PRELIMINARY CRYSTALLOGRAPHIC STUDY OF NATIVE HIV-1 PROTEASE INHIBITED BY HYDROXYETHYLAMINE MODIFIED TETRAPEPTIDES OF Boc-Phe-ψ[(R/S)-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2 TYPE

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The HIV-1 protease is essential for replication of infective virus HIV, and therefore is an attractive target for the design of specific inhibitors. In search for new inhibitors, substantial effort is devoted to understanding the nature of the inhibitor binding modes in the active site, using X-ray diffraction on crystals as the primary source of structural information. This paper describes the crystallization and preliminary diffraction study of HIV-1 (BRU) protease complexed with the

Boc-Phe-ψ[*(S)*-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2

inhibitor (SI). The SI is a four-amino-acid pseudopeptidic inhibitor, where the scissile peptide bond is replaced by the (2-hydroxyethyl)amine (HEA) isostere. Current state of crystallization of HIV-1 protease complexed with RI, (Boc-Phe-ψ[*(R)*-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2), which is a stereoisomer of SI, is also reported.

First crystallization trials were based on crystallization studies performed with complexes of HIV-1 PR with inhibitors SE, RE, RQ. These inhibitors differ from SI in the amino acid at P2' position, carrying Glu or Gln instead of Ile, and in configuration at C4, the carbon bearing hydroxygroup of the HEA group. The hanging-drop vapor diffusion technique has been used in all experiments. In the case of Glu/Gln containing inhibitors protein-inhibitor mixture of 3 mg/ml HIV PR, 0.544 mM inhibitor (four-fold molar excess over protease) in 50 mM sodium acetate pH 5.6, 1 mM EDTA, 0.05% 2-sulfonylethan-1-ol (β-mercaptoethanol, 5% DMSO was used. The optimum crystallization conditions found for these crystals are: 1M $NH_4H_2PO_4$, 100 mM sodium citrate, pH 4.5, temperature 6 - 8 °C [1].

In contrast to Glu/Gln containing inhibitors, SI inhibitor addition caused protein precipitation even in the absence of a salt precipitant. A probable reason is higher hydrophobicity of SI inhibitor. Addition of ammonium phosphate under the conditions described above produced no crystal growth and no additional precipitation. Crystals of maximum dimensions 0.5x0.05x0.05 mm were grown at pH 6-8, 25 °C and low precipitant concentration (0.1 M $NH_4H_2PO_4$, 5 mM sodium citrate, 50 mM sodium acetate), but reproducibility of the crystal growth was very low and crystals often coexisted with precipitate. Good reproducibility has not been achieved until the following measures have been undertaken to decrease protease precipitation by inhibitor:

1) decreasing the inhibitor concentration to a two-fold molar excess over protease
2) increasing the concentration of DMSO in protease-inhibitor mixture to 10%
3) avoiding an abrupt decrease in DMSO concentration in the hanging drop after mixing protein solution with the reservoir liquid
4) including 0.05% TritonX-100 in drop as well as in reservoir solution helped to prevent the crystal twinning that elsewhere became a problem at this stage
5) increasing the protein concentration resulted in a major improvement of reproducibility

These measures lead to two different crystal forms (needles and platelets). NaCl was used as precipitant in all but initial experiments.

Needle-shaped crystals of HIV-1 + SI

Needle-like crystals were grown from protein sample containing 3 mg/ml HIV-1 protease, 0.272 mM SI, 5% DMSO, 1 mM Na_3PO_4 (pH 5.6), 1 mM EDTA, 1 mM DTT in the precipitant which was in 0.77M NaCl and 50 mM sodium citrate (pH 5.0) at 19 °C. The crystals reached maximum dimensions 0.2x0.04x0.06 mm.

Diffraction data of the HIV-1 + SI were measured at the beamline 5.2 of Sincrotrone in Trieste [2] at temperature 8 °C to resolution 2.5 Å. The protease molecules crystallized in the trigonal crystal system with rhombohedral unit cell parameters $a = b = c = 67.9$ Å, $\alpha = \beta = \gamma = 66.5°$. The collected data enabled only a preliminary characterization due to the quick decay of the crystals (30 frames have been collected on 2 crystals) because of radiation damage during measurement at room temperatures.

Platelet crystals of HIV-1 + SI

Plate-shaped crystals of the same complex were obtained from a protein sample containing 6 mg/ml HIV-1 protease, 0.544 mM SI, 10% DMSO, 10 mM sodium acetate (pH 5.6), 1 mM EDTA and 0.5 mM DTT. The crystals were grown in a hanging drop (1 μl protein sample + 1μl

reservoir solution) with reservoir solution which was 1.1 M NaCl, 50 mM sodium citrate (pH 5.0) and contained 5% DMSO and 0.05% TritonX-100 at 4 °C.

A diffraction data set was collected from a crystal of dimensions 0.1 x 0.1 x 0.005 mm at the same beamline of "Sincrotrone Trieste" at 100 K using a cryoprotectant solution consisting of the precipitant solution containing 20% trehalose. The rotation method was used to collect the intensity data in 90° of reciprocal space. The platelet crystals belong to the space group $P2_12_12$ (systematic extinction h00 h=2n, 0k0 k=2n) with the cell parameters a = 58.09 Å, b = 86.58 Å, c = 46.40 Å, $\alpha = \beta = \gamma = 90°$.

There are four molecules of HIV-1 protease dimer per unit cell, implicating position of the protease dimer at the crystallographic twofold axes with inhibitor disordered in the protease tunnel. Data up to resolution 2.15 Å were collected at the sznchrotron source in Trieste. Data analysis using HKL set of programs [5] gave the results summarized in Table 1..

The structure was solved by molecular replacement from the coordinates of HIV-1 protease mutant complexed with tripeptide Glu-Asp-Leu, the PDB code 1A30 [3,4]. The model was selected for the same symmetry, similar unit cell parameters and for good quality of structure refinement. The translation function gave an obvious best solution with the correlation coefficient 60.6% and R-factor 41.5%. The factors improved to C = 73.4% and R = 35.7% after rigid body refinement with data up to 3 Å. The structure is being refined using CCP4 [6], O [7] and X-PLOR [8].

Crystals of HIV-1 + RI

HIV-1 protease was also crystallized with an inhibitor Boc-Pheψ[*(R)*-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2(RI). The best crystals so far obtained were from protein sample containing 6 mg/ml HIV-1 protease, 0.544 mM RI , 5% DMSO, 1 mM Na_3PO_4 (pH 5.6), 1 mM EDTA, 1 mM DTT, 100 mM NaCl and precipitant 275 mM NaCl, 50 mM sodium citrate (pH 5.5) at 4 °C. The crystallization conditions are still under development to get better quality of crystals for diffraction experiment.

Table 1. Overall statistics of measured data from the HIV-1 PR / SI complex

	Overall (10 Å - 2.15 Å)	Last shell (2.23 Å - 2.15 Å)
Completeness	79.9 %	82.9 %
No. reflections	10 174	1 035
Percentage of reflectionswith $I/\sigma < 2$	11.7 %	24.3 %
R_{merge}	0.066	0.140

Acknowledgement

Authors thanks to Jiří Brynda and Juraj Sedláček for providing the native HIV-1 PR and to Martin Hradílek, Milan Souček and Jan Konvalinka for providing inhibitors. The project was supported by the Grant Agency of Academy of Sciences of Czech republic A4050811 and Grant Agency of Czech Republic 203/98/K023. The diffraction data were collected in the framework of the project of synchrotron source Elettra in Trieste.

Abbreviations:

C	correlation coefficient
EDTA	ethylene diamine tetraacetic acid;
DMSO	dimethylsulfoxide
DTT	dithiothreitol
HEA	(2-hydroxyethyl)amine
HIV-1 PR	human immunodeficiency virus 1 protease
PDB	Protein Databank
R	R-factor
R_{merge}	Merging R-factor
RE	Boc-Phe[*(R)*-CH(OH)CH_2NH]-Phe-Glu-Phe-NH_2
RI	Boc-Phe[*(R)*-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2
RQ	Boc-Phe[*(R)*-CH(OH)CH_2NH]-Phe-Gln-Phe-NH_2
SE	Boc-Phe[*(S)*-CH(OH)CH_2NH]-Phe-Glu-Phe-NH_2
SI	Boc-Phe[*(S)*-CH(OH)CH_2NH]-Phe-Ile-Phe-NH_2

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