Peptidomimetic Inhibitors Complexed with HIV-1 Protease: Crystallisation for X-ray Diffraction Studies

J Dohnálek[1,2], J. Hašek[2], J Brynda[3], M. Fábry[3], J. Sedláček[3], J. Konvalinka[4], M. Hradilek[4], M. Souček[4], M.J. Adams[5] and C E. Naylor[5]

1 *Faculty of Nuclear Sciences and Physical Engineering, Czech Technical University, Břehova 7, 110 00 Praha 1, Czech Republic,*
2 *Institute of Macromolecular Chemistry AS CR Heyrovského nám 2, 162 06 Praha 6, Czech Republic*
3 *Institute of Molecular Genetics AS CR, Flemingovo nám 2, 166 37 Praha 6, Czech Republic*
4 *Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo nám 2, 166 37 Praha 6, Czech Republic*
5 *Laboratory of Molecular Biophysics, University of Oxford, South Parks Road, Oxford, OX2 3QU, UK*

Key words: HIV-1 protease, peptidomimetic inhibitors, crystallisation

HIV protease has become one of possible targets of anti AIDS treatment (Wlodawer 1993) The complexing ability of subnanomolar K_i tetrapeptide inhibitors Boc-Phe-Ψ[(S/R)-CH(OH)CH_2NH]-Phe-Glu/Gln-Phe-NH_2 (slashes denote alternatives, the four inhibitors are coded as SE, SQ, RE, RQ) (Konvalinka *et al* 1997) is a subject of investigation by X-ray structure analysis of inhibitor-protease complexes to elucidate high affinity of the inhibitors to the protease dimer, the change of affinity as a result of Glu/Gln alteration at the P2' position and of chirality of the tetrahedrally co-ordinated transition-state-analogue carbon

Details of the binding mode of these inhibitors are expected to explain the differences in affinities measured by K_i , K_i^{SE} = 0 15 nM, K_i^{SQ} = 33 0 nM, K_i^{RE} = 0 12 nM, K_i^{RQ} =14 0 nM

The series of inhibitors Boc-Phe-Ψ[(*S/R*)-CH(OH)CH_2NH]-Phe-**Glu/Gln**-Phe-NH_2 were prepared by alkylation of N-terminal amino group of tripeptides with pure diastereoisomers of N-Boc(1-amino-2-phenethyl)oxiranes at elevated temperature in a protic solvent (Konvalinka *et al* 1997) High-level expression of HIV-1 PR was achieved in an adapted T7 RNA polymerase/promoter system as detailed by Sedláček *et al* 1993

Crystallisation

Protein solution in 50 mM sodium acetate buffer, pH 5 6, 1 mM EDTA (ethylenediaminetetraacetate) and 0 05% β-mercaptoethanol was concentrated to 3 mg ml^{-1} with Centricon SR-3 concentrator (molecular weight cut-off 3 000 Da) The final protein concentration was determined from absorbance at 280nm wavelength Inhibited protease solution with approximately four-fold molar excess of inhibitor in all cases was further used in crystallisation by hanging drop vapour diffusion method (Ducruix and Giege 1992) In the course of crystallisation experiments 1μl protein drops and 0 7–1 0 ml reservoir volumes were applied

Initially crystallisation conditions published in Biological Macromolecule Crystallisation Database were investigated (ammonium sulphate and sodium chloride as precipitants)

(Gilliland *et al* 1994) These did not yield satisfactory results for our protease-inhibitor complexes The initial trials were followed by a screen of selected solutions of Crystal Screen (CS) from Hampton Research (Jancarik and Kim 1991) selection was done with respect to the nature of the buffer present in the protein solution and its pH In 2 3 days first crystals of the SE complex with needle-like shape and the longest dimension 0 8mm were grown in CS solution 9 0 2 M NH_4 acetate 0 1 M Na citrate 30% w/v PEG 4 000

The best conditions from the initial screen CS9 and CS11 (1 0 M NH_4 phosphate 0 1 M Na citrate pH 5 6) solutions were used in optimisation trials for the whole series of complexes Solutions were prepared from following chemicals PEG 4 000 Hampton Research 50% solution Na citrate Hampton Research 1 6 M solution other chemicals from SIGMA or LACHEMA Room temperature experiments for CS9 solution resulted in poor quality needle-like or hair like crystals at 60% original precipitant concentration i e 18% PEG 4 000 Experiments for CS11 solution lead to similar results at about 110% of original precipitant concentration i e 1 12 M NH_4 phosphate As rapid initiation of crystallisation process (crystals appearing in showers of hundreds of small needles) and predominantly one dimensional growth seemed to be the major problem for obtaining good quality crystals the following three factors were chosen for further optimisation additives pH level and temperature Ethanol was added to reservoir solutions in increasing concentration (0% 1% 2 5% and 3%) in CS11 concentration screens (0 8 1 2 M ammonium phosphate) for RE and RQ complexes It was shown that this additive decelerated the processes leading to crystal growth but crystal quality remained unchanged pH screen in range 3 5 10 0 of CS11 experiments for all four complexes confirmed low pH values (3 5 4 5) as an optimum with crystal shape between needle like and plates for RE and RQ inhibitors When microseeding technique (Ducruix and Giege 1992) was applied always only crystals of the same shape and quality were observed In the case of macroseeding if any observable growth of seeds could be identified the crystals grew only in the longest dimension Finally crystallisation experiments at 6 8 °C temperature resulted in growing SE and SQ crystals shape of which could be characterised as thick plates of the smallest dimension 0 1 mm and the largest up to 0 6 mm Identical conditions for RE and RQ inhibitors lead only to better quality needles For these complexes sodium chloride conditions were reinvestigated The optimum of 11 13% NaCl and 6 8 °C lead to needle like crystals of worse quality compared to CS11 Table 1 summarises crystallisation conditions found for all complexes

Crystal Characterisation

X ray diffraction was observed at selected crystals grown under the above mentioned conditions With the first crystal (SE complex) grown in CS9 (25°C) of size 0 8mm diffraction up to 0 28nm was observed (room temperature rotating anode RIGAKU R200 generator Image plate 300 mm) crystals belong to space group $P6_1$, unit cell parameters $a = 6\ 3$ nm, $b = 6\ 3$ nm, $c = 8\ 3$ nm, $\alpha = \beta = 90°$ $\gamma = 120°$ SE and RE crystals grown in CS11, 6 8 °C, pH 4 3 diffracted (100K Diffraction Beamline of Synchrotron source Elettra, Trieste Image plate 345 mm) to 0 21 nm and 0 19 nm respectively, crystals belong to space group $P6_1$, unit cell parameters $a = b = 6\ 28$ nm $c = 8\ 22$ nm, $\alpha = \beta$=90° $\gamma = 120°$ and $a = b = 6\ 29$ nm, $c = 8\ 24$ nm, $\alpha = \beta = 90°$, $\gamma = 120°$ SQ crystals from CS11, 6–8 °C, pH 4 5 diffracted (100K Synchrotron source Elettra) to 0 19 nm, the complex crystallised in space group $P2_12_12$ with unit cell parameters $a = 5\ 81$ nm $b = 8\ 63$ nm, $c = 4\ 61$ nm $\alpha = \beta = \gamma = 90°$ X-ray diffraction data have been subjected to processing and crystal structures refinement is in progress presently

Table 1. Crystallisation conditions for inhibitor HIV-1 protease complexes

Precipitant	Temperature °C	Type of inhibitor complexed with protease			
		SQ	SE	RQ	RE
CS11 (pH 4 5)[1)]	25	n	n	n	n
CS11 (pH 4 5)	6–8	X	X	N	n
CS11 (pH 5 6) +ethanol 0–3%	25	–	–	N[3)]	N[3)]
CS9 (pH 5 6)	25	n	A, n[2)]	n	n
CS9 (pH 5 6)	6–8	p	p	n	p
AS (pH 5 6)	25	n	n	n	n
NaCl (pH 4 3)	6	–	n	N	n

Notes 1) CS = Crystal screen solution, AS = ammonium sulphate, n = needles, thin, low quality, N = needles, thicker, better quality, p = precipitate only, X = good quality "three-dimensional" crystals 2) The first result with original Crystal Screen solution could not be reproduced in optimisation trials 3) 2 × slower crystallisation

Acknowledgements. This project was supported by an internal grant of Czech Technical University "Introduction of X-ray Diffraction Methods for Structural Analysis of Macromolecules Application to Enzymes" No 30497487, grant of the Czech Academy of Sciences GA AV ČR A4050811 and by the program of international cooperation of European Commission "Development of macromolecular X-ray crystallography and computer-aided modelling for genetic engineering and drug design in the Czech and Slovak Republics" No CIPA CT940189

References

Ducruix A , Giege R (1992) Crystallisation of Nucleic Acids and Proteins, A Practical Approach, Oxford University Press, New York

Gilliland G L et al (1994) The biological macromolecule crystallisation database, version 3 0 New Features, Data, and the NASA Archive for Protein Crystal Growth Data Acta Cryst **D50**, 408-413

Jancarik J , Kim S H (1991) Sparse matrix sampling a screening method for crystallisation of proteins J Appl Cryst **24**, 409-411

Konvalinka J , Litera J , Weber J , Vondrášek J , Hradilek M , Souček M , Pichova I , Majer P , Štrop P , Sedlaček J , Heuser A -M , Kottler H , Krausslich H -G (1997) Configurations of Diastereomeric hydroxyethylene Isosteres Strongly Affect Biological Activities of Series of Specific Inhibitors of human immunodeficiency virus Proteinase Eur J Biochem **250**, 559-566

Sedlaček J , Fabry M , Hořejší M , Brynda J , Luftig R B , Majer P (1993) A Rapid Screening Method for Biological Activity of Human Immunodeficiency Virus Proteinase Inhibitors by Using a Recombinant DNA-Derived Bacterial System Anal Biochem **215**, 306-309

Wlodawer A , Erickson J W (1993) structure-based inhibitors of HIV-1 Protease Annu Rev Biochem **62**, 543