Effect of L97V Substitution in the HIV-1 Protease Molecule on Its Active Site Structure

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Introduction

HIV-1 protease inhibitors have made an important contribution to AIDS treatment. The efficacy of this mode of treatment, however, has been marred by the evolution of HIV-1 mutants that are resistant to these inhibitors [1]. These mutants accumulate rapidly because of the error-prone reverse transcriptase activity and high replication rate of the HIV-1. The possible number of HIV-1 mutants is large, but we should we be concerned with only a small percentage of them because some of the potential mutants will not be viable and some will retain the wild-type phenotype and therefore will not resist drugs. We can identify the substitutions in the HIV-1 protease molecule that can lead to the evolution of drug-resistant HIV-1 mutants by examining the active site of the HIV-1 protease mutants with x-ray crystallography. By so doing, we can determine which substitutions alter the HIV-1 protease's active site. Alteration of the HIV-1 protease's active site is the structural basis for its drug resistance. The location of the substituted residue on the protease molecule plays a significant role in determining its phenotype. Different substitutions, even at the same position on the molecule, can alter the protease active site in different ways, thereby producing different phenotypes. For example, N88S and N88D show different phenotypes with regard to their responses to inhibitor drugs [2]. It is therefore imperative to determine what type of alteration in the active site of the HIV-1 protease can result from a type of substitution and what type of phenotype can result from it. Residue 97 is located at the dimer interface of the functional HIV-1 protease homodimer. We are trying to determine whether substituting the Leu at position 97 in the wild-type protease with Val (L97V) will alter the structure of the active of the HIV-1 protease.

Methods and Materials

The L97V HIV-1 mutant clone was donated to us by J. Louis of the National Institutes of Health (NIH). The L97V HIV-1 protease mutant gene was ligated into pET11 vector and transfected into *Escherichia coli* BL2 DE3.

Induction

Expression of the L97V HIV-1 protease mutant was induced at 37°C with a 2-mM concentration of IPTG (sigma) in LB-glucose (1% glucose) containing 100 ng/mL of antibiotic carbiniciline.

Purification

The purification of the L97V protease mutant was a three-step process. The first step was extraction of the inclusion bodies. Cells were pelleted by centrifugation at 12,000 rpm for 20 min at 4°C. The pellets were washed once with cold TE (TE = 10 mM Tris and 1 mM EDTA) and resuspended in 10 mL of cold solution A (TE with 5 ng per mL lysozyme) and put on ice for 10 min. The lysed cells were sonicated at 4.5 duty and 6 output (indicate speed and crushing power) five times at 1-min intervals on ice. The cell lysate was pelleted, and the supernatent was discarded by decanting. The pellet was resuspended in 15 mL of solution B (TE with 4 M urea and 1% Trixtion-100) and pelleted once again. This step was repeated one more time. The pellet (the inclusion bodies) was then washed once with TE and resuspended in 3 mL of solution C (TE with 7.5 M guanidine hydrochloride and 5 mM DTT) by pipetting. In the second step, the HIV-1 protease mutant was extracted from the inclusion body by gel filtration using Superdex-75 column (Hiload 2.6×50 cm) equalibrated with a buffer containing 50 mM of tris-HCl at pH 8.0, 4 M of guanidine hydrochloride, 5 mM of EDTA, and 5 mM of DTT, and run by the ÄKTAprime™ system. In the third step, the ÄKTAprime product was subjected to reverse-phase high-performance liquid chromatography (HPLC) by using a SOURCE™ 15RPC column from Amersham Biosciences. We then ran the HIV preparation method we programmed in the ÄKTApurifier™ system. We used 0.05% triflouroacetic in water as buffer A. Buffer B (the mobile phase) was 0.05% TFA in acetonitrile.

The L97V HIV-1 protease mutant refolding was done in two steps at 4°. First it was done in a 1-L solution made up of 0.05 M formic acid, 0.125% acetonitrile, and 5 mM of DTT for 2 h. Then it was transferred to 1 L of 0.05 sodium acetate solution for 4 h at a slower spinning speed. The protease solution was concentrated to 6 mg/mL by using a micron concentrator and centrifuged at 4000 rpm.

Crystallization

HIV-1 protease mutant L97V was crystallized in complex with inhibitor N-1270. This inhibitor is an

analog of the HIV-1 cleavage site CA-p2, Arg-Val-Leu-r-Phe-Glu-Ala-Nle, where r is the reduced peptide bond and Nle is norleucine. First, the protease-inhibitor complex was made by mixing 10 μL of 0.64 mM protease solution and 10 μL of 12 mM of the inhibitor (10 X excess inhibitor). The mixture was left on ice overnight before it was used in the drops. The protease-inhibitor complex was crystallized in a solution made up of 20% ammonium sulfate in a 0.5-M/0.25-M phosphate/citric acid buffer containing 10% DMSO and 1 mM of DTT at room temperature by the vapor drop method. Hexagonal and cubiodal crystals appeared in two of the drops after 3 days. Diffraction data were collected at the APS, and the structure was solved. The x-ray data were processed with denzo. The structure was solved by molecular replacement using AmoRe (Navazda, 1994), refined by including anisotropic B-factors using SHELX97, and refitted by using the O program, version 8.0. We then compared the active structure of the interaction between the L97V protease mutant and the inhibitor with the structure of the interaction between the wild-type HIV-1 protease and the same inhibitor.

TABLE 1. Comparison of hydrogen bond interactions involving the wild-type HIV-1 protease and those involving the L97V HIV-1 protease mutant.

	Protease	Distance (Å)	
Inhibitor	Atom	L97V	Wild-Type
Main chain atoms			
P3 Arg NH	OD Asp 29	2.81	3.25
P3 Arg C=O	HN Asp 29	2.76	2.86
P2 Val NH	O=C Gly 48	2.87	3.11
P2 Val C=O	H_2O	2.70	2.97
P1 Leu NH	O=C Gly 27	3.29	3.12
P1′ Phe C=O	H_2O	2.64	2.87
P2′ Glu NH	O=C Gly 127	3.11	3.09
P2′ Glu C=O	HN Asp 129	2.97	2.82
P3′ Ala *NH	O=C Gly 148	2.92	3.10
P3′ Ala C=O	HN Gly 148	2.77	3.48
P4′ Nle NH_2	O=C Met 146	2.99	3.40
H_2O	HN Ile 50	2.99	2.51
H_2O	HN Ile 150	2.87	2.84
Side chain atoms			
P2′ Glu OE	OD Asp 130	2.47	2.83[a]
P2′ Glu OE	HN Asp 130	2.89	2.87

[a] Proton-mediated.

Results and Discussion

Table 1 compares the hydrogen bond interactions between L97V HIV protease mutant active site atoms and the CA-p2 analog with the hydrogen bond interactions between the atoms of the active site of the wild-type HIV-1 protease and the same CA-p2 analog. The measurements below were obtained by using the Rasmol program and the command to "set picking distance."

From these measurements, we see that the HN of Gly 148 of the L97V protease mutant is 2.77 Å from P3′C = O of Ala of the inhibitor and the C = O of Met 146 of the protease mutants, and it is 2.99 Å from the P4′NH2 of Nle of the inhibitor (both within hydrogen bonding distance). These atoms are 3.48 Å and 3.4 Å, respectively, in the wild-type protease. So the protease mutant makes two more hydrogen bonds with the inhibitor than does the wild-type protease. This in itself indicates that the active site of the L97V HIV-1 protease mutant is altered relative to the wild-type protease. One can therefore deduce that the L97V substitution in the HIV-1 protease molecule alters its active site structure.

Here is a list of some of the data collection and refinement statistics.

Space group: $P2_12_12_1$
Dimers in asymmetric unit: 1
Unit cell parameters: a = 51.498, b = 57.957, and c = 61.821 Å
Inhibitors per dimer: 1
Number of unique reflections: 30,192
Rmerge 0.042 R value: 0.1456
Resolution: 1.45 Å
Resolution range for refinement: 10-1.30 Å
Completeness 99.6% R-free: 0.1567

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References

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FIG. 1. The L97V HIV-1 protease mutant in complex with inhibitor residue 97:Val.