Interactions of a Novel Inhibitor from an Extremophilic Bacillus sp. with HIV-1 Protease

IMPLICATIONS FOR THE MECHANISM OF INACTIVATION*

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The active site cleft of the HIV-1 protease (PR) is bound by two identical conformationally mobile loops known as flaps, which are important for substrate binding and catalysis. The present article reports, for the first time, an HIV-1 PR inhibitor, ATBI, from an extremophilic Bacillus sp. The inhibitor is found to be a hydrophilic peptide with Mr of 1147, and an amino acid sequence of Ala-Gly-Lys-Lys-Asp-Asp-Asp-Pro-Pro-Glu. Sequence homology exhibited no similarity with the reported peptidic inhibitors of HIV-1 PR. Investigation of the kinetics of the enzyme-inhibitor interactions revealed that ATBI is a noncompetitive and tight binding inhibitor with the IC₅₀ and Kᵢ values 18.0 and 17.8 nm, respectively. The binding of the inhibitor with the enzyme and the subsequent induction of the local conformational changes in the flap region of the HIV-1 PR were monitored by exploiting the intrinsic fluorescence of the surface exposed Trp-42 residues, which are present at the proximity of the flaps. We have demonstrated by fluorescence and circular dichroism studies that ATBI binds in the active site of the HIV-1 PR and thereby leads to the inactivation of the enzyme. Based on our results, we propose that the inactivation is due to the reorganization of the flaps impairing its flexibility leading toward inaccessibility of the substrate to the active site of the enzyme.

HIV-1 protease (PR) has been classified as an aspartic protease that functions as a homodimer, based on its primary amino acid sequence, its inhibition by pepstatin, and its crystal structure (1–3). The retroviral protease is encoded in the viral pro gene for all retroviruses, including HIV-1 (4, 5). During the replication cycle of HIV, gag and gag-pol gene products are translated as polypeptides. These proteins are subsequently processed by the virally encoded protease to yield structural proteins from microorganisms still exists. The present study deals with the isolation of an inhibitor, ATBI, of HIV-1 PR from an extremophilic Bacillus sp. and the evaluation of its kinetic parameters. Fluorescence spectroscopic studies revealed that ATBI binds in the active site of the HIV-1 PR and is the first report of a noncompetitive inhibitor from an extremophilic microorganism. It is well established that the Trp-42 is present only dynamically flexible portions of the enzyme (19). We have investigated the conformational changes induced in the flap regions of the HIV-1 PR by monitoring the intrinsic fluorescence of the Trp residues and the effects on the secondary structure of the HIV-1 PR, by circular dichroism studies, upon binding of ATBI. We have also compared the results obtained with that of the substrate and active site-directed inhibitors of the HIV-1 PR. These results demonstrated that the enzyme inactivation is caused by the loss of the flexibility of the flaps restricting the entry and exit of the polypeptide substrate and products.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The extremophilic Bacillus sp. was grown on a liquid medium containing soya meal (2%) and other nutrients at 50 °C for 48 h as described (20) (the medium was adjusted to pH 10 by the addition of sterile 10% sodium carbonate). The Escherichia coli strain harboring the recombinant plasmid containing...
the HIV-1 PR gene was grown in M9 medium supplemented with ampicillin (40 μg/ml), thiamine hydrochloride (25 μg/ml), and glucose as the carbon source at 30 °C.

**Purification and Biochemical Characterization of ATBI—**Extracellular culture filtrate (1000 ml) of the extracellular Bacillus sp. was treated with activated charcoal (65 g) and incubated at 4 °C overnight. The colorless filtrate was subjected to membrane filtration through amicon-U10 (molecular weight cut-off 10,000) and subsequently through amicon-U2 (molecular weight cut-off 2000). The resulting inhibitor sample was concentrated by lyophilization (50 ml). The residual concentrate was further purified by reverse phase high performance liquid chromatography (rp-HPLC). The concentrated inhibitor sample (100 μl) was loaded onto a prepacked Ultrapac column (Lichrosorb RP-18, LKB), which was preequilibrated with 10% acetonitrile (CH₃CN) and 0.1% trifluoroacetic acid. The fractions were eluted on a linear gradient of 0–50% CH₃CN with H₂O containing 0.01% trifluoro-acetate at a flow rate of 0.5 ml/min and monitored at a wavelength of 210 nm. The eluate was evaporated and lyophilized. The residual matter was dissolved in distilled H₂O and assayed for the anti-HIV-1 PR activity. The active fractions were rechromatographed on rp-HPLC under similar experimental conditions as described above. The active peak was finally purified by rp-HPLC using the Lichrosorb RP18 column.

The amino acid sequence of the purified peptide was analyzed with a protein sequencer (Applied Biosystems model 476A), and the sequence homology was done manually after retrieving the peptide sequences from the data bank. Molecular mass of the purified ATBI was determined on a VG Biotek Platform-II quadrupole electrospray mass spectrometer using CH₃CN-H₂O (1:1) as mobile phase. The isoelectric point of the inhibitor was determined as described (21).

**Enzyme Purification, Assay, and Kinetic Analysis—**The recombinant HIV-1 PR harbored in *Escherichia coli* was expressed by temperature induction after the onset of the log phase of bacterial growth and purified by ammonium sulfate precipitation, dialysis, and gel filtration chromatography as described (22). The HIV-1 PR activity was assayed using the synthetic substrate Lys-Ala-Arg-Val-Nle-Leu-Val-Ala (where Al is aldehyde) and pepstatin at 25 °C. Accessi-bility calculations and visualization of Trp residues were performed by Insight-II (28) from the crystallographic structure of the HIV-1 PR as described (19).² CD spectra were recorded in a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1-mm path length. Replica scans were obtained at 0.1-nm resolution, 0.1-nm bandwidth, and a scan speed of 50 nm/min. Spectra were averages of six scans with the base line subtracted spanning from 280 to 200 nm in 0.1-nm increments. The CD spectrum of the HIV-1 PR (25 μg/ml) was recorded in 50 mM sodium phosphate buffer (pH 5.6) containing 100 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol in the absence/presence of substrate (40 μM) or ATBI (20 nM). Secondary structure content of the HIV-1 PR, the HIV-1 PR-substrate complex, and the HIV-1 PR-ATBI complex was calculated using the algorithm of the K2d program (30, 31).

**RESULTS**

**Purification and Biochemical Characterization of ATBI—**The extracellular culture filtrate of the extracellular Bacillus sp. was subjected to activated charcoal treatment and ultrafiltration to remove the high molecular weight impurities. The concentrated inhibitor sample was further purified by rp-HPLC. The anti-HIV-1 PR activity was associated with the peak A (Fig. 1a), and other eluted peaks showed no inhibitory activity. Homogeneity of the active fractions was indicated by the single peak as analyzed on rp-HPLC (Fig. 1b). Further, the purified ATBI showed a single band on an analytical isoelectric focusing gel unit with a pl of 10.0. The amino acid sequence of the purified inhibitor determined by a protein sequencer was Ala-Gly-Lys-Lys-Asp-Asp-Asp-Pro-Pro-Glu and was distinctly different from the sequence of the other reported inhibitors of HIV-1 PR (32–35). The predominance of the charged amino acid residues in the inhibitor sequence indicated its hydrophilic nature. The molecular mass of ATBI as determined from electrospray mass spectrometry was 1147 Da (Fig. 2a).

**Kinetics of Inactivation of the Recombinant HIV-1 PR by ATBI—**ATBI was found to inhibit the purified recombinant HIV-1 PR with an IC₅₀ value (50% inhibitory concentration) of 18 nM (Fig. 3a). The inhibition of the HIV-1 PR followed a sigmoidal pattern with increasing concentrations of the inhibitor. However, the secondary plot (the slope of inhibition graph versus inhibitor concentration) was not linear, suggesting that the application of Michaelis-Menten inhibition kinetics was not appropriate in this study. The inhibition constant Kᵢ, determined by the classical double reciprocal plot and also by Dixon plot was 17.8 nM (Fig. 3b), which is almost equal to the IC₅₀ value of the inhibitor. The Lineweaver-Burk’s reciprocal plot (Fig. 3c) showed that ATBI was a noncompetitive inhibitor of the HIV-1 PR. For the inhibition kinetic studies, the HIV-1 PR activity was monitored in the presence of various concentrations of inhibitor and substrate as a function of time. A very

² Secondary structure as given by the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/) of HIV-1 PR as in PDB ID 1AID.
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The present paper describes a spectrofluorometric approach toward investigating the localized conformational changes induced in the HIV-1 PR upon binding of ATBI. We have shown by analyzing the kinetic parameters of the interactions of the HIV-1 PR and the inhibitor that Michaelis-Menten kinetics cannot be applied for this inhibition study. The failure of substrate protection against HIV-1 PR inhibition by ATBI and the nondissociative nature of the HIV-1 PR-ATBI complex with multiple dilutions and washings led us to apply tight binding inhibition kinetics. The short time observed for the inhibition mandated performance of the kinetics under second-order rate conditions. Observed \( \alpha \) and \( \beta \) values for ATBI were independent of the substrate concentration and relatively constant, implying that the binding of the inhibitor was not influenced by the binding of the substrate. However, a typical rectangular hyperbola resulted in a reciprocal plot of \( 1/\beta \) versus \([S]\). We have concluded, by using a diagnostic

in the HIV-1 PR upon binding of ATBI were monitored by exploiting the intrinsic fluorescence by excitation of the \( \pi-\pi^* \) transition in the Trp residues. The fluorescence emission spectra of the HIV-1 PR exhibited an emission maxima (\( \lambda_{\text{max}} \)) at ~342 nm as a result of the radiative decay of the \( \pi-\pi^* \) transition from the Trp residues, confirming the hydrophilic nature of the Trp environment. The titration of the native enzyme with increasing concentrations of ATBI resulted in a concentration-dependent quenching of the tryptophanyl fluorescence (Fig. 5). However, the \( \lambda_{\text{max}} \) of the fluorescence profile indicated no blue or red shift, revealing that the ligand binding caused reduction in the intrinsic protein fluorescence. A progressive quenching in the fluorescence of the HIV-1 PR at 342 nm was observed concomitant to the binding of substrate (Lys-Ala-Arg-Val-Nle-p-nitro-Phe-Glu-Ala-Nle-amide). Further, to throw light upon the mechanism of inactivation of the HIV-1 PR by ATBI, we have analyzed the interaction of two representative competitive inhibitors, N-acetyl-Leu-Val-Phe-Al (where Al is aldehyde) (37) and pepstatin (2) by steady-state intrinsic fluorescence measurements. The binding of the competitive inhibitors led to the decrease in the quantum yield of the tryptophanyl fluorescence as indicated by the quenching of the emission spectra of the HIV-1 PR. The comparative analysis of the intensity changes in the fluorescence spectra of the HIV-1 PR upon binding of the substrate or the known active site-based inhibitors was found to be similar to that of ATBI, suggesting that ATBI binds in the active site of the enzyme.

Secondary Structural Analysis of Enzyme Substrate-Inhibitor Complexes—To evaluate the effects of the inhibitor on the secondary structure of the enzyme, we have analyzed the CD spectra of the HIV-1 PR-ATBI complex. The secondary structure contents of the HIV-1 PR as determined from the crystallographic data were 4.04\% \( \alpha \)-helix, 47.47\% \( \beta \)-sheet, and 48.49\% of aperiodic conformation (19). The estimated secondary structure contents from the CD analysis were 5\% \( \alpha \)-helix, 48\% \( \beta \)-sheet, and 47\% aperiodic structure, which are in total agreement with the crystallographic data. The circular dichroism spectrum of the HIV-1 PR-ATBI complex showed a pronounced shift in the negative band at 220 nm of the native enzyme to 225 nm (Fig. 6). This shift reveals a subtle change in the secondary structure of the enzyme upon ligand binding. To elucidate the changes in the secondary structure of the enzyme-inhibitor complex, we have compared it with that of the HIV-1 PR-substrate complex. Interestingly, the HIV-1 PR-ATBI and HIV-1 PR-substrate complexes exhibited a similar pattern of negative ellipticity in the far-UV region, suggesting that the inhibitor causes similar structural changes and was distinctly different from that of the unliganded enzyme.

**DISCUSSION**

The present paper describes a spectrofluorometric approach toward investigating the localized conformational changes induced in the HIV-1 PR upon binding of the noncompetitive inhibitor ATBI. We have shown by analyzing the kinetic parameters of the interactions of the HIV-1 PR and the inhibitor that Michaelis-Menten kinetics cannot be applied for this inhibition study. The failure of substrate protection against HIV-1 PR inhibition by ATBI and the nondissociative nature of the HIV-1 PR-ATBI complex with multiple dilutions and washings led us to apply tight binding inhibition kinetics. The short time observed for the inhibition mandated performance of the kinetics under second-order rate conditions. Observed \( \alpha \) and \( \beta \) values for ATBI were independent of the substrate concentration and relatively constant, implying that the binding of the inhibitor was not influenced by the binding of the substrate. However, a typical rectangular hyperbola resulted in a reciprocal plot of \( 1/\beta \) versus \([S]\). We have concluded, by using a diagnostic
plot of $\Delta \beta$ versus $[S]$, that the inactivation of the HIV-1 PR by ATBI was noncompetitive.

Deciphering the crystal structure of the HIV PR and its inhibitor complexes has gained immense interest among the crystallographers in the last decade. From the available crystallographic data, it is deduced that binding of substrate or peptide-analogue inhibitors in the substrate-binding site of HIV-1 PR induces conformational changes in the flaps (3, 14, 38). The apparent function of these flaps is to force the peptide substrate into a $\beta$-sheet in the active site and to correctly position its scissile bond between the two catalytic aspartyl residues. The flaps accomplish this by the establishment of a series of hydrogen-bonding interactions between amide nitrogens and carbonoyl oxygens of the peptide substrate. Our interpretation for the changes observed in the secondary structure of the HIV-1 PR due to the binding of the substrate to the active site can be correlated to the inward movement of the flaps. It is significant to note that the secondary structure of the HIV-1 PR undergoes similar pattern of changes upon binding of the substrate or inhibitor. Thus, we have attributed the observed secondary structure changes in the HIV-1 PR-ATBI complex to the inward movement of the flaps of the HIV-1 PR. The noncompetitive nature of the inhibitor may be addressed due to the better binding affinity of the inhibitor to the active site than the substrate. This, however, does not exclude the possibility of the differential binding pockets for the inhibitor and the substrate in the active site of the enzyme.

The tryptophanyl fluorescence appears to be uniquely sensitive to shielding by a variety of ligands because of the propensity of the excited indole nucleus to emit energy in the excited state. The Trp residues (A-42 and B-42) of the HIV-1 PR are present next to the Lys-43, the first residue of the flap region,

**FIG. 2. Chemical properties of ATBI.**
a, the purified ATBI was analyzed for the determination of the molecular mass using an acetonitrile/water (1:1) system as the mobile phase on a quadrupole electrospray mass spectrometer. b, schematic representation of the chemical structure of ATBI.
which extends from Lys-43 to Arg-57 (Fig. 7) (19). There have been reports of introducing a Trp residue, which would act as a highly specific reporter to monitor the structural changes in the flap regions by substrate-inhibitor binding (14). However, the site-directed mutagenesis studies of the HIV-1 PR have revealed that the enzymatic activity is extremely sensitive to mutations in the flap regions (39). The inhibitors that bind to the active site also bind to the inner face of the flaps of the HIV-1 PR. The binding of the inhibitor-substrate and the subsequent movement of the flaps may have influence on the intrinsic fluorescence of the Trp-42 residues. Based on the above assumption, we have exploited these two Trp residues of the HIV-1 PR to investigate the localized conformational changes induced upon substrate or inhibitor binding. Our fore-

**FIG. 3.** Binding of ATBI to the HIV-1 PR and inhibition kinetics analyses. a, the proteolytic activity of the purified HIV-1 PR was determined in the presence of increasing concentrations of ATBI. The percentage inhibition of the HIV-1 PR activity was calculated from the residual enzymatic activity. The sigmoidal curve indicates the best fit for the percentage inhibition data obtained, and the IC₅₀ value was calculated from the graph. b, enzymatic activity of the HIV-1 PR (25 µg/ml) was estimated using the substrate Lys-Ala-Arg-Nle-p-nitro-Phe-Glu-Ala-Nle-amide (40 µM (A) or 50 µM (B)) at different concentrations of ATBI. Reciprocals of the reaction velocity were plotted versus the inhibitor concentration. The straight lines indicated the best fit of the data obtained. The inhibition constant Kᵢ was calculated from the point of the intersection of the plots. c, the HIV-1 PR (25 µg/ml) was incubated, without (●) or with the inhibitor at 10 nM (●) and 20 nM (●) and assayed at increasing concentrations of the substrate. The reciprocals of the rate of the substrate hydrolysis for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. Kᵢ was determined from the formula as per the noncompetitive type of inhibition.

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**FIG. 4.** Determination of the binding constants of the HIV-1 PR and ATBI interactions. a, the HIV-1 PR and ATBI were mixed, and the samples were removed at different time intervals were assayed to determine the residual activity of the enzyme. The rate constants were determined by the second order association kinetics. The hyperbola indicated the best fit of the data obtained by plotting the reciprocal of the association rate constant (β) versus the substrate concentration. b, Henderson plot of the change in the slope of Δβ for the inhibition of the HIV-1 PR by ATBI, as a function of substrate. The straight line represents the best fit for the data generated for the values of Δβ.

**FIG. 5.** Fluorescence emission spectra of the purified HIV-1 PR. The fluorescence intensity spectra of the HIV-1 PR as a function of the inhibitor are shown. Fluorescence was excited at 295 nm, and emission was monitored from 300 to 400 nm. Titration of the enzyme was performed by the addition of different concentrations of the inhibitor to the enzyme solution. The HIV-1 PR (25 µg/ml) was dissolved in 50 mM sodium acetate buffer (pH 5.6) containing 100 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol. The concentrations of ATBI were 0 nM (●), 10 nM (○), 15 nM (●), 20 nM (○), 25 nM (●), 40 nM (∇), 45 nM (∆), and 50 nM (△).
Results in the inactivation of the HIV-1 PR. The structure of the flaps, is responsible for the loss of the dynamic flexibility of the flaps, nature of ATBI, along with its multiple nonbonded interactions with the block (as described under "Experimental Procedures") and the CD spectra were recorded in the absence or in the presence of the substrate 40 μM or ATBI 20 nM from 280 to 200 nm at 25 °C. Each spectrum represents the average of six scans.

The binding of the substrate or ATBI to the active site leads to inward movement of the flaps (as indicated by the arrows), which is important for the substrate binding and catalysis. The binding of the inhibitor (as indicated by the solid block) in the active site induces inward movement of the flaps (as indicated by the arrows) further, we propose that the noncompetitive nature of ATBI, along with its multiple nonbonded interactions with the flaps, is responsible for the loss of the dynamic flexibility of the flaps, resulting in the inactivation of the HIV-1 PR. The structure of the HIV-1 PR is as described in PDB ID 1AID.

Substitution of the enzyme and is a unique example where the conformational changes in the flaps were investigated by monitoring the radiative decay of the enzyme. ATBI, by virtue of its unique sequence and noncompetitive nature of ATBI in conjunction with the multiple nonbonded interactions may be sufficient to cause the loss of the dynamic flexibility of the flaps, which is crucial for the substrate binding and catalysis of the enzyme. A schematic representation of the proposed mechanism is depicted in Fig. 7. The noncompetitive nature of the ATBI indicated that the inhibitor-complexed form of the HIV-1 PR loses its binding ability to the substrate, since the flaps can no more open up for the substrate to be aligned in the active site of the HIV-1 PR, which subsequently results in the inactivation of the enzyme. These observations are at variance with the binding of the substrate to the enzyme in the absence of ATBI, where the flexibility of the flaps can be regained after catalysis.

Inhibitors directed toward the active site of the HIV-1 PR are well documented (41, 42). Despite their loss in potency due to the spontaneous mutations occurring in the active site (29, 43) leading toward the drug resistance behavior of the virus, there is a paucity of literature on noncompetitive inhibitors. A constant search for the new class of HIV-1 PR inhibitors with high potency is a frontier area of biomedical research. The side chains of the peptidic inhibitor ATBI, which are capable of forming many nonbonded interactions (hydrogen bonding, van der Waals interactions, etc.) with the enzyme, might result in superior resistance characteristics in comparison with the competitive inhibitors. ATBI, as proposed, could interact with the backbone of the β-sheet of the flaps of the HIV-1 PR, thus eliminating the probability of drug resistance by a single mutation. ATBI, by virtue of its unique sequence and noncompetitive mode of inhibition, may represent a new class of inhibitors of the HIV-1 PR and could open up a new horizon for the development of lead molecules.

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