activity. None of the HIV-1 protease crystal structures have this turn modeled as type II′ in both subunits. In addition, replacement of the native Gly residues with the bulkier BTD does not appear to prevent formation of the correct folded structure of the active protease molecule.

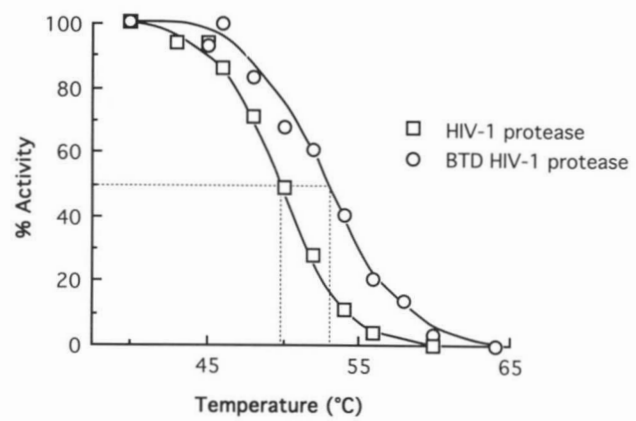

Fig. 5. Thermal inactivation of the BTD and non-BTD HIV-1 protease. Samples of enzyme were heated for 10 min at a fixed temperature between 40 and 65 °C and then assayed at the same incubation temperature. Relative fluorescence yields were corrected for temperature effects. Temperatures corresponding to a 50% reduction in activity are indicated by the dotted lines.

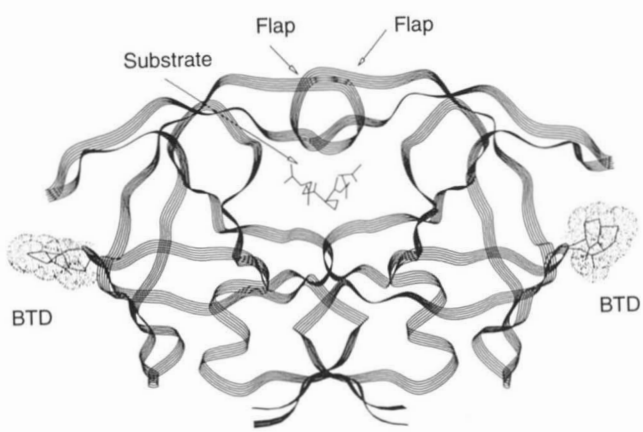

Fig. 6. Computer-generated model of the BTD HIV-1 protease based on the crystal structure of HIV-1 protease complexed to the inhibitor MVT-101 (Miller et al., 1989). The position of BTD in the enzyme molecule is indicated by a dot surface representation of the van der Waals contact radii of atoms in the BTD moiety.

Furthermore, the BTD-containing enzyme is more stable than the $Gly^{16,17}$ native-sequence enzyme. The free energy change associated with the folding of a protein into the native state can be expressed as (Schulz & Schirmer, 1979):

$$\Delta G_{\text{folding}} = \Delta H_{\text{chain}} - T \cdot \Delta S_{\text{chain}} + \Delta G_{\text{solvent}},$$

where ΔH_{chain}, ΔS_{chain}, and $\Delta G_{\text{solvent}}$ are the changes in intrachain binding energy, chain entropy, and solvation free energy upon protein folding. The change in chain entropy on folding is unfavorable, so any conformational restriction of the polypeptide chain toward the folded structure should reduce the entropic cost of folding and result in a more stable three-dimensional fold. Conclusions could not be drawn about the thermodynamic stability of the BTD HIV-1 protease because the thermal inactivation was not at equilibrium (heating for longer periods induced greater inactivation). However, the result obtained is consistent with the equilibrium thermodynamic argument presented above.

The enhanced resistance to thermal inactivation of folded BTD HIV-1 protease relative to non-BTD HIV-1 protease demonstrates the possibility of engineering more stable protein structures by the incorporation of fixed elements of secondary structure. The replacement of additional native β-turns in HIV-1 protease with conformationally constrained analogs would be expected to lead to even greater stabilization of the folded structure.

The very rapid folding of HIV-1 protease (a 6 M GnHCl solution of the enzyme diluted into assay buffer is immediately (<20 s) active [unpubl. results]) has precluded us from undertaking any simple investigation of folding kinetics. However, given the putative role of β-turns as initiation sites for protein folding (Dyson et al., 1988), it would be interesting to compare the kinetics for folding of the BTD HIV-1 protease with those of non-BTD HIV-1 protease. It has been hypothesized that the formation of turn (or other) structures in the denatured state induces the onset of the folding process (Wright et al., 1988), and this has been supported by the observation that certain short peptides can display conformational preferences in solution (Dyson et al., 1988). However, these structures are thought to be transient and in equilibrium with the randomized chain, and so the effect on folding of a fixed element of secondary structure already present in the denatured state remains to be investigated.

The full activity of BTD HIV-1 protease demonstrates that the geometry of the β-turn at residues 15–18 in both subunits is not critical for catalytic activity. The incorporation of a rigid β-turn mimic at this position resulted in enhanced stability of the folded enzyme. Perhaps most important, the successful synthesis of an enzyme analog containing a fixed element of secondary structure illustrates the potential of a "molecular kit set" approach using building blocks of fixed three-dimensional structure in the de novo construction of proteins.

Materials and methods

Protein synthesis

The target monomer sequence, 99 residues in length (SF2 strain) (Sanchez-Pescador et al., 1985), contained L-α-amino-*n*-butyric acid (Aba) as an isosteric replacement for cysteine (Wlodawer et al., 1989). BTD (bicyclic turn dipeptide: (3S,6S,9R)-2-oxo-3-*t*-butyloxycarboxylamino-7-thia-1-aza-bicyclo[4.3.0]nonane-9-carboxylic acid) (Fig. 1), a rigid bicyclic β-turn analog mimicking a type II′ β-turn (Nagai & Sato, 1985; Osano et al., 1989), was incorporated into the polypeptide in place of residues Gly^{16}–Gly^{17} in the HIV-1 protease monomer sequence (Fig. 2). Assembly of the protected peptide chain corresponding to residues 18–99 was achieved on an ABI 430A peptide synthesizer, using optimized tert-butoxycarbonyl (Boc) chemistry protocols (Kent, 1988). Boc-protected BTD was coupled manually using BOP activation (Castro et al., 1975), following which the chain assembly was completed by manually coupling residues 1–15 as hydroxybenzotriazole esters. The $Gly^{16,17}$-containing monomer sequence was synthesized from another portion of the same (residues 18–99) peptide resin on the peptide synthesizer. Each resin-bound polypeptide was treated with β-mercaptoethanol (to remove the side chain-protecting group from ImDNP-His), trifluoroacetic acid (to remove the N-terminal Boc group), and aqueous ethanolamine (to remove the side chain-protecting group from formyl-Trp). Removal of the remaining protecting groups and cleavage of the polypeptide from the resin were performed by a single-step S_N1 acidolysis in hydrogen fluoride (HF) containing 5% v/v *p*-cresol and 5% v/v *p*-thiocresol for 1 h at 0 °C. After removal of HF under reduced pressure, the peptide product was precipitated with diethyl ether, filtered, dissolved in 50% acetic acid, and lyophilized.

Product characterization and enzyme assays

Crude monomeric protein was partially purified by sequential gel filtration, preparative isoelectric focusing, and reverse-phase high pressure liquid chromatography (HPLC). The partially purified enzyme product was characterized by analytical HPLC and ion-spray mass spectrometry. Enzyme assays were performed using a continuous fluorometric assay based on the method of Toth and Marshall (1990). Enzymatic cleavage of the Nle-Phe(NO_2) bond in the fluorogenic substrate Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 (Nle = norleucine, Abz = anthranilic acid, Phe(NO_2) = 4-nitrophenylalanine) resulted in an increase in the fluorescence emission intensity at 420 nm (excitation wavelength = 325 nm) proportional to the amount of cleavage. Protease activity was

measured at 37 °C in 100 mM sodium phosphate, pH 6.5, containing 0.5 M NaCl, 10% glycerol, and 0.5 mg/mL bovine serum albumin. The substrate concentration in the assay was 50 μM. Reaction was initiated by the addition of enzyme, and the rate was followed by the increase in fluorescence over the first few minutes. Rates were taken to be the initial slope of the increase in fluorescence.

A stock solution of [BTD$^{16-17}$,Aba^{67,95}]HIV-1 protease was prepared in 6 M GnHCl, and the quantitation of active enzyme in this solution, upon dilution into assay buffer, was achieved by active site titration using the tight binding inhibitor desmethyl JG-365 (Alewood et al., 1992). A stock solution of [Aba^{67,95}]HIV-1 protease (Schnölzer et al., 1992) was similarly prepared, and subsequent dilutions of these solutions, containing equal concentrations of enzyme by active site titration, were used for comparison of BTD and non-BTD HIV-1 protease kinetics.

Properties of BTD HIV-1 protease

The comparative substrate specificities of BTD and non-BTD HIV-1 protease were assessed by the cleavage of two synthetic peptides encompassing the p17/p24 and p24/p15 cleavage sites in the viral *gag-pol* protein. The sequence and synthesis of these peptides have been reported previously (Schneider & Kent, 1988), the only difference here being that the p24/p15 peptide was synthesized with a C-terminal amide rather than free acid. The p17/p24 peptide contains one known HIV-1 protease cleavage site (Tyr-Pro), and the p24/p15 peptide has two cleavage sites (Met-Met and Leu-Ala). Solutions of both peptides (1 mg/mL in 100 mM sodium phosphate, pH 6.5, 10% glycerol, and 5% DMSO) were treated with BTD HIV-1 protease (~35 nM) for 2 h at 37 °C. The cleavage after this time was monitored by reverse-phase HPLC, and the products were characterized by ion-spray mass spectrometry.

Double reciprocal plots were used to calculate the enzymatic parameters K_m and V_{max} for cleavage of the fluorogenic substrate by both BTD and non-BTD HIV-1 protease. DMSO was used to solubilize the fluorogenic substrate. Because DMSO weakly inhibits the enzyme (D.A. Bergman, pers. comm.), its final assay concentration was standardized at 1% v/v. Protease was assayed at substrate concentrations between 20 and 100 μM, each assay being performed in triplicate. Kinetic parameters were determined by using the program ENZYME KINETICS (Gilbert, 1989).

The susceptibilities to thermal inactivation of the BTD and non-BTD proteins were evaluated by incubating enzyme (134 and 102 nM, respectively, in assay buffer containing 0.2 M GnHCl) for 10 min at a defined temperature between 40 and 65 °C and then assaying immediately at the incubation temperature. Each temperature point was performed in duplicate. Fluorescence measurements were corrected for temperature effects by reference to a temperature versus fluorescence calibration for cleaved and intact fluorogenic substrate.

Acknowledgments

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