

THESIS OF PHD DISSERTATION

***In vitro* studies on the functional characteristics of Human
Immunodeficiency Virus type-1 proteinase and Murine Leukemia Virus
proteinase**

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1. INTRODUCTION

1.1. *The role of retroviral proteases in the viral life cycle*

Retroviral proteases form a unique subclass of aspartic proteases. These homodimeric enzymes from a number of viral sources have been extensively characterized, both structurally and biochemically. The importance of such knowledge to the development of new drugs against acquired immunodeficiency syndrome (AIDS) has been the driving force behind this progress.

The function of retroviral proteases is critical for virion replication. The first identified role of retroviral proteases in the viral life cycle was the cleavage of Gag and Gag-Pol precursor proteins into functional structural proteins and enzymes. Additionally, cleavage of nucleocapsid protein was observed within the viral capsids in the early phase of virus infection. Later it turned out that many cellular proteins are also substrates of HIV proteases which can contribute to the pathogenicity of the virus.

The late phase of the retroviral life cycle starts with the transcription of viral DNA into various RNA forms by the host RNA polymerase II. A significant fraction of newly synthesized mRNA reaches the cytoplasm unspliced, to serve as template for the Gag and Gag-Pro-Pol polyproteins, and to be packaged into virions as genomic RNA. The *gag* gene encodes for the structural proteins of the virus (MA: matrix protein, CA: capsid protein, NC: nucleocapsid protein), and the *pol* gene for the replication enzymes (RT: reverse transcriptase, IN: integrase). The *pro* gene can be expressed in different ways, such as by the suppression of the *gag* termination code (MuLV) or by frameshifting (HIV-1, HTLV, BLV). The relatively low frequency of these events assures that the amount of replication enzymes in the virion is only about 5-10 % of those of the structural proteins encoded by the *gag* gene, while the PR of avian myeloblastoma virus (AMV) is encoded in the *gag* gene and therefore it is synthesized equimolarly with the structural Gag proteins. In the last stage of the late phase of the viral replication, the Gag and Gag-Pro-Pol polyproteins are assembled together with the envelope proteins and the viral genomic RNA at the plasma membrane of the infected cell, where the Env proteins concentrated, then budding yields „immature” virions with a ”doughnut-shaped” capsid structure. The PR cleaves the viral polyproteins at a limited number of sites, producing a condensed, cone-shaped core of „mature” infectious particle. This function of PR is essential for virus replication.

1.2. *General characteristics of retroviral proteases*

The research of retroviruses excited a great interest due to the appearance and rapid spread of the acquired immunodeficiency syndrome (AIDS).

Retroviral proteases consists of 99-138 residues. Their molecular weight is 11-15 kDa and they are aspartic proteases, active in homodimeric form. There is a close homology in the first and secondary structure between retroviral proteases and one domain of cellular aspartic proteases. They contain many β -sheets and one or two short α -helices depending on the enzyme. The N- and C-terminal regions of the two monomers form a four layer β -sheet. There are three specific regions in the enzyme: region of the catalytic triad (-Asp-Thr/Ser-Gly-) which is characteristic of aspartic proteases and found close to the N-terminal end; flap region, which is

rather flexible bending to the substrate during the formation of the enzyme-substrate complex; dimerisation region, for which the N- and C-terminal sequences are responsible.

The residues found in the natural cleavage sites of retroviral proteinases are usually hydrophobic, however, a general consensus sequence cannot be given. The classification of cleavage sites is possible in the case of HIV-1 PR, where type 1 cleavage sites contain Asn-Tyr/Phe↓Pro residues, while type 2 cleavage sites are hydrophobic in P2-P2'¹ positions. However, this generalization does not seem to be appropriate for other retroviral proteinases.

Both oligopeptide and polyprotein substrates have been extensively used to probe the activity and specificity of retroviral proteinases. The measurements are routinely conducted at rather high salt concentration in case of oligopeptide (2-3 M NaCl), but low ionic strength in case of polyprotein substrates. Modifying the oligopeptides makes their measurements easier by allowing the use of spectrophotometric and fluorimetric techniques.

1.3. HIV-1 proteinase as an antiviral target in AIDS therapy

The introduction of combination antiretroviral therapy (HAART, highly active antiretroviral therapy) changed the prognosis of HIV infection. The HIV-related morbidity and mortality rates in patients have significantly declined, however there are severe limits of HAART. Current antiretroviral therapies do not allow viral eradication, therefore long-term use of the drugs is required. As a consequence resistance develops in many cases. Due to the continuing emergence of viral variants that are cross-resistant to the existing inhibitors of PR there is a continuous need for designing new, more effective, broad spectrum PR inhibitors. To broaden the possibility of suppressing the virus more efficiently, we must continue to assess the influence of different genetic backgrounds on the development of resistance.

1.3.1. HIV-1 proteinase inhibitors

A common feature of HIV-1 PR inhibitors currently used in therapy is that they are peptidomimetics, they mimic the substrates of the PR. The enzyme-inhibitor interactions, similar to the enzyme-substrate interactions are primarily hydrophobic ones. Typically these inhibitors contain phenyl residue at the P1 position. Another common feature of these compounds is that they contain a nonhydrolyzable transition state mimic, like a hydroxyethylene group at the site corresponding to the cleavable bound in the substrate.

1.3.2. Problem of resistance

The main cause of the failure of the antiretroviral therapy is the appearing of resistance. In most of the cases mutations occur in the PR gene, several of these conferring resistance involve residues of the substrate binding subsites, therefore they are expected to alter the specificity and catalytic power of the enzyme. Other resistant mutations are located outside of the substrate binding site. The development of high levels of resistance to PR inhibitors, possibly requiring multiple mutations in the PR, was therefore expected to be limited

¹ Nomenclature of substrate residues and substrate binding sites is according to Schechter and Berger (1967). Substrate residues are designated from the cleavage site to the N-terminus with P1, P2, P3, etc., and to the C-terminus with P1', P2', P3', etc. The appropriate substrate binding sites are designated with S1, S2, S3, etc. or S1', S2', S3', etc.

by the functional constraints of the enzyme, which must cleave all precursor cleavage sites during viral replication. However, subsequently other regions of the viral genome are suggested to be involved in drug resistance, by compensating the reduced catalytic activity of drug resistant mutant HIV-1 proteinases.

1.4. Practical importance of the studies on murine leukemia virus (MuLV)

The murine leukemia virus (MuLV) is a prototype mammalian type C virus originally isolated from a sarcoma passaged in BALB/c mice. Injection of MuLV into newborn mice results in developing a generalized lymphocytic leukemia within 3 months. In spite of the importance of MuLV as a model system, only limited information is available on this virus, especially on the PR encoded by it.

1.4.1. Gene therapic application

Gene therapy is the treatment of different genetically inherited diseases by delivering the information encoded by an appropriate DNA sequence into the target cells. Viruses are logical tools for gene delivery. The integration machinery needed for their replication makes them suitable for more efficient gene transfer than any other approaches, such as DNA injection, lipofection, electroporation or chemical transfection. Most of the retroviral genome can be changed for the wanted DNA sequence by simple gene technological methods. Modification of the Env protein makes it possible to influence the spectrum of target cells, allowing development of selective therapeutic strategies. Besides HIV-based retroviral vectors, the ones created by modification of murine leukemia virus, became the most important in the clinical practice.

1.4.2. Inhibitor-design

The HIV-1 PR became a powerful target in AIDS therapy, because of its essential role in viral replication. Various inhibitors designed against HIV-1 proteinase are now in clinical use, however, due to the continuing emergence of viral variants that are cross-resistant to the existing inhibitors of PR indicates, that there is a continuous need for designing new, more effective, broad spectrum PR inhibitors. Comparative studies of different retroviral PRs are expected to reveal the common features of their specificity, facilitating the design of such broad spectrum inhibitors that may reduce the possibility of selection for viable mutants in therapy. Results coming from studies on the less known MuLV PR may help us in this purpose.

2. OBJECTIVES

Moderate to high level of resistance (2 to 100-fold) to PR inhibitors has been observed both *in vitro* and *in vivo*, and has been attributed to the appearance of mutations in the PR gene. Many of these mutations are located in the substrate binding site of the PR, and these mutations have considerable impact on PR activity and specificity. Other resistant mutations alter residues outside of the substrate binding site. Subsequently, a second locus was found to be involved in drug resistance to HIV-1 PR inhibitors, both *in vitro* and *in vivo*, at the nucleocapsid (NC)/p1 and p1/p6 cleavage sites. Cleavage at these sites appears to be a rate limiting step in polyprotein processing, furthermore, there is a significant sequence polymorphism at these sites, which also may have an impact on virion infectivity.

We performed kinetic studies using oligopeptides representing cleavage sites with representative, frequently occurring mutations found as sequence polymorphisms and in drug resistance, with wild type and five drug-resistant PRs (M46L, V82S/A, I84V and L90M) with mutations that appeared together with the Gag mutations *in vivo*, in PR-inhibitor therapy. Based on clinical studies, the resistant Gag mutations may occur early in PR inhibitor therapy, soon after the appearance of one (or a few) critical PR mutations, therefore we have characterized the effect of single proteinase mutations in response to Gag cleavage site mutations, in order to support the assumption, that mutation of the rate limiting cleavage sites, NC/p1 and p1/p6, can partly compensate for the reduced catalytic activity of resistant PR mutants, so they can contribute to the development of resistance.

MuLV is one of the fundamental model retroviruses, and its PR was one of the first to be purified from virion. Furthermore, majority of the retroviral vectors are based on MuLV, and have, so far, been the most widely used in clinical trials. In spite of the importance of MuLV as a model system, only limited information is available on its PR. We decided the cloning and purification of MuLV PR in fusion with maltose binding protein (MBP). Similar expression strategy has been successfully used previously for the expression of HIV-1, bovine leukemia virus (BLV) and human foamy virus (HFV) proteinases with or without flanking sequences. An advantage of expressing a protein in fusion with MBP is that it is very effective to promote the solubility of polypeptides to which it is fused. Further objective of this study was the detailed characterization of the cloned enzyme and its comparison with the viral-derived MuLV PR as well as HIV-1 PR.

3. MATERIALS AND METHODS

3.1. Construction and purification of HIV-1 PR mutants

HIV-1 PR (HIVHXB2CG) having stabilizing substitutions (Q7K, L33I, L63I, C67A and C95A) was cloned into a pET vector, expressed in *E. coli* and purified to homogeneity as described. DNA derived from this clone was used as a template for generating the mutant enzymes by site directed mutagenesis. Mutations were confirmed by nucleic acid sequencing and protein mass spectrometry. The mutant enzymes were purified as described previously for the wild type proteinase.

3.2. Cloning of various MBP-MuLV proteinase fusion protein forms

The proteinase coding region of the MuLV cDNA was amplified by PCR reactions, by using a 5' primer which containing the nucleotide sequence from the start of the PR coding region and mutations to convert the stop codon in the 5th position to a Gln codon, and a 3' primer containing the C-terminal sequence of the PR, a stop codon and an *EcoRI* restriction site. The amplified DNA fragment was cloned into the *XmnI* and *EcoRI* sites of pMal-c2 vector after the maltose binding protein (MBP) gene to produce pMBP-MPR.

The C-terminally His-tagged fusion protein was generated by modifying pMBP-MPR at its stop codon and with the insertion of an annealed oligo-pair (coding for a Factor Xa cleavage site sequence followed by a hexahistidine tag and a stop codon and having *BamHI* as well as *HindIII* restriction site overhangs) into *BamHI*/*HindIII* cleaved vector to generate pMBP-MPR-H₆. Plasmids encoding mutant MuLV PR forms were generated by the Quick-Change mutagenesis protocol with the appropriate oligonucleotide pairs. All constructs and the introduced mutations were verified by DNA sequencing.

3.3. Expression and purification of the MuLV proteinase-fusion proteins

DH5 α or BL-21 *E. coli* cells bearing the constructs for expression were grown in Luria-Bertani medium in the presence of 100 μ g/ml ampicillin. Protein expression was induced by the addition of 1 mM IPTG for 2 hr. Cells were harvested by centrifugation and suspended in lysis buffer containing an inhibitor cocktail tablet against bacterial proteinases. Cells were lysed with sonication. The suspension was clarified by centrifugation and the supernatant was loaded onto a column containing amylose resin, equilibrated with the lysis buffer, then the fusion proteins were eluted by the same buffer containing 20 mM maltose. The collected fusion proteins were processed by Factor Xa, then the PR was purified by cation exchange chromatography to remove the MBP. The pure PR was obtained as a flow-through of a second amylose affinity chromatography. Finally, the purified PR was concentrated and at the same time its buffer was changed for PL buffer (20 mM Pipes, pH 7.0, containing 1 mM EDTA, 100 mM NaCl, 10% glycerol, 5% ethylene glycol, 0.5% Nonidet P-40 and 10 mM DTT) by using a Centricon YM-10 device.

For the purification of the His-tagged construction a modified procedure was used. DH5 α or BL-21 *E. coli* cells bearing the pMBP-MPR-H₆ constructs were also transformed with the pRIL plasmid to provide rare tRNAs,

in these cases the medium was supplemented with 30 µg/ml chloramphenicol. After expression and cell lysis the suspension was clarified by centrifugation, then the supernatant was loaded onto a column containing Ni-NTA superflow affinity resin. The buffer of the eluted fusion proteins was changed, and they were processed by Factor Xa. Most of the MBP-fragments were removed by the addition of 1.5 M ammonium sulphate. After centrifugation the PR remained in the supernatant. The salt-containing buffer was changed, and the residual MBP-derived contamination was removed by cation exchange chromatography. Finally, the elution buffer was changed for PL buffer.

3.4. Extraction of MuLV proteinase from virion

Acetone precipitation of the MuLV proteinase and its extraction was performed by adding 20 volume of cold acetone to purified virus suspension, then after 30 min incubation on ice, the suspension was centrifuged. The precipitate was dried, then extracted with 20 mM Tris, pH 7.2, containing 5 mM DTT, 1 M NaCl for 30 min at 4 °C, with occasional vortexing. The suspension was centrifuged again, and then the buffer of the supernatant was changed for PL buffer.

3.5. Cloning and purification of MuLV Gag fragments

To prepare the GagΔ1 and GagΔ2 constructs, a region of the cDNA MuLV clone was amplified by PCR reaction by using 5' primers coding for the C-terminal sequence of MA and p12 and a *SacI*. restriction site, and the same 3' primer was used in both cases, which contained a mutation to convert the stop codon of the Gag to Gln and a *HindIII*. restriction site. The amplified DNA fragments were cloned into the *SacI*. and *HindIII*. sites of pET-23b. Constructs, verified by restriction analysis and DNA sequencing, were transformed into BL21(DE3) cells. Protein expression was induced by the addition of 1 mM IPTG, and the proteins were purified using metal chelate affinity chromatography, followed by a gel filtration on a Superdex-75 column. The yield of the purification procedure was 3.7 mg protein and 9.2 mg protein per liter culture for the GagΔ1 and GagΔ2 constructs. Proteolysis of these purified proteins were performed in 250 mM phosphate buffer containing 1 mM EDTA, pH 5.6, using 0.6 µM and 1.2 µM GagΔ1 and GagΔ2 constructs, respectively, 20 nM MuLV or 33 nM HIV-1 PR. The samples were analyzed by SDS-PAGE.

3.6. Oligopeptides

3.6.1. The chromogenic substrate Lys-Ala-Arg-Val-Nle-p-nitroPhe-Glu-Ala-Nle-amide (L6525, Sigma, MO) was used in the spectrophotometric assays.

3.6.2. Fluorescent substrate Arg-Glu(Edans)-Ser-Gln-Ala-Phe-Pro-Leu-Arg-Ala-Lys(Dabcyl)-Arg-OH was synthesized by Dr. Ivo Blaha.

3.6.3. Unmodified oligopeptides were synthesized by solid-phase peptide synthesis on a Model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or on the Vega Coupler 250C using Boc or FMoc chemistry, and were purified by reverse phase HPLC. Stock solutions and dilutions were made in distilled water (or in 5 mM dithiothreitol for the Cys-containing peptide), and the proper peptide concentrations were determined by amino acid analysis with a Beckman 6300 amino acid analyzer.

3.7. Assays for measurement of the activity of retroviral proteinases

3.7.1. Spectrophotometric method used for the studies on pH dependence of MuLV PR

70 nM proteinase was added to 100 - 350 μ M chromogenic substrate. The reactions were carried out in META buffer (50 mM MES, 100 mM Tris, 50 mM sodium acetate, 1 M NaCl) at various pH (3-7), and assayed over 10 minutes for the decrease in absorbance at 310 nm on a Hitachi U-3000 spectrophotometer. The absorbances were converted to substrate concentration via a calibration curve. The Michaelis-Menten curves and the bell-shaped curves of pH optimum were fitted using SigmaPlot (SPSS Inc.).

3.7.2. Fluorescent assay for inhibition studies of the MuLV proteinase

For the inhibitor assays, a microtiter plate reader assay using a fluorescent Dabcyl/Edans tagged analog of the p12/capsid substrate was used. Briefly, enzyme, substrate and inhibitor were incubated in 250 mM phosphate buffer, pH 5.6 containing 5 % glycerol, 1 mM EDTA, 5 mM DTT, 500 mM NaCl, 1% DMSO in 96-well microtiter plates. The increase of fluorescence was detected at 460 nm, using 355 nm excitation wavelength in a Victor Wallace fluorimeter-luminometer and corrected with the inner filter effect. K_i values were calculated according to Williams and Morrison.

3.7.3. HPLC-based proteinase assay

The PR assays were initiated by mixing 5 μ l of PR, 10 μ l 2x incubation buffer (0.5 M potassium phosphate buffer, pH 5.6, containing 10% glycerol, 2 mM EDTA, 10 mM DTT, 4 M NaCl) and 5 μ l of 0.5-7 mM substrate for the measurements of wild type and mutant HIV-1 PR and 0.01 to 1.32 mM substrate in case of MuLV PR. The range of substrate concentration was selected depending on the approximate K_m values. The reaction mixture was incubated at 37° C for 1 hour and terminated by the addition of 180 μ l 1 % trifluoroacetic acid (TFA), and an aliquot was injected onto a Nova-Pak C₁₈ reversed-phase chromatography column (3.9 x 150 mm, Waters Associates, Inc.) using an automatic injector (Hitachi). Substrates and the cleavage products were separated using an increasing water-acetonitrile gradient (0-100%) in the presence of 0.05% TFA. Cleavage products were identified by amino acid analysis and/or peptide sequencing. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis-Menten equation by using the Fig. P program (Fig. P Software Corp.). The standard errors of the kinetic parameters were below 25 %.

For some peptides (mutant HIV-1p1/p6 substrates and MuLV Gag cleavage site peptides measurements at low ionic strength) the k_{cat}/K_M values were determined from the linear part of the rate *versus* concentration profile. For peptides, where it was not possible to determine the K_M value from the Michaelis–Menten curve, due to the nonproductive binding at the substrate binding site (HIV-1 NC/p1, MuLV p12E/p2E), k_{cat}/K_M values were determined using competition assays and calculated from the values of substrates having known k_{cat}/K_M values.

3.8. Active site titration for the determination of the exact amount of active enzyme

The active amount of enzyme used in the assays was determined by active site titration using the potent HIV-1 PR inhibitor DMP-323 for the wild-type HIV-1PR, for the M46L, V82A, I84V, L90M mutants and for the MuLV PR, while the V82S HIV-1 PR mutant was titrated using amprenavir. Active site titrations were performed by using the HPLC method detailed above, except 0.2 μ l aliquot of the inhibitor (0 - 10 μ M) in DMSO was added to the reaction mixture. The inhibition curves were determined at three substrate concentrations, and the active enzyme concentration was determined using the DynaFit program. The standard error for the enzyme concentrations was below 20 %.

3.9. Molecular modeling

The HIV-1 models were built from the high resolution crystal structure (PDB entry 1fgc) of a mutant HIV-1 PR-inhibitor complex by altering the appropriate residues of enzyme and inhibitor. Minimization with AMMP included short runs of molecular dynamics as described previously. Analysis of hydrophobic interactions was performed with INTG.

The homolog model of MuLV proteinase, based on the crystal structure of HIV-1, was built by using Modeller3 program.

The structures were built, minimized and examined on Silicon Graphics Indigo2 or O2 computer graphics systems.

4. RESULTS AND DISCUSSION

4.1. Effect of sequence polymorphism and drug resistance on two HIV-1 Gag processing sites

4.1.1. Kinetic parameters for wild-type HIV-1 proteinase-catalyzed hydrolysis of NC/p1 and p1/p6 cleavage site substrates

The two peptides had low specificity constants, as compared to peptides representing other Gag and Gag-Pol cleavage sites tested under identical conditions, in good agreement with the hypothesis that cleavage at these sites might be the rate limiting step of Gag processing. Others also reported that peptides representing these cleavage sites are not efficient substrates of PR. Although the specificity constants for the two substrates were similar, the K_m and k_{cat} values differed remarkably: the NC/p1 peptide exhibited low K_m and k_{cat} values, while the p1/p6 cleavage site showed both higher K_m and k_{cat} values.

4.1.2. Determination of kinetic parameters for wild-type HIV-1 proteinase-catalyzed hydrolysis of natural and mutant NC/p1 and p1/p6 cleavage site substrates

Increasing the concentration of the NC/p1 substrate above the K_m resulted in a decreased velocity, suggesting the possibility of increased nonproductive binding at the substrate binding site. Nevertheless, the specificity constants determined with a competition assay for the NC/p1 substrate, as well as under pseudo first order conditions for the p1/p6 substrate were in good agreement with the values calculated from the Michaelis-Menten curve (Table 1).

4.1.3. Processing of peptides representing NC/p1 cleavage site sequences by wild-type and mutant HIV-1 proteinases

Natural variations of this cleavage site exhibited either decreased or increased specificity constants with the wild-type PR. When compared to peptides having the same natural sequence background, the Ala to Val mutation at P2, seen in resistance, increased the specificity constant by two to tenfold. Based on molecular modeling, the P2 Val fits much better than Ala in the S2 binding site due to more favorable van der Waals contacts with Val 32, Ile 47 and Ile 84, and P3 Arg could interact favorably with different residues of the enzyme and substrate, which may contribute to its beneficial effect in specificity.

The same substrate set was also tested with the mutant PRs. While the combined P3 Arg and P2 Val mutations provided the best combination for the M46L mutant, the P2 Ala to Val mutation alone provided the best specificity constant for the V82 mutants. In these mutants the positive effect of P3 Arg is offset due to the loss of favorable hydrophobic interaction with the smaller Ala or Ser side chain.

V82S and V82A mutants gave lower, L90M higher specificity constants for each substrate as compared to the wild-type enzyme, as expected, however activity of I84V and M46L mutants did not follow the prediction, that active site mutations decrease while nonactive site mutations increase the catalytic power of HIV-1 PR.

4.1.4. Processing of peptides representing p1/p6 cleavage site sequences by wild-type and mutant HIV-1 proteinases

The effect of natural sequence variations on the susceptibility towards PR cleavage has not been reported yet. The natural variations we examined did not substantially change the specificity constants for the wild-type PR, except for the P1 Leu substitution, which provided a very inefficient processing. This result raises the question of whether viral proteins having this mutation could be processed at this site and whether viruses harboring this mutation could be replication competent. In contrast to the natural variations, the P1' Phe substitution, which is seen only in resistant viruses, provided a substantially better substrate for the wild-type and for mutant enzymes. The specificity constant showed a strong correlation with the volume of the P1' residue and with the number of hydrophobic contacts the P1' side chain formed with residues of the S1' subsite. These results suggest that the maximization of the van der Waals interactions of P1' with S1' residues may be the most important feature determining the efficiency of cleavage, similarly to the effects observed for P2 substitution in the NC/p1 site.

4.2. Cloning and purification of Murine Leukemia Virus (MuLV) proteinase and the comparison of the recombinant enzyme with viral-derived MuLV proteinase and HIV-1 proteinase

4.2.1. Cloning and purification of MuLV proteinase

We have cloned the cDNA sequence coded for MuLV PR into pMalc2 plasmid, than BL21 cells were transformed with the construction (pMBP-MPR).

A protocol was developed for the purification of the enzyme, in which amylose affinity chromatography was used to separate the expressed fusion proteins from bacterial proteins. Elution yielded the fusion protein having the expected size, but also various lower migrating protein products. Processing of the fusion protein by Factor Xa yielded some active PR, together with processed MBP. Cation exchange chromatography was used to eliminate the majority of the MBP contamination, but its complete elimination as well as elimination of remaining MBP-MPR fusion protein required a second amylose affinity chromatography. Although the maltose binds tightly to the MBP, therefore usually preventing the second use of amylose affinity chromatography to remove the processed MBP after Xa cleavage, separate experiments showed that the 1 M NaCl used for elution of the proteinase is sufficient to remove maltose from MBP.

Extensive protein degradation seemed to occur during expression. Although applying bacterial proteinase inhibitor cocktail during preparation did not change the profile of the obtained fusion proteins, it protected the purified fusion protein from degradation during storage. Supplementing rare tRNAs by using the pRIL plasmid also did not prevent the observed degradation, suggesting that it is not due to the different codon preference. Immunoblot analysis of the lysate of the bacterial cells expressing the fusion protein coded by pMBP-MPR suggested, that the bands which sizes did not correlate with the fusion proteins bound to the amylose resin are presumably proteins

truncated at the N-terminal part. These proteins, having full length PR sequence, were proteolytically active but lost in the effluent of the column. To increase the yield of PR by

allowing isolation of the N-terminally degraded fusion proteins, a hexahistidine tag with an additional Factor Xa cleavage site was cloned after the PR, and a nickel chelate affinity chromatography was used as the first purification step. Besides the full-length fusion protein, the eluent also contained degraded forms, which were intact at the C-terminus. Factor Xa cleavage eliminated the N-terminal MBP or its fragments as well as the C-terminal histidine-tag. Then an ammonium sulfate precipitation was used to remove additional protein contaminants, and a final cation-exchange chromatography step yielded the pure PR. The yield of this procedure was substantially higher than that obtained with the original protocol.

In degradation studies when D32A active center mutant MuLV PR was expressed in a his-tagged form, the level of MBP degradation was comparable to the level of degradation observed when his-tagged MBP was expressed alone, and it was apparently much lower than the level of degradation obtained in case of the wild-type enzyme. On the other hand, purified MuLV PR was unable to degrade purified MBP. These results suggest that during expression the MuLV PR its activity may be responsible for the activation of bacterial proteolytic system(s), which could degrade substantial amount of the expressed fusion proteins.

4.2.2. Detailed characterization of the Murine Leukemia Virus (MuLV) proteinase

4.2.2.1. Comparison of specificity of recombinant purified proteinase with the proteinase extracted from virions

Although it is widely assumed that bacterially expressed retroviral proteinases behave identically to the virion-derived enzymes, this assumption is seldom verified experimentally. Therefore, the active amount of proteinase was also determined in viral extraction, and the enzymes obtained from the two (bacterial and viral) sources were compared by using oligopeptides representing naturally occurring retroviral cleavage sites. Their specificity was found to be identical within the experimental error of the measurements.

4.2.2.2. Comparison of the MuLV proteinase with HIV-1 proteinase

4.2.2.2.1. pH-dependence of the kinetic parameters

The catalytic system of the aspartic proteinase family is characterized by a bell-shaped pH dependence with respect to the specificity rate constant (k_{cat}/K_m) as previously determined for the HIV proteinase. The pH optimum of the MuLV PR was found to be 5.0 with the same chromogenic substrate used for HIV-1 PR, substantially higher than the pH 4.0 optimal value determined for HIV-1 PR. There are two Asp residues (Asp29' and Asp30') in the S2' binding site of the HIV-1 proteinase, one of them was shown to share a proton with P2'Glu of the substrate. However, the corresponding residues in MuLV PR are Gln36' and His37', and based on molecular modeling studies, these residues are not expected to provide hydrogen bond interactions with P2' Glu as seen in HIV-1 PR. Therefore P2' Glu is preferred by the PR of HIV-1 more than by the PR of MuLV at lower pH. At its optimal pH (5.0) MuLV PR showed the highest k_{cat} and the lowest K_m values.

4.2.2.2.2. Comparison of the specificity of MuLV proteinase and HIV-1 proteinase

Oligopeptides representing naturally occurring cleavage sites in the HIV-1 and MuLV Gag, Gag-Pol and Env polyproteins were tested as substrates for MuLV and HIV-1 PRs. The range of specificity constants for MuLV PR ($1.7 - 15.0 \text{ mM}^{-1} \text{ s}^{-1}$) is narrower than that measured for HIV-1 PR ($0.02 - 202 \text{ mM}^{-1} \text{ s}^{-1}$), but they are in a similar catalytic range, unlike those measured for AMV PR, which were substantially lower. This is in good agreement with the relative amounts of the PR in the virions. The PR of the MuLV and HIV-1 is produced by a different mechanism (suppression of translational termination as well as -1 frameshifting, respectively), the amount of PR is about 5-10% of Gag in both cases, while the PR of AMV is encoded in the *gag* gene and therefore it is synthesized equimolarly with the structural Gag proteins.

The MuLV based peptides were also assayed as substrates of HIV-1 PR, but only three of them were substrates of this enzyme; while with one exception, MuLV PR was able to cleave the HIV-1 cleavage site substrates, although the kinetic parameters were typically lower than those obtained with HIV-1 enzyme. These results indicate the broad specificity of the MuLV proteinase in comparison with HIV-1 PR, due to that most of the MuLV cleavage sites have Leu(Val,Ala)-Leu at the P2 and P1 positions, which are relatively good substrates for the MuLV PR, but previous studies demonstrated that HIV-1 PR prefers rather smaller, more hydrophilic residues at this position .

Although in most cases their own enzyme better hydrolyzed the peptides, there were two exceptions: the MuLV p12/CA cleavage site peptide was a substantially better substrate for HIV-1 PR, and the HIV-1 “in p6” site was better substrate for the MuLV PR.

Strikingly, the MLV p12E/p2E cleavage site peptide was cleaved by the HIV PR one residue upstream from the residue where the cleavage was observed with MuLV PR, which is very rare among the retroviral proteinases. The MuLV Env was found to be cleaved by HIV-1 PR in a cell culture study, but the cleavage site was not determined, based on these results one residue shift at the site of cleavage is not expected to cause a functional defect.

4.2.2.2.4. Cleavage of recombinant MuLV Gag fragments by MuLV and HIV-1 proteinases

To further characterize the specificity of the two proteinases, MuLV Gag fragments containing subsets of cleavage sites were cloned and expressed in bacteria, purified to homogeneity and used as substrates for MuLV PR and HIV-1 PR.

The cleavage assay was performed in the presence of low salt, therefore the kinetic parameters for the peptides representing the cleavage sites incorporated into these Gag fragments were also assayed under identical conditions. All of the peptides were less efficiently hydrolyzed at lower salt concentration, but the tendency seemed to be similar.

The time-dependent cleavage of the shorter Gag fragment expressed from the Gag Δ 2 plasmid suggested that the first processing occurs either at the p12/CA site by generating a 40 kDa protein or at the CA/NC site

generating a 33 kDa protein, while the final mature CA was obtained by processing at the alternating site of each intermediary fragment. Based on these results the cleavage rate at the p12/CA and CA/NC sites is comparable at the protein level, even if the peptide corresponding to the p12/CA cleavage site showed much lower specificity constants, independently of the ionic strength of the assay. At these sites the rate of hydrolysis at the protein level may also be a function of steric effects. Gel filtration experiments suggested that both Gag fragments we have studied oligomerized at the assay conditions, and oligomerization might hinder the accessibility of some cleavage sites.

Using the larger construct Gag Δ 1, again multiple intermediate forms appeared even within a short time of incubation, indicating that the rate of cleavage at the MA/p12 site is comparable to the rate of cleavage at the other sites. These results suggested that the processing at the MuLV cleavage sites occurs at a similar rate, as also could be inferred from the relatively small range of the specificity constants, in sharp contrast with the findings about HIV-1. However, studies of processing within the virus suggested that another cleavage site, the p12/CA site was first to be cleaved. Therefore, caution should be made when kinetic data obtained either with oligopeptides or with recombinant proteins are extrapolated to events occurring within the virion.

The same fusion proteins were also tested as substrates for HIV-1 PR. In good agreement with the lack of cleavage of peptides representing the CA/NC and NC/PR sites, only the 40 kDa CA-NC-C-terminal extended proteins were observed even after 2 h of incubation, in the absence of proteinase inhibitor. The altered cleavage of these proteins with HIV-1 PR also suggested a substantial difference in specificity of the two enzymes.

4.2.2.2.4. Inhibition profile of MLV proteinase

We have also tested different HIV-1 PR inhibitors on the MuLV proteinase using a fluorescent substrate, which was based on the p12/CA cleavage site of MuLV. Amprenavir was able to inhibit MuLV PR under moderate ionic strength of the assay with the lowest K_i value. DMP 323 was also a good inhibitor of the enzyme; furthermore, at high ionic strength, it was useful for active site titration (with a K_i of 0.8 nM). All of the compounds used in AIDS therapy inhibited HIV-1 PR by a K_i value lower than 1 nM in the fluorescent assay. MuLV PR seemed to be less sensitive against these drugs, but could be inhibited by them more strongly than by KH-164, which was previously reported to be the most potent inhibitor of this enzyme.

5. SUMMARY

I have had the opportunity to study the proteinases of two retroviruses having serious practical importance during my Ph.D. work.

In my study on HIV-1 proteinase we have focused on the resistance developing against PR inhibitors used in AIDS therapy, and it was examined in respect of proteinase- and Gag cleavage site mutations.

Based on the general assumptions, mutations located in the binding cleft of the enzyme can lead to the development of drug resistance by increasing K_i of the inhibitors at the expense of impaired proteinase function, while non-active-site mutations may act by enhancing the catalytic efficiency. In our set of mutations the active site mutant I84V enzyme had higher specificity constant than the wild-type PR, while the nonactive-site M46L mutation did not substantially improve the catalytic efficiency of the PR. The activity of L90M, V82S and V82A mutations, however, followed the prediction. We have studied the hydrolysis of oligopeptides representing NC/p1 and p1/p6 cleavage sites with representative mutations found as natural variations or that arise as resistant mutations. Wild type and five drug resistant PRs with mutations appearing together with the Gag mutations within or outside the substrate binding site were tested. While the natural variations showed either somewhat increased or decreased susceptibility of peptides toward the proteinases, the resistant mutations always had a beneficial effect on catalytic efficiency. Comparison of the specificity changes obtained for the various substrates suggested that the maximization of the van der Waals contacts between substrate and PR is the major determinant of specificity: the same effect is crucial for inhibitor potency. The natural nucleocapsid/p1 and p1/p6 sites do not appear to be optimized for rapid hydrolysis, therefore it is possible to increase the cleavage rate by mutation at these sites when the PR activity is diminished due to the accumulation of PR mutations. Consequently, mutation of these rate limiting cleavage sites can partly compensate for the reduced catalytic activity of drug resistant mutant HIV-1 proteinases.

In our study on MuLV proteinase we have cloned the enzyme into pMal-c2 vector and expressed as a maltose binding protein (MBP)-proteinase fusion protein. The fusion protein was purified by amylose affinity chromatography, processed by Factor Xa, and then cation-exchange chromatography was used to remove majority of MBP. A second amylose affinity chromatography step yielded pure proteinase. Substantial degradation of the fusion protein was observed during expression, which severely diminished the yield. To increase the yield by allowing isolation of the N-terminally degraded fusion proteins, not capable to bind to the amylose resin, a hexahistidine tag with an additional Factor Xa cleavage site was cloned after the proteinase and nickel chelate affinity chromatography was used as the first purification step. The isolated full-length and N-terminally degraded fusion proteins were processed by Factor Xa, the majority of contaminating fragments as well as MBP were eliminated by an ammonium sulfate precipitation, then the proteinase was finally purified by cation-exchange chromatography. The modified procedure resulted in substantially higher yield as compared to the original procedure.

We have also studied the possible cause of protein degradation in our expression system. It was very low in case of hexahistidine-tagged active site mutant MuLV, comparable to that obtained with hexahistidine-tagged MBP, but purified MuLV proteinase alone was not able to degrade purified MBP, suggesting that during expression the active MuLV proteinase may activate bacterial proteinases which appear to be responsible for the degradation of the fusion proteins.

The MuLV expressed in *Escherichia coli* was characterized using various assay methods including HPLC-based, photometric and fluorometric activity measurements. The specificity of the bacterially expressed proteinase was similar to that of virion-extracted proteinase. Compared to HIV-1 proteinase, pH optimum of the MuLV enzyme was higher. Specificity of the MuLV proteinase was further compared to that of HIV-1 proteinase using various oligopeptides representing naturally occurring cleavage sites in MuLV and HIV-1 as well as by using bacterially expressed proteins having part of the MuLV Gag. Inhibitors designed against HIV-1 PR were also active on MuLV proteinase, although all of the tested ones were substantially less potent on this enzyme than on HIV-1 PR. These studies indicated that the two proteinases are only distantly related in their specificity.

6. REFERENCES

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