THESIS OF PHD DISSERTATION

Characterization of the Bovin Leukemia Virus Protease and Comparison with Other Retroviral Proteases

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1. INTRODUCTION

1.1. THE RETROVIRUSES

The existence of retroviruses was already known even in the beginning of the last century, however until the discovery of human T-cell leukemia virus (HTLV) and human immunodeficiency virus (HIV) their capability to infect human was not obvious. The research of retroviruses excited a great interest due to the appearance and rapid spread of the acquired immunodeficiency syndrome (AIDS). There are examples for infections of all vertebrates now and they can have many different outcomes: viremia without illness, tumor formation, alterations in nervous system, anemia and immunodeficiency.

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators that include structure, composition, and replicative properties. The virions are 80–100 nm in diameter, and their outer lipid envelope incorporates and displays the viral glycoproteins. The shape and location of the internal protein core are characteristic for various genera of the family. The virion RNA is 7–12 kb in size, and it is linear, single-stranded, nonsegmented, and of positive polarity. The hallmark of the family is its replicative strategy which includes as essential steps reverse transcription of the virion RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell.

Retroviruses are broadly divided into two categories—simple and complex—distinguishable by the organization of their genomes. All retroviruses contain three major coding domains with information for virion proteins: *gag*, which directs the synthesis of internal virion proteins that form the matrix, the capsid, and the nucleoprotein structures; *pol*, which contains the information for the reverse transcriptase and integrase enzymes; and *env*, from which are derived the surface and transmembrane components of the viral envelope protein. An additional, smaller, coding domain present in all retroviruses is *pro*, which encodes the virion protease. Simple retroviruses usually carry only this elementary information, whereas complex retroviruses code for additional regulatory nonvirion proteins derived from multiply spliced messages.

Retroviruses are further subdivided into seven groups defined by evolutionary relatedness, each with the taxonomic rank of *genus*. Five of these groups represent retroviruses with oncogenic potential, and one of the other two groups is the *Lentiviridae*

genus, with the HIV-1.

1.2. THE RETROVIRAL LIFE CYCLE

Viruses enter the cell with receptor mediated endocytosis or direct fusion. The genome RNA positive and regular like the eukaryotic mRNA, although it is not used for protein synthesis. The viral RNA transcripted to DNA by the reverse transcriptase (RT), maybe in the citoplasm inside the core structure. During *in vitro* incubation of equine infectious anemia virus (EIAV) capsids the NC was further processed into smaller fragments by the incorporated PR. Later the HIV-1 NC was found to be processed in a similar manner by HIV-1 PR. It is possible, that active PR which enter the cell within the „core" has a crucial role in reverse transcription and integration.

The new DNA-nucleoprotein complex get into nucleus, when the viral DNA integrate to the host cell's genome (provirus formation).

The late phase of the retroviral life cycle starts with the transcripion of viral DNA into various RNA forms by the host RNA polymerase II. A significant fraction of newly sythesized 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* gen 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 supression of the *gag* termination code (Moloney murine leukemia virus (MMLV)) 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.

While the late-phase function of the PR is well established , it is function int he early phase is controversial and much debated. It was demonstrated for EIAV and later for HIV that

the PR is part of the core structure, which enters the infected cell. In both the receptor – mediated andocytotic and macropinocytotic routes, the incoming core is surrounded by an acidic environment, which has an optimal pH for the retroviral protease. The journey of the core towards the nucleus requires interaction with the actin microfilaments, therefore proteolysis of the components of the actin filament may be an important step in regulation of this process.

Cellular proteins were also detected in virions. For example the peptidyl prolyl isomerase cyclophilin A (Cyp A) that is incorporated into the virion by binding to CA and it is presence enhances viral infectivity. Actin, and various actin-binding proteins have also been detected. The fate of these proteins after entering the cells is mostly unknown, but many were already fragmented in the virions.

Among the proteins of the core, the HIV-1 RT, RNaseH and Nef have been demonstrated to be substrates of the HIV-1 PR. Furthermore, the PR itself undergoes a self-degradation.

1.3. THE BOVIN LEUKEMIA VIRUS (BLV)

The BLV and human T-cell lymphotropic viruses (HTLV) belong to the family of retroviruses, and together with simian T-cell lymphotropic viruses (STLV) to the subfamily of HTLV-BLV group. The characteristics of this group is substantially different from other retroviruses, however the members of the group are closely related, they share a common genome organization, presence of regulatory proteins Tax and Rex, and nucleotide sequence similarity.

BLV is considered to be an important model system for understanding and inhibiting human T-lymphotropic virus 1 (HTLV-1). BLV infection of rabbits can be used as a model system to evaluate vaccination strategies against lymphotropic retroviruses. HTLV-1 PR, like HIV-1 PR, is a target for chemotherapy, especially in the case of tropical spastic paraparesis where active replication is thought to be involved in pathogenesis. The availability of an infectious molecular clone of BLV, together with the cattle and sheep models provides an excellent model system for the development of anti-HTLV-1 therapies. Therefore, it is important to determine to what degree BLV can serve as a model for *in vivo* studies using PR inhibitors. Furthermore, although it has been generally accepted that BLV cannot infect,

replicate and induce cancer in humans, recent findings with state-of-the-art immunological techniques have shown that antibodies reactive against BLV are frequently present in humans, emphasizing the importance of further studies.

1.4. THE RETROVIRAL PROTEASES

The function of retroviral proteinases is critical for virion replication. The first identified role of retroviral proteinases 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 proteinases which can contribute to the pathogenicity of the virus.

Retroviral proteinases consists of 99-138 residues. Their molecular weight is 11-15 kDa and they are aspartic proteinases, active in homodimeric form. There is a close homology in the first and secondary structure between retroviral proteinases and one domain of cellular aspartic proteinases. 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 proteinases 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.

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. Binding of about 6-7 amino acid residues of substrate are necessery for the effective hydrolization.

The residues found in the natural cleavage sites of retroviral proteinases are usually hydrophobic, however, a general consensus sequence cannot be given. Retroviral protease cleavage sites are currently classified into two groups. Type 1 cleavage sites have aromatic residue and Pro, and type 2 sites have hydrophobic residues (excluding Pro) at the site of cleavage. The P2 position is also critical in determining the type of cleavage site. In type 1

cleavage sites of primate lentiviruses, like HIV-1, there is a preference for Asn at P2, while in type 2 cleavage sites the P2 position is typically β-branched. The type 1 cleavage site is very important for several reasons. No other protease, except pepsin, is known to act at the imino side of a Pro residue. Proline residues, especially after Tyr or Phe in the sequence (as the case in type 1 cleavage sites) have a relatively high probability of forming the *cis* isomer rather than the *trans* isomer of the preceding peptide bond. Conformational selectivity of the HIV-1 PR towards the *trans* isomer of the cleaved peptide bond was demonstrated by NMR and kinetic studies.

2. OBJECTIVES

Retroviral (like HIV-1, HTLV-1) is associated with a number of human diseases; therefore its protease - like the human immunodeficiency virus type-1 (HIV-1) protease (PR) - is a potential target for chemotherapy. Residues that confer inhibitor resistance to HIV-1 PR are frequently seen in equivalent positions of other retroviral PRs, as demonstrated in case of HTLV-1 PR. Therefore, understanding the specificity differences of PRs may help the design of inhibitors effective against the mutant HIV-1 PR forms appearing in resistance. The 3-dimensional structures of several different retroviral proteases (from HIV-1, human immunodeficiency virus, type 2 (HIV-2), simian immunodeficiency virus (SIV), Rous sarcoma virus (RSV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV)) have been determined, with the latest addition of the HTLV-1 PR structure. The primary and secondary structures of all retroviral proteases resemble a single domain of the bilobal cellular aspartic proteases. Comparison of modeled PR structures of HIV-1, FIV and EIAV proteases with subsequently determined crystal structures revealed that the models were essentially correct in prediction of the structure of the substrate binding sites. Therefore, molecular modeling of retroviral proteases can serve as an important tool in the absence of crystal structures.

Bovine leukemia virus (BLV) is a valuable model system for understanding the human T-lymphotropic virus 1 (HTLV-1); the availability of an infectious BLV clone together with animal model systems will help to explore anti-HTLV-1 strategies. Nevertheless, the specificity and inhibitor sensitivity of the BLV protease (PR) has not been characterized in detail. To facilitate such studies, we wanted to build a molecular model for the enzyme and to

investigate the specificity of the BLV PR with a set of oligopeptides representing naturally occurring cleavage sites in various retroviruses and a series of peptides containing amino acid substitutions in a sequence representing a naturally occurring HTLV-1 PR cleavage site ((Lys-Thr-Lys-Val-Leu↓Val-Val-Gln-Pro-Lys oligopeptide (arrow denotes the site of cleavage)). Our aim was to replace some amino acid residues of the BLV PR substrate binding sites by the equivalent ones of HIV-1 PR and to test inhibitors of HIV-1, HTLV-1 and other retroviral proteases on the BLV PR.

Previously a large series of peptides containing single amino acid substitutions in the P4-P3' region of the Val-Ser-Gln-Asn-Tyr↓Pro-Ile-Val-Gln oligopeptide was used to characterize the specificity of the protease of various retroviruses including that of HIV-1, HIV-2, EIAV, MMLV and AMV. Activities relative to the unmodified peptide were determined for substituted peptides (except for MMLV PR) to compare the specificity of the enzymes. Based on these studies, the P2 position was found to be one of the most critical ones in determining the substrate specificity differences of retroviral proteases. We wanted to extend these studies for the P2 position with the BLV and other proteases (Mason-Pfizer monkey virus (MPMV), mouse mammary tumor virus (MMTV), HTLV-1, human foamy virus (HFV) and walleye dermal sarcoma virus (WDSV) proteases) which set now contains at least one member of each genera of the *retroviridae*.

3. MATERIALS AND METHODS

3.1. RETROVIRAL PROTEASES

Purified proteases were prepared as described previously. The relative activities for the HIV-1 PR, EIAV PR and AMV PR have been reported previously.

3.2. OLIGOPEPTIDES

Oligopeptides were synthesized by solid-phase peptide synthesis on a Model 430A automated peptide synthesizer or on the Vega Coupler 250C using Boc chemistry, and were purified by reversed-phase high-performance liquid chromatography. Stock solutions and dilutions were made in distilled water (or in 10 mM dithiothreitol for the Cys-containing

peptide), and the proper peptide concentration was determined by amino acid analysis with a Beckman 6300 amino acid analyzer. Peptides were obtained from Dr. Stephen Oroszlan or from Dr. Terry D. Copeland (Molecular Virology and Carcinogenesis Laboratory, NCI-FCRDC, Frederick, MD, USA).

3.3. ASSAYS WITH OLIGOPEPTIDE SUBSTRATES

Protease assays of activity were performed using purified retroviral proteases (5µl) and chemically synthesized oligopeptides (5µl, 1.6 mM) in 0.25 M potassium phosphate buffer (10µl), pH 5.6, containing 7.5% glycerol, 5 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40 and 2 M NaCl. The reaction mixtures were incubated at 37 °C for 1-4 hours or 24 hours in case of HFV and WDSV proteases, and were stopped by the addition of 9 volumes 1% trifluoroacetic acid (TFA) then injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 x 150 mm, Waters Associates, Inc.) using an automatic injector. Substrates and the cleavage products were separated using acetonitrile gradient (0-100%) in water, in the presence of 0.05% TFA. The cleavage of peptides was monitored at 206 nm, and the peak areas were integrated. Amino acid analysis of the collected peaks was used to confirm the site of cleavage with HIV-1 PR. For the other retroviral proteases the cleavage products were identified by the retention time, which was found to be identical to that obtained with HIV-1 PR. Relative activities were calculated from the molar amounts of peptides cleaved per unit time at less than 20% substrate turnover, by dividing the activity on a given peptide by the activity on the Val-Ser-Gln-Val-Tyr↓Pro-Ile-Val-Gln substrate, which has the smallest hydrocarbon side chain at P2 site among the peptides that were hydrolyzable by all studied proteases. Measurements were performed in duplicate and the average values were calculated. The standard error was less than 10%.

Determination of the kinetic constants were performed as mentioned above, with the following changes: we used 5 µl (12 - 7800 nM) purified retroviral proteases and 5 µl (0.03 – 2.90 mM) substrate in the reaction mixtures. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis-Menten equation (or by linear fitting for k_{cat}/K_m values determined under pseudo first order conditions) by using the Fig. P program (Fig. P Software Corp). The standard errors of the kinetic parameters were below 20 %. For some peptides the k_{cat}/K_m values were determined from the linear part of the rate *versus* concentration profile, or using competition assays. Amino acid analysis of the collected peaks was used to confirm the site of cleavage with at least one PR (typically with

the PR of the same retrovirus) and to quantitate the amount of substrate cleaved; for other proteases the site of cleavage was assumed to be identical if cleavage products eluted with the same retention time and gave the same relative integration values as those identified by analysis. In case of ambiguity, cleavage sites were also verified by mass spectrometric analysis.

3.4. MUTAGENESIS OF THE BLV PR, AND STUDIES OF THE SELF-PROCESSING AND FOLDING ABILITY.

The pMAL-BLVPr clone coding for the BLV PR with an N-terminal but without a C-terminal flanking region was used as a template for the mutagenesis. Mutants were generated by the Quick-Change mutagenesis protocol with the appropriate oligonucleotide pairs. Mutations were verified by DNA sequencing performed with ABI-Prism dye terminator cycle sequencing kit and an Applied Biosystems Model 373A sequencer.

Protein expression was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3.5 h to DH5α cells harboring the plasmid coding for the wild-type or mutant MBP-BLV PR fusion proteins in 5 ml cultures. After expression, cells were collected in 50 mM Tris-HCl, pH 8.2, containing 1 mM EDTA, 1 mM DTT, 1 % Triton X-100 and disrupted with sonication. Protein samples were separated by SDS-PAGE and transferred to a PVDF membrane. Immunoblots were developed using an antiserum of a rabbit immunized with chemically synthesized BLV protease and a peroxidase-conjugated anti-rabbit antibody, using an ECL detection kit. Mutant proteases that showed substantial self-processing ability were purified after large scale expression (500 ml) by ion exchange chromatography as described for the wild-type enzyme while the other mutants were purified in the fusion form using amylose affinity chromatography. All of the mutants were at least 95% pure based on Coomassie staining of SDS polyacrylamide gels.

Self-processing activity was calculated from densitometric analysis of immunoblots of small culture expressions, while folding efficiency was calculated from the ratio of active enzyme determined by using active site titration and total protein amount. Active site titration of the wild type and mutant BLV proteinases were performed using peptide KTKVL-r-VVQPK (IB268) where -r- represents a reduced peptide bond. Reduced peptide bond-containing inhibitors used in this study (IB268 and IB269: APQVL-r-PVMHP) were synthesized by Dr. Ivo Blaha (Ferring Leciva).

3.5. INHIBITOR PROFILING

For the inhibitor assays, a microtiter plate reader assay was used with a fluorescent Dabcyl/Edans tagged analog of the capsid↓nucleocapsid substrate of HTLV-1 PR (RE(Edans)TKVL↓VVQPK(Dabcyl)R). The 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 Wallac 1420 Victor^2 fluorimeter-luminometer. The inner filter effect correction and K_i values were calculated with the KiDet program

3.5. MOLECULAR MODELING

The crystal structure of the HTLV-1 PR with a substrate-based statine inhibitor was the basis for the model for BLV PR. The amino-acid residues of HTLV PR were replaced by those of BLV PR. The BLV PR dimer was modeled with the peptide substrates TKVL↓VVQP and SQNY↓PIVQ using the program AMMP as described previously for HTLV-1 PR. The C-terminal residues 118-126 were not included in the model. A conserved water molecule was included between the flaps and the peptide, and a proton was used to stabilize the charged oxygen atoms of the two catalytic Asp residues. The positions for all new atoms were generated with the sp4 potential set using 15 cycles of the Kohonen algorithm followed by conjugate gradients minimization of the non-bonded and geometrical terms. Finally, the entire PR-substrate complex was minimized using 400 steps of conjugate gradients. Structural models were examined using the molecular graphic programs Sybyl or RasMol run on Silicon Graphics workstations or Linux PCs. The models have been deposited in the Protein Data Bank with codes 2IM6 for BLV PR in complex with TKVL↓VVQP, and 2IM7 for BLV PR with SQNY↓PIVQ.

Structure-based alignment of the HIV-1, HIV-2, SIV, EIAV, FIV and RSV proteases were used as a template for the alignment of BLV, HTLV-1, MPMV, MMTV, MMLV, HFV and WDSV protease sequences for comparative investigation of the S2 subsites of the different retroviral proteases. Structural alignment was made by Whatif, and the initial multiple sequence alignment was made by ClustalW and was corrected by hand based on the structural alignment. Phylogenetic tree was made by ClustalW and Phylip.

The program Modeller was used to build the initial models of all studied retroviral proteases. The program allows the use of multiple template crystal structures as an input, and

can create multiple homologous models as an output. We used the highest (1.2-2.4 Å) resolution crystal structures of HIV-1, HIV-2, EIAV, FIV and RSV proteases (either as a wild-type or a mutant form) available at the time of modeling as templates (PDB accession codes [and resolutions]: 1K1T [1.2 Å], 1IDA [1.7 Å], 1FMB [1.8 Å], 4FIV [1.8 Å], 1BAI [2.4 Å], respectively) and generated 3 simultaneous models for each protease. RSV protease structure was used to interpret the AMV protease results: AMV and RSV proteases differ only in two residues, which are not expected to be involved in the enzyme-substrate interactions. A model of QVY↓PIV hexapeptide was docked into the substrate binding site of each retroviral protease models in forward and in reverse direction, and a water molecule was also placed in a conserved position between the flaps of the protease and the substrate. This sequence has the smallest hydrocarbon side chain at P2 site among the peptides that were hydrolyzable by all studied proteases and the appropriate substrate was also used as a reference substrate for activity measurements.

Unfavorable van der Waals interactions were removed by short minimizations using Sybyl with the following parameters: Kollman all atom force field as implemented in Sybyl, 8 Å cutoff, 20 Simplex and 100 Powell iterations with distance constraints between the carboxyl groups of the two catalytic aspartates and between the H-bond donor and acceptor atoms of the enzyme and the substrate in the P3-P3' region to maintain the conserved H-bond network of substrate binding site. Another 100 Powell iterations were applied with distance constraints only between the catalytic aspartates.

The structures were examined on a Silicon Graphics Indigo2 or O2 computer graphics system using the programs Sybyl or Whatif. Root mean square (RMS) deviations were calculated by structural superposition modul of Whatif. Cavities were calculated on the minimized structures containing P2 Gly substrate using SiteID modul of Sybyl, and they were averaged on 3 models and 2 directions for each enzyme. At least 3 values were included in the averaging procedure for each enzyme. Volume of the amino acid residues was retrieved from the literature. Connolly-type molecular surfaces and Poisson-Boltzman electrostatic potentials were calculated by MolCAD modul of Sybyl. The residues forming the S2 subsites were predicted previously for HIV-1, HIV-2, EIAV, AMV, MMLV and HTLV-1 proteases and the corresponding residues in BLV, MMTV, MPMV, HFV and WDSV proteases were obtained from the sequence alignment and verified using the crystal structures/homologous models.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF THE BLV PROTEASE AND ITS COMPARISON WITH THE HTLV-1 AND HIV-1 PROTEASES

4.1.1. *Modeled structure of the BLV protease*

In the absence of a crystal structure for the BLV PR, a model was built with two peptide substrates in order to understand the molecular basis for the specificity. The amino acid sequence of BLV PR was aligned with the sequences of other retroviral proteases of known structure to determine the best starting structure for building the model. Although numerous crystal structures are available for the HIV-1 PR, the HTLV-1 PR structure was determined only recently. The BLV, HTLV-1 and HIV-1 proteases have different lengths. The HIV-1 PR is the shortest with 99 residues, followed by the HTLV-1 PR (125 residues), while BLV PR has 126 residues. In spite of the differences in length, the crystal structures of HIV-1 and HTLV-1 proteases share a conserved core region that includes the substrate binding site and the dimer interface. Therefore, the molecular model of BLV PR was predicted to share the conserved core region of HIV-1 and HTLV-1 proteases. The BLV PR showing 55% sequence similarity with the HIV-1 and 89% with the HTLV-1 PR in the substrate binding region. The extra amino acids of BLV and HTLV-1 proteases form longer loop structures on the surface of the molecules. In addition, BLV and HTLV proteases have C-termini that are extended by 10 additional amino acid residues compared to the HIV-1 PR. The function of the C-terminal extension (residues 116-125) is controversial. Based on some previous studies, these residues were not required for activity of HTLV-1 PR, while five of the C-terminal residues (116-120) appeared to be important in another study. The final model of the BLV PR dimer with substrate had a RMS (root mean square) difference of 0.79 Å for 223 pairs of C_α atoms compared to the crystal structure of HTLV-1 PR, and 1.72 Å for 175 pairs of C_α atoms compared to the crystal structure of HIV-1 PR. These values are comparable to the rms difference of 1.25 Å observed for 194 pairs of C_α atoms in the dimers of RSV S9 PR and HIV-1 PR with inhibitor.

4.1.2. *Studies on the specificity of the BLV protease using oligopeptides representing naturally occurring cleavage sites in different retroviruses*

To compare the specificity of BLV PR to those of HTLV-1 and HIV-1 proteases, a large set of oligopeptides representing naturally occurring cleavage sites in HIV-1, HIV-2, EIAV, RSV, MMTV, MMLV, HTLV-1 and BLV was used. These peptides have been characterized previously. They were found to be hydrolyzed by the respective PR coded within the same virus, and have been used to compare the specificity of HTLV-1 and HIV-1 proteases. The majority of the peptides was hydrolyzed by both BLV (40/50) and HIV-1 (40/50) proteases. However, only two peptides were not substrates for both enzymes and sixteen were substrates of one of them. Selecting 1 $mM^{-1}s^{-1}$ as an arbitrary threshold to separate efficient substrates from inefficient ones, the majority of the substrates can be considered as efficient ones in both cases. However, only 20 out of the 50 peptides were substrates for the HTLV-1 PR, and only half of them were efficiently hydrolyzed. In most cases the lack of hydrolysis could not be attributed to the lack of binding of the peptides, since they were able to inhibit the enzyme activity when tested with the HTLV-1 P1 Phe-substituted capsid/nucleocapsid (CA/NC) peptide. The specificity results on these peptides indicated a substantially broader specificity of the BLV and HIV-1 proteases as compared to the HTLV-1 PR. Interestingly, one residue shift from the authentic site of cleavage was observed in three cases. One of these peptides was cleaved at the authentic site (DLVL↓LSAE) by HIV-1 PR but at a shifted site (SDLV↓LLSA) by the BLV PR, while it was not a substrate for HTLV-1 PR. The other one was cleaved also at a shifted site (TSTL↓LIEN) besides the concomitant cleavage at the authentic site (STLL↓IENS) by BLV PR, while this peptide was not substrate for the other two proteases. On the other hand, a BLV cleavage site was cleaved at a shifted site by HIV-1 PR (ELEC↓LLSI) as compared to the authentic one (LECL↓LSIP). Although these shifts are in agreement with the substantially more hydrophilic nature of the S4, S2 and S2' subsites of HIV-1 (as discussed below), they cannot be readily explained by the specificity of the proteases, due to its strong sequence context dependence. Only a few peptides were efficiently cleaved substrates with significant specificity constants ($k_{cat}/K_m > 1$ $mM^{-1}s^{-1}$) for all three proteases: these include the peptides representing the HIV-1 RT/IN, HIV-2 RT/IN, RSV p3/NC, MMTV p3/p8, and HTLV-1 MA/CA, CA/NC and PR/pX cleavage sites. Common characteristics of these generally efficient substrates are that all of them contain beta-branched residues (Val or Ile) at P2, and also at P2', with the exception of the two HIV sites, and, with one exception, they contain Leu at P1.

4.1.3. *Characterization of the specificity of the BLV protease with a series of peptides representing the capsid/nucleocapsid cleavage site of HTLV-1*

A peptide series representing the CA/NC cleavage site of HTLV-1 was previously successfully utilized to compare the specificity of the HIV-1 and HTLV-1 proteases, as well as to determine the specificity changes exerted by HIV-1 PR-like mutations in HTLV-1 PR. This set contained peptides with N-terminally shortened sequences and peptides with single amino acid substitutions in the P4-P1' positions. This peptide set proved useful in characterizing the specificity of BLV PR in this study. The kinetic, together with the specificity constants (k_{cat}/K_m) previously determined for the HTLV-1 and HIV-1 proteases. The specificity constants obtained for the BLV PR were in the same range as those determined for the other two proteases, however, hydrolysis with BLV PR generally resulted in relatively low K_m and k_{cat} values, as also was observed for the natural cleavage site peptides.

4.1.3.1. Size of the substrate binding site of the BLV PR

The original unmodified peptide was a good substrate of all three proteases. It is important to note that this peptide was also a good substrate for several other retroviral proteases (our unpublished data), therefore it can be considered as a general PR substrate. Interestingly, the shortened peptides were even better substrates than the full-length peptide for the BLV PR. In this aspect the BLV PR appears to be more similar to the HIV-1 PR than to the HTLV-1 PR, for which the best substrate was the full-length decapeptide. This result suggested that the BLV PR and HIV-1 PR have less extended substrate binding sites than does HTLV-1 PR.

4.1.3.2. Characterization of the S4 substrate binding site of the BLV PR

All the P4 substituted peptides were good substrates for the BLV PR. The best substrates were obtained when the original Thr was exchanged to hydrophobic amino acid residues, such as Val or Leu, similar to the results with the HTLV-1 PR. However, unlike these two enzymes, HIV-1 PR preferred the more hydrophilic Ser (and Thr) in this position.

The S4 subsite of retroviral proteases is close to the surface, and more or less open to the solvent. A side chain at P4 may either interact with the solvent molecules or may form interactions with residues of the binding pockets. A unique feature of the HIV-1 and other

primate lentiviral proteases is that they contain Asp30, while most of the retroviral proteases including BLV PR and HTLV-1 PR contain uncharged residues at the equivalent position. The BLV PR has Asn at this position, which still may form hydrogen bonds with the Thr residue of the original HTLV-1 substrate, while the corresponding HTLV-1 PR residue is Met. Besides the critical Asn residue, the other residues of S4 form a more hydrophobic pocket in BLV PR as compared to HIV-1 PR. Therefore, BLV PR S4 more closely resembles the S4 subsite of other (nonprimate) retroviral proteases. Interestingly, the Asp 30 to Asn mutation occurs frequently in HIV-1 PR with drug resistance to PR inhibitors.

4.1.3.3. Characterization of the S3 substrate binding site of the BLV PR

All of the studied P3-substituted peptides were well hydrolyzed by the BLV PR. The range of specificity constants was smaller for the BLV PR than for the other two enzymes: the only large deviation was with the Ala substitution. Based on these results various amino acid residues can be accommodated at this subsite by the enzymes.

The S3 subsites of all retroviral proteases characterized so far consist of a relatively open and deep pocket that is near the surface. The side chains of amino acids in the P3 position have freedom of movement to either interact with hydrophobic residues near the S1 subsite or polar residues at the surface of the enzyme. Furthermore, in BLV PR Leu 58 and Trp 99' can interact with hydrophobic P3 residues (as predicted for the identical residues in HTLV-1 PR). Val 82' of HIV-1 PR, which is structurally equivalent to the Trp residues of the deltaretroviral proteases, provides a deeper pocket, which explains why the substrates containing P3 Phe or Leu were much better for HIV-1 PR as compared to the substrates with small or medium sized side chains, like Ala or Val.

4.1.3.4. Characterization of the S2 substrate binding site of the BLV PR

Using the HTLV-1 cleavage site peptide series, substitution of Val to other hydrophobic residues provided similar or even better substrates for BLV PR. The best substrates were obtained with Phe and Ile substitutions. The same substitutions exerted various effects on the other two proteases; they were typically very unfavorable for HIV-1 PR, but some of them were well tolerated by HTLV-1 PR. The preference for larger hydrophobic P2 residues is consistent with the presence of smaller S2 residues Ala and Val in both BLV and HTLV-1 proteases instead of Ile47 and Ile50 in HIV-1 PR. Unlike HTLV-1

and HIV-1 proteases, BLV PR tolerated well the hydrophilic Asn residue at this position, even though Asn is the typical P2 residue in type 1 but not in type 2 cleavage sites.

The S2 subsites of all PRs are sterically more restricted than the S4 and S3 subsites and are predicted to accommodate mainly hydrophobic residues. Based on the side chains forming S2, the BLV PR is expected to have a somewhat larger pocket as compared to that of HIV-1 PR. The ability of HIV-1 PR to accommodate more polar residues may be related to the presence of Asp 30. Except for Asn 38 and Tyr 68, which are Met and Phe, respectively, in HTLV-1 PR, all other residues forming the S2 subsite in BLV PR S2 are identical to those in HTLV-1 PR, but substantially different from those forming the S2 site in HIV-1 PR. However, due to the presence of Asn 38 and Tyr 68, the S2 subsite of BLV PR is more hydrophilic compared to the corresponding S2 subsite of HTLV-1 PR, in good agreement with the relatively good kinetic efficiencies obtained for peptides with Asn, Asp or Ser at the P2 position.

4.1.3.5. Characterization of the S1 substrate binding site of the BLV PR

Interestingly, various peptides with P1 substitutions, including charged or hydrophilic residues, in the HTLV-1 cleavage site peptide set provided fairly good substrates for BLV PR, while HTLV-1 and HIV-1 proteases tolerated only hydrophobic residues and Gly at this position. P1 Tyr or Phe substitutions were the best for the proteases, followed by other hydrophobic residues in varied order.

Based on structural analysis, the S1 subsite is mainly hydrophobic, and buried inside the protein. Many of the residues forming the S1 site of the retroviral proteases are conserved. However, there are some nonconserved changes. The Thr 80'-Pro 81'-Val 82' part of HIV-1 PR and the corresponding regions of other retroviral proteases provide a 'roof' over the S1 binding site and these residues are involved in determining the size of the pocket. In HTLV-1 and BLV proteases, these three residues are replaced by a Trp residue, since the other two side chains cannot interact directly with the substrate. Trp 99' of BLV PR renders the S1 pocket smaller than that of HIV-1 PR which has Val 82 in the equivalent position. It is an interesting feature of the retroviral proteases that a bulky P1 side chain is predicted to occupy a region that is also a part of the S3 pocket. The S1 and S3 subsites seem to overlap much more than S4-S2, S2-S1', S1-S2' and S1'-S3' pockets. This could be a major factor in the sequence context dependence of the results of specificity studies based on different peptide sequences.

4.1.3.6. Characterization of the S1' substrate binding site of the BLV PR

Hydrophilic amino acid residues at the P1' position were relatively well tolerated by the BLV PR as compared to the other two proteases. The Pro substitution provided a large increase in K_m for HTLV and HIV-1 PRs, while the relatively large k_{cat} in the case of BLV PR yielded a relatively high specificity constant. Due to the symmetry of the PR, the S1' subsite is structurally very similar to the S1 subsite, which is formed by the corresponding residues of the other subunit of the dimer. Therefore, the better tolerance of the BLV PR for various P1' residues is in good agreement with the predicted participation of Trp 99 in this binding site, rather than the loop of Thr80, Pro81 and Val82 as in HIV-1 PR.

4.1.4. *Sensitivity of the BLV protease towards substrate binding site mutations*

To further understand the specificity of BLV PR, and its tolerance to mutations, several residues in the substrate binding site were mutated to the corresponding HIV-1 PR residue, individually or in combination. Many of the mutants appeared to be defective in self-processing ability, indicating the lack of proper folding or activity of the folded mutants. The mutant containing Asp in place of Asn38 behaved aberrantly since a partial self-processing resulted a truncated protease: this is predicted to be due to the creation of a new cleavage site by the mutation. The sensitivity towards mutation may extend to residues outside the substrate binding site. Based on the molecular models, Asn97 and Lys98 of BLV PR cannot participate directly in ligand binding, unlike the corresponding Thr80 and Pro81 of HIV-1 PR, nevertheless, mutation of Lys98 to Pro also completely blocked the activity of the enzyme as was observed with the Asn97 to Thr mutation. Lack of activity of the mutants was also verified by HPLC-based PR assay of highly purified, concentrated MBP- PR fusion proteins with an oligopeptide substrate. Only one mutant, W99V, was able to completely self-process itself from the fusion protein. Previous studies with HTLV-1 PR also indicated a high sensitivity towards mutations, while HIV-1 PR activity showed much better toleration of mutations of the substrate binding residues.

It is important to note that while HIV has a high mutation rate while HTLV-1 (and predictably BLV) do not utilize the error-prone reverse trancriptase to generate substantial sequence diversity, since the virus chiefly maintains its high proviral load through clonal expansion of the HTLV-infected cells. As a consequence, BLV, like HTLV-1 has not undergone the extensive mutational changes that might have optimized the HIV-1 PR for both

high catalytic efficiency as well as flexibility in tolerating mutations under selective pressure.

4.1.5. *Inhibition profile of the BLV protease*

Several potent inhibitors that target HIV-1 PR are currently used in clinical therapy. We have tested the clinical inhibitors saquinavir, ritonavir, nelfinavir, amprenavir and indinavir, as well as other retroviral PR inhibitors on BLV PR. Most of these inhibitors were previously tested on HIV-1 and HTLV-1 proteases using the same fluorescent assay or by the more conventional HPLC-based assay; for comparison, the data obtained for HTLV-1 and HIV-1 PR with the fluorescent measurements are also shown. While all of the HIV-1 PR inhibitors inhibited HIV-1 PR with a K_i value less than 30 nM, most of them were weak inhibitors or practically inactive on BLV PR; only amprenavir and compound 3 inhibited this enzyme to an appreciable manner. Interestingly, all four HTLV-1 PR sequence-based inhibitors inhibited BLV PR more strongly than HTLV-1 PR, while they were inactive against HIV-1 PR. This effect appears to correlate with the lower K_m values of the corresponding substrates for the BLV PR. One of these inhibitors, IB-268 was very potent against BLV-1 PR, having an unusually low K_i value considering the moderate ionic strength used in the assay, and it is predicted to be useful for *in vivo* inhibition studies on BLV PR. This compound was even more potent at the high ionic strength conditions used in the HPLC-based assay, and was utilized to perform the active site titration of the enzyme. This strong dependence of the K_i values on ionic strength has been observed earlier. With the exception of amprenavir, which substantially discriminated between BLV and HTLV-1 proteases, there is a correlation ($R = 0.91$) between the inhibition values obtained for the two deltaretrovirus proteases.

4.2. COMPARISON OF THE S2 SUBSTRATE BINDING SITES OF ELEVEN DIFFERENT RETROVIRAL PROTEASES

Previously we have characterized the specificity of HIV-1, HIV-2, EIAV, AMV proteases using an oligopeptide substrate series based on the naturally occurring type 1 cleavage site (Val-Ser-Gln-Asn-Tyr↓Pro-Ile-Val-Gln) between the matrix (MA) and capsid (CA) proteins of HIV-1. Previous studies indicated a strong correlation between the relative activities and the specificity constants, therefore the determined activity values can be considered as a measure of the k_{cat}/K_m values.

In this study we extended these studies for the protease of MPMV, MMTV, MMLV, HTLV-1, BLV, HFV and WDSV to have at least one member of each *genera* of the retroviruses.

4.2.1. *Determination and comparison of relative activities obtained on a series of oligopeptide substrates having amino acid substitutions in P2 position of a naturally occurring type 1 cleavage site*

Substitution of the P2 Asn in the original peptide to medium-sized hydrophobic residues resulted in peptides that were well hydrolyzed by most proteases. The largest effects were observed with HTLV-1 and HFV proteases, where the original nonhydrolyzable peptide was converted to hydrolyzable ones, and with BLV PR, where the best substrate was the P2 Leu containing peptide, which was almost 200-fold better substrate as compared to the unmodified peptide. P2 Leu containing peptide was also the best substrate for MMLV PR, as described in a previous study. The peptide having Ile substitution was the best substrate for MMTV and HTLV-1 proteases. The small hydrophobic amino acid, Ala was preferred by AMV and MPMV proteases. EIAV PR preferred the slightly polar Cys residue, while HIV-1 PR preferred the more polar Asn residue, giving the best value with the original peptide. Although in case of HIV-2 and WDSV proteases, Cys was the best residue in this position, a similar value was obtained for Asn. HFV PR showed equally high activity on substrates containing Ala, Val or Cys at P2 site within the experimental error of the measurement. Therefore, in this sequence context, the preference for Asn by the HIV-1 PR is rather exceptional, since the other studied retroviral proteases showed higher preference for hydrophobic or less polar residues. It seems to be a common result for retroviral proteases, that Phe or Gly at P2 of this peptide series forms a poor substrate: molecular modeling suggested that the Phe side chain is too bulky, while Gly is too small for this subsite.

4.2.2. *Building of homologous models of the S2 substrate binding sites and interpretation of measured enzyme activities at submolecular level*

To understand the specificity similarities and differences between retroviral proteases, molecular models were built for all studied enzymes by using the same procedure. The goodness of the models was estimated by calculating their RMS deviations from the five high-resolution crystal structures used as templates. Naturally the smallest values (0.3-0.5 Å) were calculated for HIV-1, HIV-2, EIAV and RSV models from their own crystal structures templates, and their

range showed similar values as can be seen in comparison of different crystallographic determination of the same enzyme. Other models showed similar RMS deviations (0.9-2.9 Å) to the values calculated between the templates themselves (1.0-2.7 Å) and they also showed similar relationship between the RMS deviation and the sequence homology as can be seen in comparison of crystal structures.

Based on the molecular models, the S2 binding sites of retroviral proteases are usually small, hydrophobic pockets. S2 is sterically more restricted than the other subsites, especially S4 and S3. About half of the subsite forming residues of S2 are conserved, however, in place of Asp 30 of HIV-1 PR and HIV-2 PR, which appears to be the reason why these enzymes could accommodate more polar residues into this subsite, other enzymes contain hydrophobic residues or hydrophilic ones but pointing outward from the pocket. At the tip of the flap most enzymes contain branched side chains (Ile, Val, Leu), but MMLV, BLV and HTLV-1 PRs contain smaller Ala residue, which may at least partly responsible for larger Ile and Leu preference at S2 sites of these enzymes. Previously we have observed that the two residues which seem to be crucial in determining the preference for Val over Leu at P2 in our substrate series are residues of the retroviral proteases corresponding to Val 32 and Ile 47 of HIV-1: in both positions Ile favors Val at P2 in the substrate, while Val favors Leu. BLV, HTLV-1 and MMLV PRs have Val at both positions, and the relative preference for Leu over Val is in good agreement with the previously proposed rule. This suggests that size complementarities are the main specificity determining features of the S2 subsite of retroviral PRs, and electrostatic contributions play role only in case of proteases of primate lentiviruses (HIVs and SIVs). To demonstrate this suggestion, cavity volumes were calculated in cases of P2 Gly substrate complexes and were plotted against the averaged volume of the best two P2 residues measured experimentally. The data points (except of HIV-1 PR) showed linear correlation ($r = 0.96$) and were clustered into two regions of the plot according the P2 preference of the enzymes. This correlation was further supported by phylogenetic analysis of sequences of retroviral proteases.

4.2.3. *Investigation of amino acid residues in P2 position of naturally occurring type 1 cleavage site sequences*

Naturally occurring type 1 cleavage site sequences of the studied retroviruses have P2 residues which are usually in good agreement with the findings of S2 mapping study. Asn can be found at P2 position in 5 out of 6 cleavage sites of HIV-1 and HIV-2, and the peptide having P2 Leu showed a very low cleavage efficiency. The S2 specificity of EIAV PR appear to show a

transition between the specificity of primate lentiviruses (preference for polar residues) and the alpha-, beta- and gammaretroviruses (preference for small hydrophobic residues): the preferred residues are Cys and Ala in the context of VSQXY↓PIVQ sequence, while Glu and Thr are found in the natural cleavage sites of EIAV. The best P2 residue (Ala) observed for AMV protease in the mapping study appears in the the P2 position of its naturally occurring type 1 cleavage site sequence, however, the MPMV and the MMTV sites contain less favorable Ile and Thr, respectively, at this position. In one of the MMLV cleavage sites the most preferred Leu appears in P2, while the other contains a less preferred Ala. Discrepancies between the most preferred P2 residues and those observed in the naturally occurring cleavage sites might be due to the different sequence context, which has been shown to have a profound effect on the subsite preference of HIV-1 PR including S2 as well as due to the possibility that not all retroviral cleavage sites are optimized evolutionarily for rapid processing.

The importance of the proper van der Waals interactions and filling in the S2 binding site is also demonstrated by mutations appearing in drug resistance. One of the slowest HIV-1 cleavage sites, the NC/p1 cleavage site (Arg-Gln-Ala-Asn↓Phe-Leu-Gly-Lys) contains a small Ala residue at P2, which does not fit optimally into this pocket, as demonstrated by kinetic, modeling and crystallographic studies; this residue is frequently mutated to Val in drug resistance to provide a substantially better fit.

5. SUMMARY

During my Ph.D work I had the opportunity to study the BLV proteinase in details and to compare its features with other retroviral proteases (mainly with HIV-1 and HTLV-1 proteases).

We built a molecular model of the BLV PR. The specificity of the BLV PR was characterized using a large set of oligopeptides representing naturally occurring cleavage sites in various retroviruses. Based on the results, the BLV PR appears to have a broad substrate specificity, similar to HIV-1 PR, but unlike the related HTLV-1 PR. The broader specificity of the BLV PR, compared to the HTLV-1 PR, was also verified by mapping the individual substrate binding sites using a set of oligopeptides with single amino acid substitutions. Furthermore, the substrate binding site of BLV PR appeared to be less extended compared to that of HTLV-1 PR. While both BLV and HTLV proteases showed a preference for larger hydrophobic P2 and P1 residues, BLV PR much better tolerated hydrophilic or even charged residues at these positions. Nevertheless, in most aspects the specificity of individual subsites

of BLV PR more closely resembled that of HTLV-1 PR, in good agreement with the more similar sets of residues predicted to be involved in substrate binding, as compared to those of HIV-1 PR. It appears to be a common characteristic of BLV and HTLV-1 proteases that their folding capability and/or catalytic efficiency are much more sensitive towards mutations than many other retroviral proteases, especially HIV proteases. Inhibition profile of the BLV PR resembles more to that of HTLV-1 PR as compared to HIV-1 PR. However, BLV PR was substantially better inhibited as compared to the HTLV-1 PR. This effect appears to correlate with the generally lower K_m values observed for BLV PR as compared to HTLV-1 PR.

The specificity of the critical S2 substrate binding site of the proteases of eleven retroviruses representing each of the seven genera of *retroviridae* was studied using a series of oligopeptides having amino acid substitutions in P2 position. Despite the previous experiment, when we employed oligopeptides based on naturally occurring type 2 cleavage site sequences, the S2 binding site of BLV PR appeared to be a relatively large pocket and a preference for Leu was observed, without considerable hydrophilic residue tolerance. However, it is important to note that the specificity of retroviral proteases appears to be strongly context dependent, as reviewed for HIV-1 PR.

The specificity distinction of the proteases correlated well with the phylogenetic tree of retroviruses prepared solely based on the PR sequences. Molecular models for all studied proteases were built, and they were used to interpret the results. While size complementarities appear to be the main specificity-determining features of the S2 subsite of retroviral proteases, electrostatic contributions may play a role only in case of HIV proteases. In most cases the P2 residues of naturally occurring type 1 cleavage site sequences of the studied proteases agreed well with the observed P2 preferences.

In conclusion, based on our studies, despite the specificity differences, in terms of mutation intolerance and inhibitor susceptibility of the PR, BLV and the corresponding animal model systems may provide good models for the test of PR inhibitors that would be developed by in vitro studies against the protease of HTLV-1. Understanding the specificity similarities and differences of the retroviral enzymes may help to design broad-spectrum inhibitors against HIV-1 PR.

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