THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 274, No. 10, Issue of March 5, pp. 6660–6666, 1999
Printed in U.S.A.

Stabilization from Autoproteolysis and Kinetic Characterization of the Human T-cell Leukemia Virus Type 1 Proteinase*

(Received for publication, September 22, 1998, and in revised form, December 14, 1998)

John M. Louis‡§, Stephen Oroszlan¶, and József Tözsér§‖

From the ‡Laboratory of Chemical Physics, NIDDK, National Institutes of Health, Bethesda, Maryland 20892, and ‖Department of Biochemistry and Molecular Biology, University Medical School of Debrecen, H-4012 Debrecen, Hungary, and ¶Molecular Virology and Carcinogenesis Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

We have developed a system for expression and purification of wild-type human T-cell leukemia virus type 1 (HTLV-1) proteinase to attain sufficient quantities for structural, kinetic, and biophysical investigations. However, similar to the human immunodeficiency virus type 1 (HIV-1) proteinase, HTLV-1 proteinase also undergoes autoproteolysis rapidly upon renaturation to produce two products. The site of this autoproteolytic cleavage was mapped, and a resistant HTLV-1 proteinase construct (L40I) as well as another construct, wherein the two cysteine residues were exchanged to alanines, were expressed and purified. Oligopeptide substrates representing the naturally occurring cleavage sites in HTLV-1 were good substrates of the HTLV-1 proteinase. The kinetic parameters k_{cat} and K_m were nearly identical for all the three enzymes. Although three of four peptides representing HTLV-1 proteinase cleavage sites were fairly good substrates of HIV-1 proteinase, only two of nine peptides representing HIV-1 proteinase cleavage sites were hydrolyzed by the HTLV-1 proteinase, suggesting substantial differences in the specificity of the two enzymes. The large difference in the specificity of the two enzymes was also demonstrated by inhibition studies. Of the several inhibitors of HIV-1 or other retroviral proteinases that were tested on HTLV-1 proteinase, only two inhibit the enzyme with a K_i lower than 100 nM.

The human T-cell leukemia virus type 1 (HTLV-1)[1] is a retrovirus that has been etiologically associated with human adult T-cell leukemia (1, 2), HTLV-1-associated myelopathy, tropical spastic paraparesis (3, 4), and a number of other chronic diseases (5). Although it has not been shown to be directly linked to the development of leukemia, several recent studies indicate that viral replication is critical for the development of HTLV-1-associated myelopathy. Initial studies have reported the treatment of this syndrome using 5′-azidothymidine (6).

All replication-competent retroviruses including HIV and HTLV-1 code for an aspartic proteinase (PR). Unlike HIV-1, HTLV-1 PR is coded through a mechanism of expression in separate *gag-pro* and *gag-pro-pol* open reading frames (7). The level of expression of the Gag and Gag-Pro polyproteins in HTLV-1 is comparable with that of Gag and Gag-Pol polyproteins in HIV-1. The function of the mature 125-amino acid-long HTLV-1 PR is crucial for virion replication (for a review, see Ref. 8). The HIV-1 PR proved to be a promising target of antiretroviral therapy of AIDS, and various PR inhibitors are now in clinical use (for a review, see Ref. 9).

As in the case of treatment of patients with reverse transcriptase inhibitors, selection of viral variants that are resistant to inhibitors of PR also develops rapidly (9). Some of the amino acid changes in the HIV-1 PR that are responsible for drug resistance are found in equivalent positions of other retroviral proteinases, including HTLV-1 (see Fig. 1). A comparative study of various retroviral proteinases is expected to reveal the common features of their specificity. These studies will aid in the rational design of inhibitors effective against different retroviral proteinases, which may reduce the frequency of occurrence of drug-resistant mutants. In this respect, many inhibitors designed against HIV-1 PR have much reduced potency against the closely related HIV-2 PR (10) as well as against the PR of lentiviral equine infectious anemia virus (11). Furthermore, a recent study demonstrated that all four HIV-1 PR inhibitors currently used in therapy failed to block HTLV-1 Gag processing *in vitro* (12).

Although several studies report expression of HTLV-1 PR in *Escherichia coli* (13–17) as well as its chemical synthesis (18), its biochemical and kinetic properties and specificity have not been well described. This can be attributed to the difficulty in protein purification and to the autoproteolysis that is known to occur with native retroviral proteinases (19, 20). Peptides representing some of the natural cleavage sites of HIV-1 are hydrolyzed by HTLV-1 PR (16, 18), although no kinetic parameters were reported for these peptides.

The crystal structures of HIV-1 and several other retroviral proteinases have been determined (21–23). However, to date no structural information is available for the HTLV-1 protease. Furthermore, insight into the mechanism of autocatalytic maturation of the mature PR from the precursor is limited to HIV-1 (24, 25). Here we report the high level expression in *E. coli* and rapid purification of wild type and autoproteolysis-resistant HTLV-1 proteinases. The specificities of these two enzymes were compared utilizing synthetic peptides that correspond to known Gag and Pol cleavage sites of HIV-1 and HTLV-1. Sev-

* This research was sponsored in part by the Hungarian Science and Research Fund (OTKA T 16396, OTKA T 22140), by the Hungarian Ministry of Culture and Education (FKFP 1318/97), by the National Institutes of Health (NCI) Department of Health and Human Services under contract with Advanced BioScience Laboratories and by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health.

§ Contributed equally to this research. To whom correspondence should be addressed. J. Tözsér: Tel./Fax: 36-52-416-432; E-mail: tozser@indi.biochem.dote.hu or J. M. Louis: Tel./Fax: 301-594-3122/301-496-0825, E-mail: jmlouis@helix.nih.gov.

[1] The abbreviations used are: HTLV-1, human T-cell leukemia virus type 1; HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2, respectively; DTT, dithiothreitol; HPLC, high performance liquid chromatography; PR, proteinase; PIPES, 1,4-piperazinediethanesulfonic acid; MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; C2A-PR, stabilized HTLV-1 PR containing substitution mutations L40I,C90A,C109A. The nomenclature of viral proteins is according to Leis *et al.*(51).

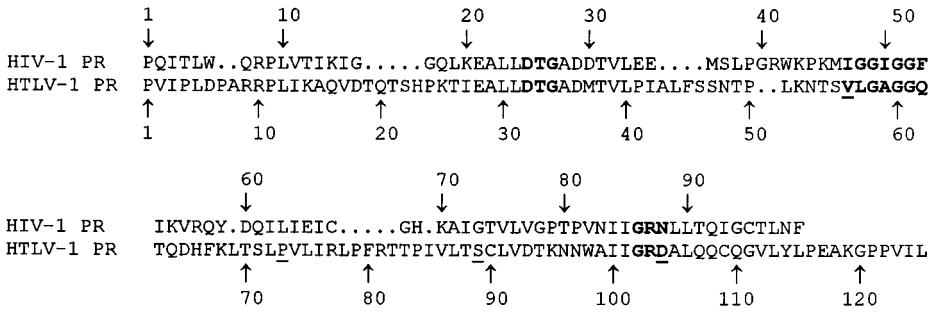

FIG. 1. **Sequence comparison of HIV-1 and HTLV-1 proteinases.** The three conserved regions of proteinases of HIV-1_{HXB2} and HTLV-1 (from clone CS95) are shown in *bold*. Residues of HTLV-1 PR that are found in equivalent positions of drug-resistant variants of HIV-1 proteinases are *underlined*. Additionally, three frequently occurring drug-resistant mutations in HIV-1, G48V, A71V, and V82F, are represented by residues *L*, *L*, and *W* in equivalent positions in HTLV-1 PR.

eral potent inhibitors of HIV-1 PR and other retroviral proteinases have been assessed for their inhibition of the HTLV-1 PR. We also report the effect of substituting the two cysteine residues in HTLV-1 PR and define a chromogenic substrate for assaying this enzyme.

MATERIALS AND METHODS

Cloning, Expression, and Site-directed Mutagenesis of the HTLV-1 Proteinase—The PR coding region of the *Sac*I fragment of an infectious HTLV-1 clone, pCS-HTLV-I (26), derived by polymerase chain reaction amplification was cloned at the *Nde*I and *Bam*HI sites of pET-11a vector (Novagen, WI). Mutagenesis of the Leu^{40} to Ile was performed by using the overlap extension method (27). The C90A and C109A mutations were introduced into the L40I-mutated HTLV-I PR using the Quick-Change mutagenesis protocol (Stratagene, CA). Triple mutant L40I,C90A,C109A PR was prepared by using the L40I,C109A double mutant as the template.

Cells bearing the construct for the expression of the wild-type HTLV-1 PR and its mutants were grown in Luria-Bertani medium supplemented with 1% glucose at 37 °C, 30% pO2, pH 7.0, in a 3-l Braun model MD fermentor. When the cell suspension reached an absorbance of 0.5–0.6 at 600 nm, protein expression was induced by the addition of 2 mM isopropyl β-D-thiogalactopyranoside for 3–4 h. Cells (4 g) were suspended in 20 volumes of buffer A (50 mM Tris-HCl, pH 8.2, 1 mM DTT, 1 mM EDTA) containing 100 μg/ml lysozyme and lysed by sonication. The insoluble recombinant protein (inclusion bodies) was washed twice by resuspension in buffer A containing 1 M urea and 1% Triton X-100 and pelleted by centrifugation at 20,000 × *g* for 30 min. The final pellet was solubilized in 50 mM Tris-HCl, pH 8.0, 7.5 M guanidine HCl, 5 mM DTT, and 5 mM EDTA and applied to a Superdex 75 column (HiLoad 26 × 60, Amersham Pharmacia Biotech, NJ) equilibrated in 50 mM Tris-HCl, pH 7.5, 4 M guanidine HCl, 5 mM EDTA, and 5 mM DTT at a flow rate of 3 ml/min. Peak fractions were further purified by reverse-phase HPLC on POROS RII resin (PerSeptive Biosystems, MA). HPLC fractions were combined, and aliquots were dried, assayed for protein content as described previously (28), and subjected to mass spectroscopic and N-terminal sequence analyses.

Protein Folding—The HPLC-purified protein fractions were dialyzed in large excess of 25 mM formic acid, pH 2.8, and subsequently into 50 mM sodium acetate buffer, pH 5.0, 1 mM, DTT, 1 mM EDTA. For kinetic studies, a portion of the protein in 25 mM formic acid was quenched with at least 100-fold excess of 20 mM PIPES buffer, pH 7.0, containing 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, and 0.5% Nonidet P-40.

Oligopeptide Synthesis and Characterization—Oligopeptides were synthesized by standard *tert*-butoxycarbonyl or 9-fluorenylmethyloxycarbonyl chemistry on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or a semiautomatic Vega peptide synthesizer (Vega-Fox Biochemicals). All peptides were synthesized with C-terminal amides. Amino acid composition of the peptides was determined with a Beckman 6300 amino acid analyzer. Stock solutions and dilutions were made in distilled water (or in 10 mM DTT for peptides containing Cys or Met residues), and the peptide concentrations were determined by amino acid analysis.

Proteinase Inhibitors—Substrate-based inhibitors of HIV-1 PR assayed as inhibitors of HTLV-1 PR were Ro-31-8959 (29), DMP 323 (30), UK-88,897 (31), KH 164 (32), and Compound 3 (33). Pepstatin A was obtained from Sigma, and reduced peptide bond inhibitor (N-1460) was purchased from Bachem BioScience Inc., King of Prussia, PA. Dr. Ivo Bláha (Ferring Leciva, Prague) prepared the statine- and phenylstatine-containing peptide inhibitors.

Enzyme Assay with Oligopeptide Substrates—Purified HIV-1 PR was prepared as described previously (34).[2] Active-site titration for the HIV-1 PR was performed with Compound 3 (33). The enzyme concentrations for HTLV-1 PR preparations were determined by amino acid analysis of the stock solutions, and k_{cat} values were calculated by assuming 100% activity for the PR dimers. The PR assays were initiated by mixing 5 μl of HTLV-1 or HIV-1 PR (8–140 nM), 10 μl of 2× 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 μl of 0.01 to 0.40 mM substrate. The range of substrate concentration was selected depending on the approximate K_m values. The reaction mixture was incubated at 37 °C for 1 h and terminated by the addition of guanidine HCl to give a final concentration of 6 M. The solution was acidified by the addition of trifluoroacetic acid, and an aliquot was injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 × 150 mm, Waters Associates, Inc.) using an automatic injector. Substrates and the cleavage products were separated using an increasing water-acetonitrile gradient (0–100%) in the presence of 0.05% trifluoroacetic acid. Cleavage products were identified by amino acid analysis. 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.). To assay the inhibitors, the volume of substrate KTKVL ↓ VVQPK was reduced to 4.8 μl (0.25 mM final concentration), and 0.2 μl of inhibitor (in water or in Me_2SO) was added. Inhibitors were initially tested at 10 μM or higher concentration. Me_2SO was also added at the same concentration to the appropriate control experiments. When inhibition was observed in initial testing, K_i values were determined according to Dixon (35). A competitive mode of inhibition was observed, and the standard error for K_i values was estimated to be below 20%.

RESULTS AND DISCUSSION

Expression and Purification of HTLV-1 Proteinase—The 375-base pair coding region derived from an infectious clone of HTLV-1 by polymerase chain reaction was cloned into pET-11a vector and expressed in *E. coli*. Fig. 2 (*lanes 1* and *2*) shows the analysis of the total cell lysate before and after isopropyl β-D-thiogalactopyranoside induction. Two products were observed, the larger molecular weight product corresponding to the full-length mature PR and a smaller product with an apparent size of roughly 7 kDa. The latter product was expected to be the result of self-degradation (autoproteolysis) of the mature PR rather than by premature termination during expression.

As in the case of HIV-1 PR (28, 36), expression of HTLV-1 PR results in its accumulation in the form of inclusion bodies. *Lanes 3*, *4*, and *5* show the supernatant upon lysis of cells and the supernatant and the pellet derived after 1 M urea treatment of the initial insoluble pellet, respectively. A comparison of *lanes 4* and *5* shows that 1 M urea partially solublizes the full-length PR but not the truncated protein. The insoluble fraction after 1 M urea treatment of the inclusion bodies (see *lane 5*) was solubilized in guanidine hydrochloride and subjected to size-exclusion chromatography under denaturing conditions. The peak fractions of both the full-length PR and the truncated protein were further subjected to reverse-phase HPLC chromatography. The two proteins that were >98% pure

[2] J. M. Louis, unpublished data.

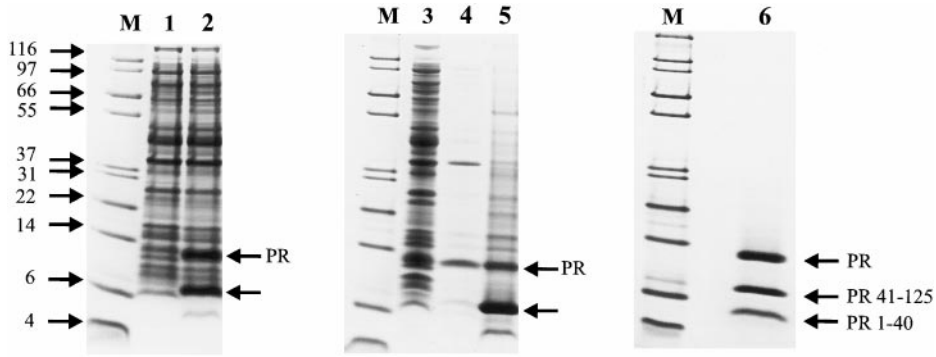

FIG. 2. **Expression and purification of the wild-type HTLV-1 proteinase and demonstration of its intrinsic autoproteolysis.** Analyses of total cell lysate prepared from *E. coli* bearing the construct for the expression of the wild-type HTLV-1 PR before and after isopropyl β-D-thiogalactopyranoside induction are shown in *lanes 1* and *2*, respectively. *Lane 3* shows the soluble supernatant derived after lysis of induced cells in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM DTT, and 100 μg/ml lysozyme. The insoluble pellet attained from this step was treated with 1 M urea solution in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM DTT, 0.5% Nonidet P-40. The soluble supernatant and the pellet (inclusion bodies) that were derived from this step are shown in *lanes 4* and *5*, respectively. The inclusion bodies were solubilized, and the mature PR was purified to homogeneity by size-exclusion followed by reverse-phase HPLC chromatography. The purified PR that was renatured from 50 mM formic acid into 50 mM sodium acetate buffer, pH 5.5, 1 mM DTT, and 0.05% Triton-100 is shown in *lane 6*. Proteins were electrophoresed on 10–20% gradient polyacrylamide gels (Novex, CA), and bands were visualized by Coomassie staining. *M* denotes molecular mass standards in kDa. Mature PR and truncated PR 41–125 were confirmed by N-terminal sequencing and mass spectrometry. *Arrows* indicate the truncated PR 41–125 before its characterization.

were analyzed for their mass and N-terminal sequence. The full-length mature PR clearly corresponded to the expected N-terminal sequence and mass (m/z = 13475). The truncated protein showed its N-terminal sequence to start with residues Pro-Ile-Ala-Leu-Phe-Ser-Ser, and the expected mass was close to the experimental value (m/z = 9171). From these results, we confirmed our prediction that the truncated protein is derived via autoproteolysis between residues Leu-40/Pro-41 of the full-length mature PR with a sequence spanning amino acids 41 through 125, now termed PR 41–125. The pure full-length protein was also renatured from 25 mM formic acid into 50 mM sodium acetate buffer, pH 5.5, 1 mM DTT, 1 mM EDTA, and 0.05% Triton X-100 at a final concentration of 6.7 μM and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2, *lane 6*). As expected, the full-length PR gave rise to two products, with apparent mobilities corresponding to ~9 and 4.5 kDa. The latter protein is termed PR 1–40. Also, similar to the HIV-1 PR, autoproteolysis of the HTLV-1 PR proceeds roughly to about 50%. It is likely that either one or both products inhibit further autoproteolysis. This site of autoproteolysis in HTLV-1 PR is analogous to a less susceptible site in HIV-1 PR (Leu^{33}-Glu^{34}). The major cleavage in HIV-1 PR occurs between residues Leu^5 and Trp^6 (19). Recently the feline immunodeficiency virus PR was also shown to undergo autoproteolysis at four primary sites in its 116-amino acid sequence (20).

Autoproteolysis-resistant HTLV-1 Proteinase—To prevent autoproteolysis of the mature HTLV-1 PR, the Leu^{40} residue was substituted with an Ile. This substitution was chosen because it has been shown by using synthetic peptides and proteins as substrates that HIV-1 and other retroviral proteinases cannot process at the C-terminal side of β-branched residues (37, 38). Furthermore, autoprocessing at an equivalent position (Leu^{33}) and another position (Leu^{63}) in HIV-1 was effectively reduced by substituting these residues with Ile without impairing the k_{cat}/K_m for several substrates (36). The expression of the HTLV-1 PR bearing the mutation L40I compared with the wild-type PR is shown in Fig. 3. Clearly as observed in duplicate clones, there is a single product that accumulates with a substantially higher yield corresponding to the size of the full-length PR. 1.6 g (wet weight) of cells derived from 0.4 liters of culture yields ~19 and 14 mg of PR after size-exclusion and reversed-phase HPLC chromatography, respectively.

Kinetic Characterization of the Wild-type and L40I HTLV-I Proteinases—Based on the sequence comparison with HIV-1 PR and homologous molecular modeling,[3] the Leu^{40} residue of HTLV-1 PR is not expected to interact with the substrate or inhibitor. Therefore we predicted that substituting this residue with Ile would not change the kinetic parameters but would prevent its autolysis as compared with the wild-type enzyme. To verify this assumption and to characterize the HTLV-1 PR specificity, kinetic analysis was performed using oligopeptide substrates. The proteinases were renatured by quenching the denatured protein in 50 mM formic acid into 100-fold excess of PIPES buffer at pH 7.0 under conditions similar to those previously described for refolding HIV-2 and equine infectious anemia virus proteinases (39). Kinetic data for the wild type and L40I mutant PR-catalyzed hydrolysis of synthetic substrates are shown in Table I. Both enzymes exhibit nearly identical kinetic parameters. The nearly identical values of k_{cat} for the wild-type and mutant enzymes also suggest that under

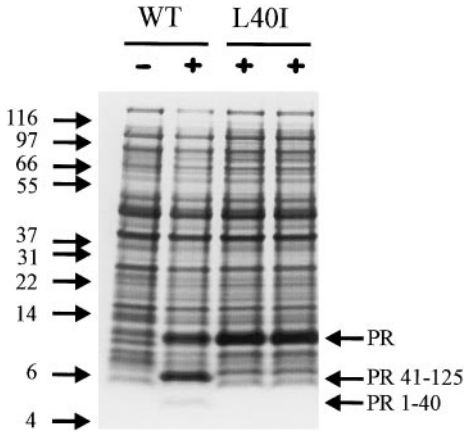

FIG. 3. **Expression of the stabilized (L40I) HTLV-1 proteinase.** Analyses of total cell lysate prepared from *E. coli* bearing the construct for the expression of the stabilized (L40I) HTLV-1 PR after isopropyl β-D-thiogalactopyranoside induction are shown in + *lanes*. Cell lysates prepared from the wild type (*WT*) clone before (− lane) and after (+ *lane*) induction are shown as controls. *M* denoted molecular mass standards in kDa. Proteins were electrophoresed on 10–20% gradient polyacrylamide gels (Novex), and *bands* were visualized by Coomassie staining.

[3] P. Bagossi, G. Zahuczky, and J. Tozser, unpublished results.

TABLE I

Kinetic parameters for hydrolysis of naturally occurring HTLV-1 cleavage site peptides by wild-type and L40I mutant HTLV-1 proteinases

wt, wild type.

Cleavage site	Substrate sequence	Enzyme	K_m	k_{cat}	k_{cat}/K_m
			mM	s^{-1}	$mM^{-1}s^{-1}$
MA/CA	APQVL ↓ PVMHP	HTLV-1 (wt)	0.115 ± 0.018	8.67 ± 0.60	75.4
		HTLV-1 (L40I)	0.098 ± 0.020	6.78 ± 0.50	69.2
CA/NC	KTKVL ↓ VVQPK	HTLV-1 (wt)	0.033 ± 0.004	5.15 ± 0.10	156.1
		HTLV-1 (L40I)	0.032 ± 0.004	5.18 ± 0.12	161.8
TF1/PR	DPASIL ↓ PVIP	HTLV-1 (wt)	0.230 ± 0.035	0.70 ± 0.05	3.0
		HTLV-1 (L40I)	0.244 ± 0.033	0.62 ± 0.04	2.5
PR/p3	KGPPVIL ↓ PIQAP	HTLV-1 (wt)	0.030 ± 0.006	6.91 ± 0.39	230.3
		HTLV-1 (L40I)	0.034 ± 0.005	6.26 ± 0.30	184.2

these refolding and assay conditions there is no significant autoproteolysis of the wild-type PR. Stabilization of the enzyme can be attributed to 1) the refolding procedure, which involves quenching the denatured protein with a large excess of buffer (pH 7) to a final concentration of 0.1 μM as compared with dialyzing the protein to pH 5.0 at a higher protein concentration and 2) presence of Nonidet P-40 and glycerol in the quench buffer.

Mutation of the Cysteine Residues of the Stabilized HTLV-1 Proteinase—Because one of our major objectives was to produce quantities of enzyme suitable for structural studies by solution NMR and x-ray crystallography, we next addressed the role of the two cysteines (Cys^{90}, Cys^{109}) on enzymatic activity and specificity of the PR. It was shown that a Cys to Ala substitution at residue 95 of HIV-1 mature PR greatly reduced the tendency of the protein to form an intermolecular disulfide bond (40). Subsequent studies have shown that both the Cys^{67} and Cys^{95} residues of HIV-1 can be exchanged to Ala without significantly affecting the structure and kinetic parameters (41).[2]

Constructs bearing either or both of the two cysteines substituted by Ala were prepared in a L40I HTLV-1 PR construct as described under "Materials and Methods." We chose to compare the triple mutant L40I,C90A,C109A (termed C2A-PR) with the wild-type PR. C2A-PR was purified as described before for the wild-type HTLV-1 PR. Fig. 4 shows the enzyme before and after refolding. Clearly the refolded enzyme was stable up to 24 h at a concentration of 0.6 mM at ambient temperature. The triple mutant was used to compare the specificity of HTLV-1 and HIV-1 proteinases on naturally occurring cleavage sites of HTLV-1 Gag-Pol polyprotein (Table II).

Kinetic Characterization of the Stabilized C2A-PR Mutant HTLV-1 Proteinase and Comparison of Its Specificity with HIV-1 Proteinase—To characterize the specificity of C2A-PR, we first used oligopeptide substrates that represent naturally occurring cleavage sites in HTLV-1 Gag-Pol polyprotein. Comparison of the kinetic parameters using C2A-PR (Table II) to those obtained with wild-type and L40I mutant enzymes (Table I) suggest that substitution of the Cys residues to Ala does not alter its catalytic activity. Three of the four peptides tested were excellent substrates of HTLV-1 PR, with catalytic constants comparable with the best naturally occurring HIV-1 cleavage site substrates with HIV-1 PR (42). Thus HTLV PR is an enzyme with high specific activity, similar to HIV-1 PR but unlike avian myeloblastosis virus PR, which has much lower k_{cat} for its specific substrates. This interpretation is consistent with the mechanisms that regulate the level of expression of the Gag and Gag-Pol products. HIV-1 and HTLV-1, wherein the PR is coded in a *Pol* open reading frame at ~10-fold lower level than Gag proteins, have higher specific activity for the PR as compared with avian myeloblastosis virus PR, which is

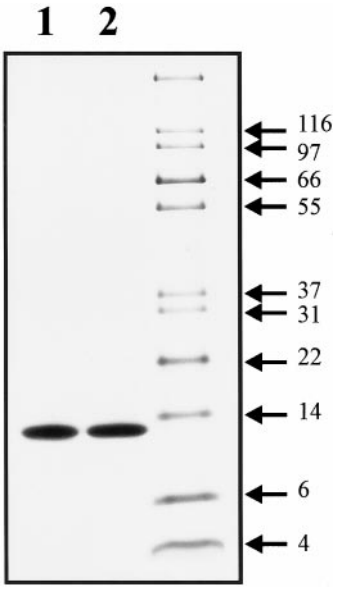

FIG. 4. **Analysis by SDS-polyacrylamide gel electrophoresis of the triply mutated (L40I, C90A, C109A, or C2A-PR) HTLV-1 PR before and after protein folding.** Triply mutated HTLV-1 PR was purified similar to the conditions described for the wild-type enzyme. Protein was renatured similar to conditions described under "Materials and Methods" and concentrated to 0.6 mM. A fraction of the enzyme before and after refolding that was subjected to 10–20% gradient SDS-polyacrylamide gel electrophoresis is shown in *lanes 1* and *2*, respectively. *M* denoted molecular mass standards in kDa.

coded in a *Gag* open reading frame. Another interesting similarity with the HIV-1 and HTLV-1 proteinases is that among the least efficient sites for hydrolysis is the one that yields the free N terminus of PR (42). Studies with an analog of the HIV-1 PR precursor indicate that the cleavage at the N terminus of the PR occurs intramolecularly, concomitant with a large increase in enzymatic activity, and this cleavage has been suggested to be the rate-limiting step in the maturation cascade of Gag-Pol polyprotein (24, 43). When HTLV-1 cleavage site substrates were used as substrates for HIV-1 PR (Table II), three of four peptides were found to be hydrolyzed. The kinetic parameters of HIV-1 PR-catalyzed hydrolysis of these HTLV-1 substrates were in the same range as those for substrates that correspond to analogous sites in HIV-1 (42). On the other hand, only two of the nine HIV-1 cleavage site substrates were cleaved by the HTLV-1 PR (Table III) but with substantially lower values of k_{cat}/K_m than those obtained with HTLV-1 substrates.

The lack of cleavage of HIV-1 Gag substrates by HTLV-1 PR is in good agreement with previous findings that HTLV-1 PR was not able to process HIV-1 Gag precursor in a vaccinia virus expression system (44). In the same expression system, HIV-1

TABLE II

Kinetic parameters for hydrolysis of naturally occurring HTLV-1 cleavage site peptides by C2A-PR and wild-type HIV-1 PR

ND, cleavage products not detected in the assay performed as described under "Materials and Methods" using 0.3 mM substrate and 100 nM enzyme (k_{cat} is estimated to be smaller than 0.01 s^{-1}).

Cleavage site	Substrate sequence	Enzyme	K_m	k_{cat}	k_{cat}/K_m
			mM	s^{-1}	$mM^{-1}s^{-1}$
MA/CA	APQVL ↓ PVMHP	C2A-PR	0.069 ± 0.020	5.88 ± 0.59	85.2
		HIV-1 PR	0.023 ± 0.004	0.37 ± 0.01	16.1
CA/NC	KTKVL ↓ VVQPK	C2A-PR	0.051 ± 0.005	7.68 ± 0.17	150.6
		HIV-1 PR	0.182 ± 0.033	2.60 ± 0.20	14.3
TF1/PR	DPASIL ↓ PVIP	C2A-PR	0.221 ± 0.031	0.84 ± 0.05	3.8
		HIV-1 PR	ND		
PR/p3	KGPPVIL ↓ PIQAP	C2A-PR	0.041 ± 0.010	11.82 ± 0.89	288.3
		HIV-1 PR	0.025 ± 0.002	0.21 ± 0.01	8.4

TABLE III

Hydrolysis of oligopeptides representing naturally occurring HIV-1 cleavage sites and chromogenic substrates by the C2A-PR

ND, cleavage products not detected in the assay performed as described under "Materials and Methods" using 0.3 mM substrate and 600 nM C2A-PR (k_{cat} is estimated to be smaller than 0.002 s^{-1}). Φ, 4-nitrophenylalanine.

Cleavage site	Substrate sequence	K_m	k_{cat}	k_{cat}/K_m
		mM	s^{-1}	$mM^{-1}s^{-1}$
HIV-1 MA/CA	VSQNY ↓ PIVQ		ND	
CA/p2	KARVL ↓ AEAMS		ND	
p2/NC	TATIM ↓ MQRGN		ND	
NC/p1	ERQAN ↓ FLGKI		ND	
p1/p6	RPGNF ↓ LQSRP		ND	
in p6	DKELY ↓ PLTSL	>0.5		0.02
TF/PR	VSFNF ↓ PQITL		ND	
PR/reverse transcriptase	CTLNF ↓ PISP		ND	
Reverse transcriptase/IN	IRKIL ↓ FLDG	0.283 ± 0.038	0.21 ± 0.01	8.4
HIV-1 CA/p2	KARVL ↓ ΦEAM		ND	
HTLV CA/NC	KTKVL ↓ ΦVQPK	>0.3	<0.01	0.04
HTLV CA/NC	KTKVΦ ↓ VVQPK	0.188 ± 0.013	1.23 ± 0.04	6.5

PR was also not able to process HTLV-1 Gag (44) even though the peptides representing HTLV-1 Gag cleavage sites were hydrolyzed by HIV-1 PR *in vitro*. This result *in vivo* may at least partially be because of reduced specificity of this enzyme toward these sites as compared with HTLV-1 PR.

Hydrolysis of Chromogenic Substrates by HTLV-1 Proteinase—To develop a more convenient chromogenic assay, we synthesized and assayed analogs of the HTLV-1 CA/NC cleavage site substrate that contained a Phe (NO_2) substitution in either the P1 or P1′ positions (Table III). Although the substrate with P1′ substitution was an inefficient substrate, the substrate containing the P1 substitution was hydrolyzed to about 4% the level of the native substrate. This substrate may serve to screen for ideal-folding conditions of C2A-PR in simple buffer systems for structural studies. In good agreement with the lack of hydrolysis of the HIV-1 CA/p2 peptide by HTLV-1 PR, its chromogenic derivative, which is a widely used chromogenic substrate of HIV-1 PR was not hydrolyzed (Table III).

Daenke *et al.* (16) reported kinetic parameters for peptides representing naturally occurring cleavage sites of HTLV-1 in which the P1′ residue were substituted by a chromogenic reporter, Phe (NO_2). They have reported K_m and k_{cat} values within a range of 0.003–0.031 mM and 14–33 s^{-1}, respectively. These K_m values are lower than our observed values (0.030–0.230 mM, see Table I) using entirely natural peptides with no substitutions whereas the k_{cat} values were substantially higher, assayed under similar conditions. The difference in K_m values may be because of the introduction of the bulky aromatic residue in the P1′ position. This may also be the reason why the naturally occurring cleavage site sequences representing the autocatalytic maturation sites (TF1/PR and PR/p3) in HTLV-1 as compared with the corresponding chromogenic derivatives do not have substantially higher affinities toward HTLV-1 PR than the peptides representing other sites, as noted for the chromogenic derivatives by Daenke *et al.* (16). The reason for the differences in the reported k_{cat} values is less obvious. When we introduced Phe(NO_2) group in either the P1 or P1′ positions of the naturally occurring cleavage site sequence, a substantial decrease in hydrolytic rate was observed instead of the expected increase (see Table III). Our observations which suggest that bulky residues at the site of cleavage are unfavorable are in accordance with the interpretation of Pettit *et al.* (12) that lack of inhibition by HIV-1 PR inhibitors on *in vitro* HTLV-1 Gag processing might be because of the presence of Trp98 in HTLV-1 rather than Val in equivalent position of HIV-1 reducing the size of S1 binding pocket. A two-fold increase in NaCl concentration used in our assay conditions as compared with that reported (16) does not account for this large difference in k_{cat} values. The specific activity reported using the chromogenic substrate representing the CA/NC site (1 μmol/min/mg; Ref. 16) suggests a much smaller catalytic rate.

Inhibition of the HTLV-1 Proteinase—Several inhibitors of HIV-1 PR were assayed as inhibitors of the HTLV-1 PR. DMP 323, KH 164, and Compound 3 have previously been shown to be potent inhibitors of the HIV-1 PR. Only Compound 3 inhibited HTLV-1 PR with a K_i of 80 nM, whereas the others did not inhibit up to 10 μM (Table VI). Another potent inhibitor of HIV-1 PR, Ro-31-8959 was also inactive against HTLV-1 PR (16). These results also emphasize the large differences in specificity of the two enzymes. Interestingly, Compound 3 was also a good inhibitor of HIV-2 PR (45), equine infectious anemia virus PR (39), murine leukemia virus, and mouse mammary tumor virus proteinases (46) and, therefore, may serve as a lead compound for general inhibitors of retroviral proteinases. However, the most potent inhibitor of the enzyme in our assay system was a statine-based inhibitor, which was based on the MA/CA cleavage site sequence with a K_i of 50 nM (Inhibitor 10 in Table IV). Potent, active-site inhibitor of Mason-Pfizer monkey virus PR (Inhibitor 9 in Table IV; Ref. 47) and avian myeloblastosis virus PR (Inhibitor 8 in Table IV; Ref. 50) also

TABLE IV
Inhibition of C2A-PR and HIV-1 PR by inhibitors specific to HIV-1 and other retroviral proteinases and by statine-based HTLV-1 substrate analogs

PR	Inhibitor	K_i	
		HTLV-1 PR	HIV-1
		μM	
1	Compound 3	0.080	0.0004[a]
2	DMP 323	>10	0.0003[b]
3	Ro-31–8959 (Saquinavir)	>10	0.0004[b]
4	UK 88,947	>10	0.012[b]
5	KH 164	>10	0.400[c]
6	LP-149	>10	0.0017[d]
7	N-1460	>10	0.050[c]
7	Pepstatin	>10	>10[f]
8	Pro-Pro-Cys-Val-PheSta-Ala-Met-Thr-Met[g]	>10	Not determined
9	Pro-Tyr-Val-PheSta-Ala-Met-Thr	>10	12.5[h]
10	Ala-Pro-Gln-Val-Sta-Val-Met-His-Pro[i]	0.050	0.13
11	Lys-Thr-Lys-Val-Sta-Val-Gln-Pro-Lys[i]	>10	>10

[a] Compound 3 inhibits HIV-2, equine infectious anemia virus, murine leukemia virus, and mouse mammary tumor virus proteinases with a K_i of 0.4 nM, 0.2 nM (39), 134 nM, and 90 nM (47, 49), respectively.
[b] Potent, competitive inhibitors of HIV-1 PR (29–31).
[c] KH 164, a modest statine-containing inhibitor of HIV-1 PR (compound D in Ref. 32), inhibits murine leukemia virus PR (K_i = 62 nM (46)).
[d] LP-149, a statine-containing inhibitor designed for HIV-1 PR that also inhibits feline immunodeficiency virus PR (K_i = 260 nM (22)).
[e] H-Arg-Val-Leu-r-Phe-Glu-Ala-Nle-HN_2, where r and Nle denote a reduced peptide bond and norleucine, respectively, inhibitor of HIV-1 PR (25).
[f] A well known inhibitor of aspartic proteases (32).
[g] Inhibitor of avian myeloblastosis virus PR (K_i = 5 nM (50)).
[h] Inhibitor of Mason-Pfizer monkey virus PR and avian myeloblastosis PR with a K_i of 3 nM and 10 nM, respectively (47).
[i] Statine-containing inhibitors based on naturally occurring HTLV-1 cleavage sites (see Table I).

did not inhibit the HTLV-1 PR. N-1460, a low nM peptide analog inhibitor of HIV-1 PR, and LP-149, inhibitor of HIV-1 and feline immunodeficiency virus proteinases, do not inhibit HTLV-1 PR up to 10 μM.

Acknowledgments—We thank Dr. Jane M. Sayer for critical review of the manuscript, Dr. Barbara Felber for providing the pCS-HTLV-1 clone, Deanna Gotte for help in the early stage of the cloning of the HTLV-1 PR, Dr. Lewis K. Pannell for mass spectroscopic analyses, Suzanne Specht for help with the amino acid analysis, Eva Majerova for peptide synthesis, and Dr. H. Parikh for contract services provided at Braton Biotech, Rockville, Inc., MD. The UK 88,947 and Ro-31-8959 inhibitors were generous gifts from Dr. Peter Whittle, KH 164 and LP-149 from Dr. Kwan Y. Hui, and DMP 323 from Dr. N. Hodge.

REFERENCES

1. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Dunn, P. A., Minna, J. D., and Gallo, R. C. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 7415–7419
2. Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S., and Miyoshi, I. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6476–6480
3. Gessain, A, Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., and de-The, G. (1985) *Lancet* **2,** 407–410
4. Osame, M., Usuku, K., Izumo, S., Ijichi, N., Amitani, H., Igata, A., Matsumoto, M., and Tara, M. (1986) *Lancet* **1,** 1031–1032
5. Kaplan, J. E., and Khabbaz, R. F. (1993) *Rev. Med. Virol.***3,** 137–148
6. Sheremata, W. A., Benedict, D., Squilacote, D. C., Sazant, A., and DeFreitas, E. (1993) *Neurology* **43,** 2125–2129
7. Hatfield, D. L. Levin, J. G., Rein, A., and Oroszlan, S. (1992) *Adv. Virus Res.* **41,** 193–239
8. Oroszlan, S., and Luftig, R. B. (1990) *Curr. Top. Microbiol. Immunol.* **157,** 153–185
9. Flexner, C. (1998) *N. Eng. J. Med.* **338,** 1281–1292
10. Tomasselli, A. G., Hui, J. O., Sawyer, T. K, Staples, D. J., Bannow, C., Reardon, I. M., Howe, W. J., DeCamp, D. L., Craik, C. S., and Heinrikson, R. L. (1990) *J. Biol. Chem.* **265,** 14675–14683
11. Powell, D. J., Bur, D., Wlodawer, A., Gustchina, A., Payne, S. L., Dunn, B. M., and Kay, J. (1996) *Eur. J. Biochem.* **241,** 664–674
12. Pettit, S. C., Sanchez, R., Smith, T., Wehbie, R., Derse, D., and Swanstrom, R. (1998) *AIDS Res. Hum. Retroviruses* **14,** 1007–1014
13. Kobayashi, M., Ohi, Y., Asano, T., Hayakawa, T., Kato, K., Kakinuma, A., and Hatanaka, M. (1991) *FEBS Lett.* **293,** 106–110
14. Hayakawa, T., Misumi, Y., Kobayashi, M., Ohi, Y., Fujisawa, Y., Kakinuma, A., and Hatanaka, M. (1991) *Biochem. Biophys. Res. Commun.* **181,** 1281–1287
15. Saiga, A., Tanaka, T., Orita, S., Sato, A., Sato, S., Hachisu, T., Abe, K., Kimura, Y., Kondo, Y., Fujiwara, T., and Igarashi, H. (1993) *Arch. Virol.* **128,** 195–210
16. Daenke, S., Schramm, H. J., and Bangham, C. R. M. (1994) *J. Gen. Virol.* **75,** 2233–2239
17. Ding, Y. S., Owen, S. M., Lal, R. B., and Ikeda, R. A. (1998) *J. Virol.* **72,** 3383–3386
18. Hrusková-Heidingsfeldová, O., Bláha , I., Urban. J., Strop, P., and Pichova, I. (1997) *Leukemia (Baltimore)* **3,** 45–46
19. Rose, J. R., Salto, R., and Craik, C. S. (1993) *J. Biol. Chem.* **268,** 11939–11945
20. Laco, G. S., Fitzgerald, M. C., Morris, G. M., Olson, A. J., Kent, S. B. H., and Elder, J. H. (1997) *J. Virol.* **71,** 5505–5511
21. Wlodawer, A., and Erickson, J. W. (1993) *Annu. Rev. Biochem.* **62,** 543–585
22. Wlodawer, A., Gustchina, A., Reshetnikova, L., Lubkowski, J., Zdanov, A., Hui, K. Y., Angleton, E. L., Farmerie, W. G., Goodenow, M. M., Bhatt, D., Zhang, L., and Dunn, B. M. (1995) *Nat. Struct. Biol.* **2,** 480–488
23. Gustchina, A., Kervinen, J., Powell, D. J., Zdanov, A., Kay, J., Wlodawer, A. (1995) *Protein Sci.* **8,** 1453–1465
24. Louis, J. M., Nashed, N., Parris, K. D., Kimmel. A. R., and Jerina, D. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7970–7974
25. Wondrak, E. M., Nashed, N. T., Haber, M. T., Jerina, D. M., and Louis, J. M. (1996) *J. Biol. Chem.* **271,** 4477–4481
26. Ciminale, V., Pavlakis, G. N., Derse, D., Cunningham, C. P., and Felber, B. K. (1992) *J. Virol.* **66,** 1737–1745
27. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 51–59
28. Wondrak, E. M., and Louis, J. M. (1996) *Biochemistry* **35,** 12957–12962
29. Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Duncan, J. B., Galpin, S. A., Handa, B. K., Kay, J., Kröhn, A., Lambert, R. W., Merrett, J. H., Mills, J. S., Parkes, E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J., and Machin, P. J. (1990) *Science* **248,** 358–361
30. Lam, P. Y. S., Jadhav, P. K., Eyermann, C. J., Hodge, C. N., Ru, Y., Bacheler, L. T., Meek, J. L., Otto, M. J., Rayner, M. M., Wong, Y. N., Chang, C. H., Weber, P. C., Jackson, D. A., Sharpe, T. R., and Erickson-Viitanen, S. (1994) *Science* **263,** 380–384
31. Baboonian, C., Dalgleish, A., Bountiff, L., Gross, J., Oroszlan, S., Rickett, G., Smith-Burchnell, C., Troke, P., and Merson, J. (1991) *Biochem. Biophys. Res. Commun.* **179,** 17–24
32. Hui, K. Y., Manetta, J. V., Gygi, T., Bowdon, B. J., Keith, K. A., Shannow, W. M., and Lai, M.-H. T. (1991) *FASEB J.* **5,** 2606–2610
33. Grobelny, D., Wondrak, E. M., Galardy, R. E., and Oroszlan, S. (1990) *Biochem. Biophys. Res. Commun.* **169,** 1111–1116
34. Louis, J., McDonald, R., Nashed, N., Wondrak, E. M., Jerina, D., Oroszlan, S., and Mora, P. (1991) *Eur. J. Biochem.* **199,** 361–369
35. Dixon, M. (1953) *Biochem. J.* **55,** 170–171
36. Mildner, A. M., Rothrock, D. J., Leone, J. W., Bannow, C. A., Lull, J. M., Reardon, I. M., Sarcich, J. L., Howe, W. J., Tomich, C.-S. C., Smith, C. W., Heinrickson, R. L., and Tomasselli, A. G. (1994) *Biochemistry* **33,** 9405–9413
37. Dunn, B. M., Gustchina, A., Wlodawer, A., and Kay, J. (1994) *Methods Enzymol.* **241,** 254–278
38. Tomasselli, A. G., and Heinrickson, R. L. (1994) *Methods Enzymol.* **241,** 279–301
39. Tözsér, J., Friedman, D., Weber, I. T., Bláha, I., and Oroszlan, S. (1993) *Biochemistry* **32,** 3347–3353
40. Yamazaki, T., Hinck, A. P., Wang, Y. X., Nicholson, L. K., Torchia, D. A., Wingfield, P., Stahl, S. J., Kaufman, J. D., Chang, C. H., Domaille, P. J., and Lam, P. Y. (1996) *Protein Sci.* **5,** 495–506
41. Wu, J., Adomat, J. M., Ridky, T. W., Louis, J. M., Leis, J., Harrison, R. W., and Weber, I. T. (1998) *Biochemistry* **37,** 4518–4526
42. Tözsér, J., Bláha, I., Copeland, T. D., Wondrak, E. M., and Oroszlan, S. (1991)

FEBS Lett. **281,** 77–80
43. Tessmer, U., and Kräusslich, H.-G. (1998) *J. Virol.* **72,** 3459–3463
44. Luukkonen, B. G. M., Tan, W., Fenyö, E. M., and Schwartz, S. (1995) *J. Gen. Virol.* **76,** 2169–2180
45. Tözsér, J., Weber, I. T., Gustchina, A., Bláha, I., Copeland, T. D., Louis, J. M., and Oroszlan, S. (1992) *Biochemistry* **31,** 4793–4800
46. Menendez-Arias, L., Gotte, D., and Oroszlan, S. (1993) *Virology* **196,** 557–563
47. Hrusková-Heidingsfeldová, O., Andreansky, M., Fábry, M., Bláha, I., Strop, P., and Hunter, E. (1995) *J. Biol. Chem.* **270,** 15053–15058
48. Deleted in proof
49. Menendez-Arias, L., Young, M., and Oroszlan, S. (1992) *J. Biol. Chem.* **267,** 24134–24139
50. Strop, P., Konvalinka, J., Stys, D., Pavlickova, L., Bláha, I., Velek, J., Travnicek, M., Kostka, V., and Sedlacek, J. (1991) *Biochemistry* **30,** 3437–3443
51. Leis, J., Baltimore, D., Bishop, J. M., Coffin, J., Fleissner, E., Goff, S. P., Oroszlan, S., Robinson, H., Skalka, A. M., Temin, H. M., and Vogt, V. (1988) *J. Virol.* **62,** 1808–1809