PROTEASES OF HIV-1 AND MAV HYDROLYZE SPECIFICALLY HUMAN APO-HEMOPEXIN

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The specificities of HIV-1 (Human Immunodeficiency Virus Type 1) and MAV (Myeloblastosis Associated Virus) proteases have been evaluated for their ability to split two-domain protein human apo-hemopexin. Both proteases hydrolyze only one peptidic bond Leu_{240}-Ser_{241} located in the connecting region between two domains. The ability of viral proteases to cleave Leu-Ser bond was confirmed by cleavage of synthetic octapeptide His-Leu-Val-Leu-Ser-Ala-Leu-Thr-NH_2 covering the susceptible area of human apo-hemopexin. The results demonstrate that the cleavage of Leu-Ser bond is not due to its location in the interdomain region of apo-hemopexin. The cleavable bond Leu-Ser has never been found either in viral or in non-viral proteins. According to the vector projection method this octapeptide was considered as non-hydrolyzable.

Key words: HIV-1 protease; MAV protease; Proteolytic cleavage; Proteases; Hemopexin; Enzyme catalysis; Viral Proteins; Viruses.

Viral encoded proteases play essential roles in the life cycle of retroviruses. The high selectivity and specificity of the cleavage of viral polyprotein are the crucial condition for the generation of infectious virus particles. Selectivity of viral proteases is governed first by the susceptibility of the appropriate peptide bond. The comparison of primary structures of susceptible bonds in natural substrates and their surroundings has not brought a clear picture of the specificity of retroviral proteases. Several attempts have been made to extend these data by evaluation of non-viral proteins as substrates of retroviral proteases. It has been shown that only small number of non-viral proteins is susceptible to the proteolytic attack of viral proteases[1]. The natural substrate, the viral polyprotein, contains probably several distinct domains. It is possible to presume that protein substrates of retroviral

proteases are cleaved in the connecting regions between structural domains. The ability of the enzyme to recognize the conformation rather than to entirely rely on the primary structure of the substrate could be a possible explanation of the stability of many non-viral proteins. Multidomain non-viral proteins could mimic in their interdomain regions the natural polyprotein substrate. This approach to the problem was successful in three-domain Pseudomonas exotoxin (PE 66) and related proteins[2]. On the other hand, several other multidomain proteins such as CD 4, tissue plasminogen activator, citrate synthetase, 3-phosphoglyceric phosphokinase and adenyl kinase were found not to be susceptible to the proteolytic attack of retroviral proteases. Thus, a particular conformation and the presence of susceptible bond could be crucial for proteolytic cleavage and therefore, it is valuable to look for susceptible bonds among multidomain proteins.

We have chosen human apo-hemopexin, a single chain protein synthesized by liver, as a model for our study. Computer assisted analysis of the internal homology in amino acid sequence indicates that apo-hemopexin contains two domains separated by positively charged histidine and glycine-rich hinge-like region. The limited tryptic cleavage site of apo-hemopexin is also located in the interdomain region[3]. We can show that this two-domain protein can be split by action of two retroviral proteases within the interdomain area and the cleavage of susceptible Leu-Ser bond is not influenced by the presence of the domains.

EXPERIMENTAL

Abbreviation used. ALV, avian leukemia virus; CD4, soluble extracellular fraction of T4 surface antigen; Gla, galactosamine; Glc, glucosamine; HIV, human immunodeficiency virus; MAV, myeloblastosis associated virus; PE 66, Pseudomonas endotoxin; PR, proteinase; PTH, phenylthiohydantoin; RSV, Rous sarcoma virus; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

Materials. HIV-1 and MAV proteases were cloned, expressed and purified as described by Weber *et al.*[4] and Pichová *et al.*[5]. Human apo-hemopexin was prepared from Cohn fraction IV by precipitation with rivanol and by affinity chromatography using hemin bound to the Biogel P 100 (ref.[6]). Carboxymethyl apo-hemopexin was prepared by reduction with 2-sulfanylethanol and subsequent substitution by iodoacetic acid in 6 M guanidin hydrochloride[6]. Cyanogen bromide fragment CB5 of carboxymethyl apo-hemopexin (residues 232–352) was purified by reverse-phase chromatography[6]. Peptide substrate His-Leu-Val-Leu-Ser-Ala-Leu-Thr-NH_2 was synthesized by the solid-phase method[7] using MBHA (4-methylbenzhydrylamine resin). All amino acids were protected with *N-tert*-butyloxycarbonyl group. Side-chain protection was as follows: benzyl (Ser,Thr), *tert*-butylcarbonyl (His). The cleavage of peptide from the resin and of protecting groups from amino acid side chains was accomplished by liquid HF in the presence of anisole (0 °C,

1 h). Crude products were purified by reverse-phase water–methanol gradient on a column of Sepharon-RPS (10 μ, Tessek, Praha). Peptide was characterized by amino acid analysis and by sequencing.

Digestion of proteins with proteases. Apo-hemoxin and its derivatives were digested in 0.2 M sodium phosphate, 2 M NaCl, pH 6.0. In a typical preparative experiment, 40 nmol (2 mg) of protein was dissolved in 1.5 ml of phosphate buffer and after addition of 0.70 nmol of retroviral protease, the concentration of NaCl was adjusted to 2 mol l^{-1}. The progress of reaction at 30 °C was monitored by SDS-PAGE (ref.[8]) for 16 h. The final stage of the cleavage was characterized by sequencing of the reaction mixture.

Digestion of synthetic substrates. Peptidic substrate as well as the cyanogen bromide fragment of apo-hemopexin were dissolved in the above-mentioned buffer. The enzyme–substrate ratio was 1 : 100. Digestion reaction mixtures were incubated at 30 °C for 16 h. Reactions were stopped by the addition of trifluoroacetic acid and the mixture was subjected to HPLC on a Vydac C_{18} column with gradient 0–80% methanol. The effluent from the column was monitored at 220 nm. Collected fractions were dried and subjected to amino acid analysis and sequencing.

Analytical methods. Amino acid analysis was performed by conventional automated ion-exchange chromatography in a Durrum D-500 model analyzer. Samples were hydrolyzed in vacuum in 6 M HCl at 110 °C for 24 h. Automated Edman degradation of protein and peptides was carried out in an Applied Biosystems model 470 Gas-phase Sequencer using program 03RPTH, fitted with on-line HPLC analyzer for phenylthiohydantoin (PTH) amino acids. A Shimadzu-2A integrator was used for integration of peaks.

SDS-PAGE–Laemmli[8] 12.5% gels were run in the presence of reducing agents and stained with Coomassie Blue G-250 for detection of proteins. The molecular weight of apo-hemopexin calculated from amino acid sequence is 49 295, however the band corresponding to apo-hemopexin migrates with a higher mobility (M_r about 63 kDa), due to the carbohydrate content[3].

Kinetic constants k_{cat} and K_m were determined from a Michaelis–Menten plot by nonlinear regression program Enzfitter.

Prediction. Prediction of cleavability of the synthetic substrate by HIV-1 protease was calculated by the vector projection method[9].

RESULTS AND DISCUSSION

We have paid our attention to multidomain proteins as substrates for retroviral proteases. We have found a model in two-domain protein, human apo-hemopexin, which can be in native form split by action of HIV-1 and MAV proteases. The identical gel pattern of apo-hemoxin fragments produced by hydrolysis with HIV-1 and MAV protease is shown in Fig. 1. A considerable increase of molecular weight of apo-hemopexin and its fragments in comparison with calculations from amino acid sequence is due to the carbohydrate content[3]. The cleavage of apo-hemopexin by MAV protease is slower than that by HIV-1 PR. The lower catalytic efficiency of MAV protease compared to HIV-1 protease is a known characteristic of the enzyme. MAV and the closely related proteases RSV and ALV are roughly ten-

fold less active on optimal substrates than HIV-1 PR (ref.[1]). Since the gene encoding PR in avian retroviruses is in the C-terminus of *gag* reading frame, the level of PR in virions is *ca* 20-fold higher than in HIV-1 in which the gene encoding PR is in the N-terminus of *pol* reading frame. Thus, the lower activity of avian proteases is compensated with their high level in virions.

The sequence analysis of products generated with proteolysis of apo-hemopexin by MAV or HIV-1 proteases confirmed that two sequences are present in the digestion mixture in equimolar ratio. The sequence Thr-Pro-Leu-Pro-Pro-Thr-Ser corresponds to the N-terminal sequence of apo-hemopexin and the second sequence Ser-Ala-Leu-Thr-Ser-Asp-Asn was identified in the interdomain region very close to the C-terminal domain of apo-hemopexin (Fig. 2). Number of fragments obtained by proteolysis as well as the presence of only one sequence Ser-Ala-Leu-Thr-Ser-Asp-Asn in the primary structure of apo-hemopexin[3] confirm that the susceptible bond for both proteases is between Leu_{240}-Ser_{241}. A prolonged incubation of the above-mentioned fragments with both proteases did not show any change in the number of fragments. The connecting region of apo-hemopexin is also susceptible to limited trypsinolysis[3] (after Arg_{216}). However, the N-terminal fragment of apo-hemopexin released by action of trypsin is susceptible to further fragmentation.

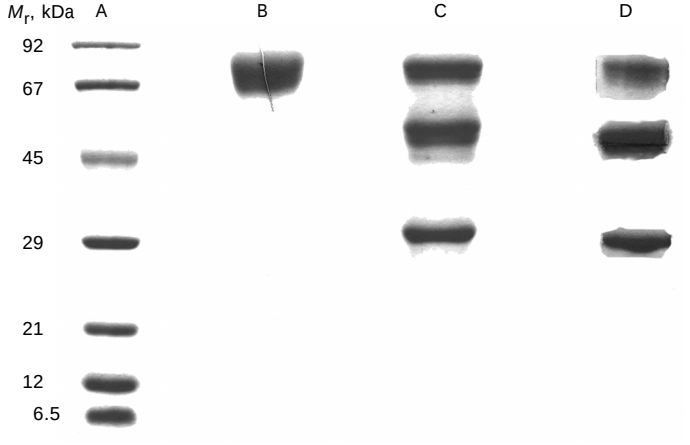

FIG. 1

Digestion of human apo-hemopexin by HIV-1 and MAV proteases. SDS-PAGE electrophoresis, gel 12.5%, staining with Coomassie Blue G 250. Lane A, molecular weight standards; lane B, human apo-hemopexin; lane C, hydrolyzate of apo-hemopexin with HIV-1 protease and apo-hemopexin (ratio 1 : 60) after 4 h of incubation at 30 °C; lane D, hydrolyzate of apo-hemopexin with MAV protease (ratio 1 : 60) after 6 h of incubation at 30 °C

The susceptibility of Leu_{240}-Ser_{241} bond can be caused by the specific conformation of the connecting region or by the Leu-Ser bond itself. To distinguish between these possibilities we have prepared the fully reduced and *S*-carboxymethyl derivative of apo-hemopexin, apo-hemopexin denatured by heating at 80 °C for 30 min, as well as the cyanogen bromide fragment of *S*-carboxymethylated apo-hemopexin, covering the region Arg_{232}–$Homoserin_{352}$. Drastic conditions of preparation could change the tertiary structure and eliminate the susceptibility of the peptidic bond. We have found that all derivatives were split by both HIV-1 and MAV PRs in the same way as the native protein. As we have no direct proof of the influence of the preparation method on the tertiary structure of apo-hemopexin, we have prepared an octapeptide His-Leu-Val-Leu-Ser-Ala-Leu-Thr-NH_2 corresponding to the retroviral protease-sensitive region. The substrate was readily soluble in water and it was completely cleaved by HIV-1 and MAV protease in two fragments His-Leu-Val-Leu and Ser-Ala-Leu-ThrNH_2. The fragments were isolated by chromatography on the reverse phase and characterized by amino acid and sequence analysis.

The cleavage of peptide His-Leu-Val-Leu-Ser-Ala-Leu-ThrNH_2 by HIV-1 protease was kinetically characterized as rather slow (k_{cat} = 0.05 s^{-1}, K_m = 240 ± 30 μmol l^{-1}). However, the value is comparable to those obtained for other peptidic substrates representing cleavage sites in viral polyproteins (see Table I) and may reflect the inability of a short peptide to stay in the

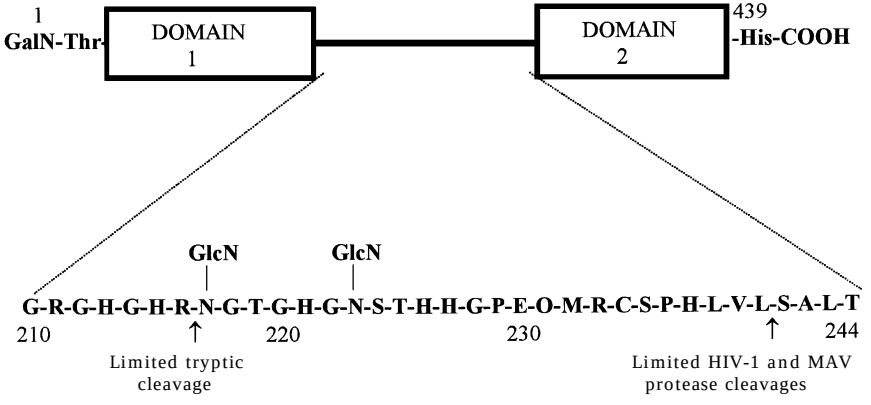

FIG. 2
Schematic representation of the molecule of apo-hemopexin with cleavage sites indicated for trypsin and retroviral proteases (HIV-1, MAV). Both cleaved bonds are located within the connecting region between domains

optimal conformation during its binding in the active site of retroviral proteases. The efficient cleavage of substrates by retroviral proteases is dependent on multiple interactions between side chains of amino acids upstream and downstream of the scissile bond in the substrate with the residues in corresponding subsites of proteases. Thus, the catalysis of splitting of the substrate derived from apo-hemopexin is also influenced by the specificities of HIV-1 and MAV PRs. According to the vector projection method[9] octapeptides having $\Gamma \geq 0$ are classified as hydrolyzable by HIV-1 PR with a rate of correct prediction 88.2%. Although the calculated value for the octapeptide derived from apo-hemopexin was $\Gamma = -0.021$, we have shown that this peptide is cleaved with HIV-1 and MAV PRs. This discrepancy confirms the limited applicability of computational methods for prediction of the cleavage of peptidic substrates with retroviral proteases.

The results of this study demonstrate that HIV-1 and MAV proteases are able to cleave the Leu-Ser bond in apo-hemopexin. In spite of common occurrence in proteins, the peptide bond Leu-Ser has never been identified as susceptible to the proteolytic attack of retroviral proteases. The presence of the Leu-Ser bond in the interdomain region of apo-hemopexin reveals its susceptibility toward the proteolytic attack. The cleavage of the derivatives of apo-hemopexin, the CNBr-fragment, and the synthetic peptide, repre-

TABLE I
Comparison of kinetic parameters of synthetic peptides representing cleavage sites in *gag* or *gag–pol* viral polyproteins (**10**) and human apo-hemopexin cleaved by HIV-1 protease

Peptide sequence	Representing cleavage site in	K_m mmol l^{-1}	k_{cat} s^{-1}	k_{cat}/K_m s^{-1} mmol^{-1} l
V-S-Q-N-Y↓P-I-V-Q	viral polyprotein	0.15	6.8	45.3
K-A-R-V-L↓A-E-A-M-S	viral polyprotein	0.01	0.09	90
T-A-T-I-M↓M-Q-R-G-N	viral polyprotein	0.05	3.7	74
V-S-F-N-F↓P-Q-I-T-L	viral polyprotein	0.01	0.06	6.9
R-P-Q-N-F↓L-Q-S-R-P	viral polyprotein	0.53	0.3	0.6
C-T-L-N-F↓P-I-S-P	viral polyprotein	0.07	1.5	24.1
A-E-T-F↓Y-V-D-G-A-A	viral polyprotein	0.04	0.4	10
I-R-K-I-L↓F-L-D-G	viral polyprotein	0.006	1.2	202
H-L-V-L↓S-A-L-T	human apo-hemopexin	0.24	0.05	0.2

senting the susceptible region of apo-hemopexin, shows that this bond is cleaved not only due to its location in the interdomain region of apo-hemopexin. Our results support the validity of the idea of Tomasselli *et al.*[2] that the extended structures of nonviral multidomain proteins resemble the natural viral polyprotein and can be used for evaluation of the susceptibility of peptide bonds toward proteolytic attack of viral proteases.

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