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The *gag* precursor contains a specific HIV-1 protease cleavage site between the NC (P7) and P1 proteins

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The predicted protease cleavage site (p7/p1; [J. Virol. 66 (1992) 1856–1865]) within the nucleocapsid precursor protein (p15) of human immunodeficiency virus, type 1, was confirmed using an in vitro assay employing recombinant HIV-1 protease and a chemically synthesized 72 amino acid polypeptide containing the p7 and p1 protein domains of the native *gag* polyprotein. The cleavage occurred between amino acid 55 (N) and amino acid 56 (F) of the polypeptide, as determined by N-terminal sequencing. The hydrolysis was optimal at pH 6.0 and at high salt concentration. The kinetic parameters K_m, k_{cat} and k_{cat}/K_m were 99 μM (±8), 0.152 s^{-1} (± 0.002) and 1.56 $mM^{-1} \cdot s^{-1}$ (± 0.11), respectively. Reconstituted as well as denatured polypeptides were cleaved at approximately the same rate, demonstrating that the conformation of the p7 protein, as a result of the Zn^{2+}-binding, had no significant effect on the rate of hydrolysis of the p7/p1 cleavage.

Human immunodeficiency virus; HIV; Nucleocapsid protein; NC; p7; Protease

1. INTRODUCTION

The human immunodeficiency virus, type 1 (HIV-1), genome is translated into the $Pr165^{gag\text{-}pol}$ and $Pr55^{gag}$ polyproteins [1], which are ultimately cleaved by the viral protease (PR). Cleavage of $Pr55^{gag}$ generates the mature structural proteins, p17, p24, p7 and p6, found in the virus; p17 represents the matrix antigen (MA), p24 the core antigen (CA), and p7 the nucleocapsid (NC) protein (for review see [2–6]). Recently, two small proteins, p2 and p1, have been identified in HIV preparations, the respective coding sequences of which align up-stream and down-stream of the p7 region in the HIV-1 genome [7,8]. This raised the question of whether these small proteins represent products of the maturation event characterized by the polyprotein processing by the HIV PR. Synthetic oligopeptides corresponding to the p2/p7 cleavage site were readily cleaved in vitro by PR [9,10]. These two arguments, specific in vitro cleavage by HIV PR and identification of the products in mature viruses, classifies the p2/p7 as a maturation site. However, a synthetic 11-amino acid oligopeptide spanning the predicted p7/p1 site was cleaved neither by HIV-1 PR nor by HIV-2 PR. This could indicate that p1 is generated by cellular proteases or, as was suggested, sequences additional to the p7/p1 cleavage site are necessary for this processing [10]. In this report, we confirm and characterize the specific in vitro cleavage of the p7/p1 site of a chemically synthesized 72-amino acid polypeptide containing the p7 and p1 protein domains by recombinant HIV-1 PR.

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2. MATERIALS AND METHODS

A 72-amino acid polypeptide containing the p7 and p1 protein domains was chemically synthesized as previously published [11]. The lyophilized powder was dissolved in 10 mM DTT at a concentration of 50 mg/ml, aliquoted under nitrogen and stored in a sealed tube at −80°C.

HIV-1 PR was obtained as a solution of 5 μg/ml in 50 mM 2-morpholinoethanesulfonic acid (MES) buffer, pH 6.5, containing 5% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) as described [12].

Protease assays containing 5 μg of denatured 72-amino acid polypeptide (without Zn^{2+}) as substrate were carried out in the presence of 6 nM HIV-1 protease at 37°C in buffer A (250 mM potassium phosphate, pH 6.0, 750 mM ammonium sulfate, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol) in a total volume of 10 μl. Reactions were terminated by adding 90 μl of 1% tri-fluoroacetic acid (TFA) and subjected to RP-HPLC on a DELTA PAK C_4-300 Å column (Waters) using a gradient of 20–30% acetonitrile in 0.05% TFA over 6 min followed by a 2 min gradient of 30–32% acetonitrile. Control reactions employing a synthetic nona-peptide substrate corresponding to the p17/p24 cleavage site of HIV-1 were carried out as described earlier [13]. The amount of products generated by proteolytic cleavage were evaluated by integration of the peak areas. Protease concentration in the assay was determined by active site titration

using the HIV protease inhibitor, SR 47247 (SANOFI) and by Henderson analysis [14]. For kinetic analysis, protease activity was titrated with the 72-amino acid polypeptide at concentrations of 47–588 μM in two independent assays incubated for 6 and 15 h, respectively. Kinetic parameters were determined by Lineweaver–Burk and Eadie Hofstee analysis.

3. RESULTS AND DISCUSSION

The HIV *gag* polyprotein contains at last 4 different cleavage sites. For conducting kinetic analysis, we used a chemically synthesized 72-amino acid polypeptide spanning just the p7 and p1 proteins (Fig. 1A). No hydrolysis was observed in the absence of HIV-1 protease (Fig. 1B). The 72-amino acid polypeptide was hydrolyzed by HIV-1 PR, giving rise to two products as shown in the HPLC chromatogram (Fig. 1C). The hydrolysis of this synthetic polypeptide was linear up to 24 h of incubation according to the appearance of product peaks by RP-HPLC (Fig. 2A). Additionally, protease inhibitors, such as JLK80/1MC, inhibited hydrolysis of the synthetic polypeptide demonstrating specific hydrolysis mediated by the HIV-1 protease (data not shown).

One cleavage product of the synthetic polypeptide eluting at 23% acetonitrile on a C_4 reverse-phase column (Fig. 1C), peak 1) was identified as the N-terminus of p7 by N-terminal sequencing of the first 35 amino acids, starting with MQRGN. The second cleavage product, which elutes slightly after the substrate at 31% acetonitrile (Fig. 1C, peak 2) was sequenced up to 13 amino acids. The sequence starting with FLGKI, as well as its molecular weight of 1980.4 Da, as determined by mass spectroscopy, indicate hydrolysis of the peptide bond between amino acid 55 (N) and 56 (F) of

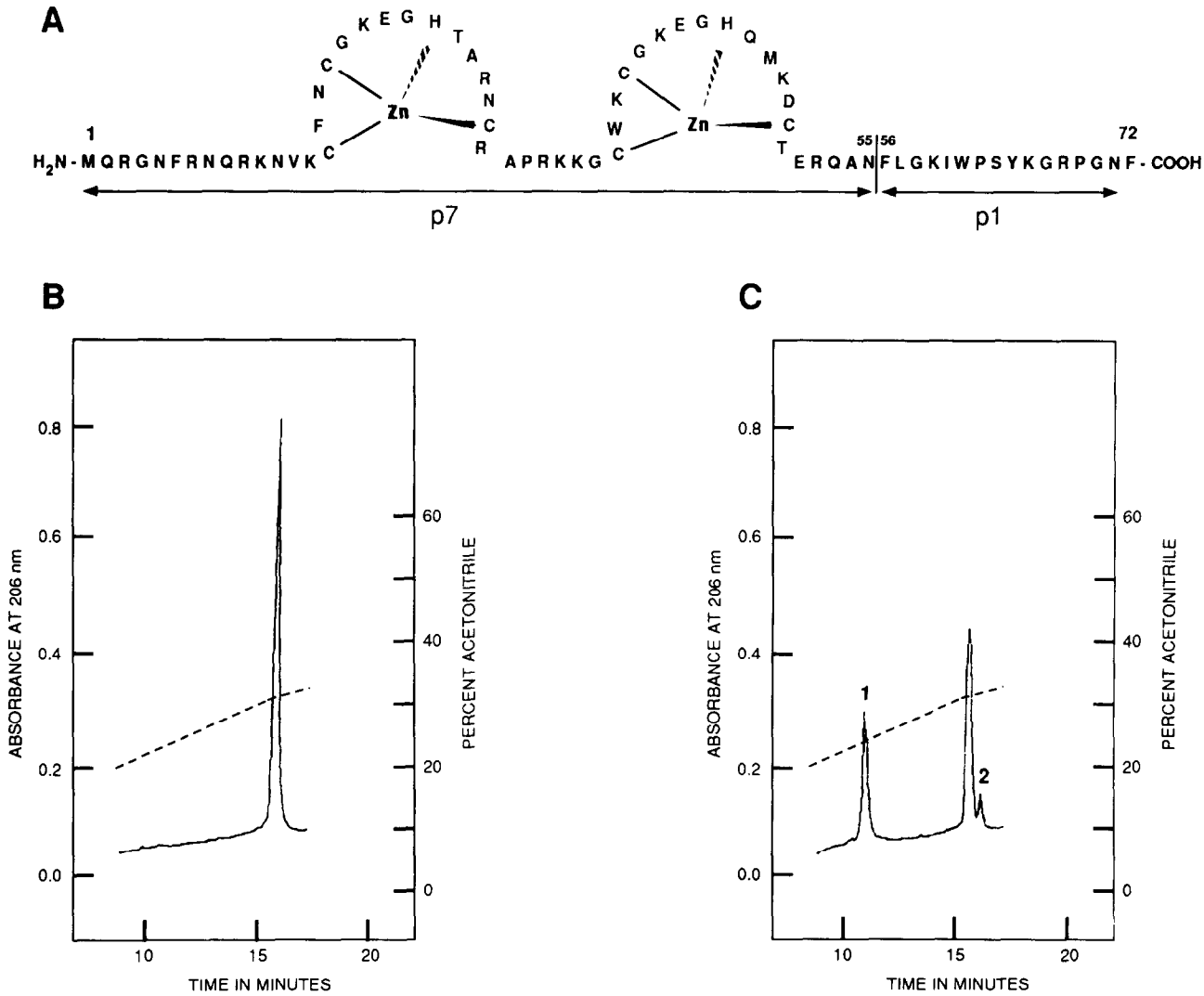

Fig. 1. (A) Structure of the 72-amino acid polypeptide. The 72-amino acid polypeptide was chemically synthesized and assayed in the absence of Zn^{2+}, unless indicated otherwise. The p7/p1 cleavage site was deduced by N-terminal sequencing and mass spectrometric analysis of the hydrolysis products after RP-HPLC purification. (B,C) HPLC elution profiles of the 72-amino acid polypeptide and its cleavage products. 5 μg of the 72-amino acid polypeptide was assayed in the absence (B) and presence (C) of 5.3 ng of HIV-1 protease. The time scale is given in minutes after injection, and the indicated gradient (----) is corrected for the void volume. Peaks 1 and 2 in C denote the product peaks, p7 and p1, respectively.

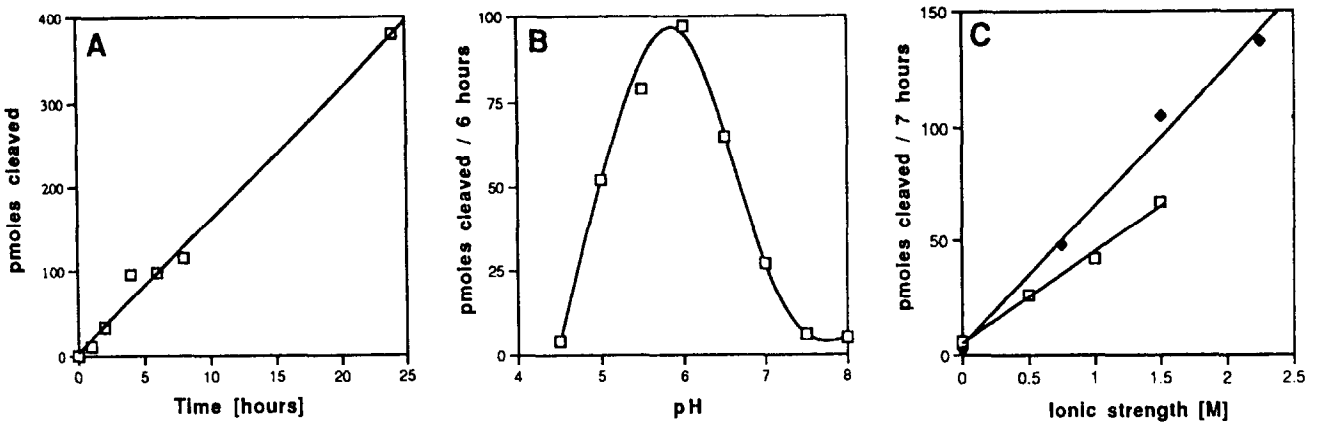

Fig. 2. (A) Time–course of p7/p1 cleavage. An 8-fold volume of the standard assay, as described in section 2 containing 5,970 pmol of the synthetic polypeptide was incubated in a sealed tube at 37°C and in a closed incubator to minimize evaporation. After mixing, 10 μl were removed at the indicated time points, added to 90 μl of 1% TFA solution and analyzed by RP-HPLC. (B) pH titration of p7/p1 cleavage. Enzyme activity at different pH values was tested at a final concentration of 4 M KCl, which was used instead of 0.75 M ammonium sulfate. (C) Salt titration of p7/p1 cleavage. Increasing concentrations of KCl (□) and ammonium sulfate (■), expressed in ionic strength, are plotted against enzyme activity.

the polypeptide (see Fig. 1A). This confirms the previously predicted p7/p1 cleavage site [7,8]. Under the conditions used, we did not detect any other cleavage products.

The pH optimum for proteolytic hydrolysis of the p7/p1 cleavage site is 6.0 (Fig. 2B). The rate of cleavage increases with increasing ionic strength, with a more pronounced effect when salts possessing a high salting-out effect were used (Fig. 2C), which is in accordance with published data for HIV-1 PR employing synthetic oligopeptide substrates [15,16]. For the p7/p1 hydrolysis, we obtained values of $K_m = 99$ μM (± 8) and $k_{cat} = 0.152$ s^{-1} (± 0.002), which are within the range reported for cleavage of various peptide substrates of HIV-1 PR (K_m = 5–1,250 μM and k_{cat} = 0.01–6.8 s^{-1} [10]). However, the turnover rates (k_{cat}/K_m) of the p7/p1 and the p1/p6 cleavage sites are small, 1.56 $mM^{-1} \cdot s^{-1}$ (± 0.11) and 0.6 $mM^{-1} \cdot s^{-1}$ [10], respectively, indicating a rather low rate of cleavage of its parent protein, p15 into p7, p1 and p6, as compared to other sites within the $pr55^{gag}$. However, incubation of the *gag* polyprotein synthesized in vitro with purified HIV PR shows that the p6 and p7 proteins are processed faster than the p17 and p24 proteins during the sequential cleavage reaction [17]. This apparent contradiction between the fast appearance of p7 and p6 during polyprotein processing, and the low turnover rates of synthetic peptides corresponding to these cleavage sites, indicates that additional or other parameters determine the order in which the proteins are processed. Therefore, it was of interest to test whether the secondary structure of p7 has an effect on the rate of cleavage.

In the absence of EDTA and at neutral pH, addition of ≥ 2.2 molar equivalents of $ZnCl_2$ reconstitutes the structure of the mature p7 protein [11,18–20]. One zinc atom is coordinated within each of the two Cys-X_2-Cys-X_4-His-X_4-Cys motifs of p7 (Fig. 1A), and the two zinc domains were found to be spatially close [21]. As shown in Table I, addition of 2.5 or 12.5 equivalents of zinc had no substantial effect on the hydrolysis of the p7/p1 cleavage. It is possible that an enhanced enzyme activity induced by conformational changes of p7 complexed to Zn^{2+} was not detected due to concomitant inhibition of the PR by Zn^{2+} [22] present in the assay. To test this, the polypeptide was substituted with 80 μM EDTA to maintain a stoichiometric number of Zn^{2+} binding (chelation) sites in the assay, and PR activity was measured by hydrolysis of a synthetic nona-peptide [13]. Under these conditions, inhibition of PR activity was not detected up to a concentration of 1 mM $ZnCl_2$ (data not shown). Therefore, we conclude that the hydrolysis of the p7/p1 cleavage site is independent of the Zn^{2+}-induced conformation of the p7 protein.

The data presented here confirm the p7/p1 cleavage site to be a specific maturation site of HIV. For hydrolysis, the native conformation of the p7–Zn^{2+} complex is not required in vitro. Furthermore, the ability of the HIV-1 PR to hydrolyse the p7/p1 scissile bond in the synthetic polypeptide, but not in a synthetic 11-amino acid oligopeptide substrate [10], suggests that amino

Table I

p7/p1 cleavage of denatured and reconstituted p7

$ZnCl_2$ (mM)[a]	$ZnCl_2$ (eq. of p7/p1)[b]	enzyme activity (pmol/15 h)[c]	% of substrate cleaved
0	0	163	41
0.1	2.5	197	49
0.5	12.5	142	36

[a]Final concentration in the assay.
[b]$ZnCl_2$ concentration in relation to the polypeptide concentration (40 μM = 1 molar equivalent (eq.))
[c]Enzyme assays were carried out for 15 h at 37°C in the absence of EDTA.

acids relatively far away from the cleavage site contribute to the recognition of an amino acid sequence as a substrate. Taken together, recognition of this cleavage site by the HIV-1 PR is determined by the primary structure of the substrate. The Zn^{2+}-induced conformation of the p7 protein has no effect on the hydrolysis of its C-terminal cleavage site.

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