Sensitivity, Soluble Chromogenic Substrates for HIV-1 Proteinase*

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By replacement of the P1 residue in a capsid/nucleocapsid capsid site mimic with 4-N02-phenylalanine (Nph), an excellent chromogenic substrate, Lys-Ala-Arg-Val-Leu*Nph-Glu-Ala-Met, for HIV-1 proteinase (kcat = 20 s^-1, Km = 22 mM) has been prepared. Substitution of the Leu residue in P1 with norleucine, Met, Phe, or Tyr had minimal effects on the kinetic parameters (kcat and Km/Km) determined at different pH values, whereas peptides containing Ile or Val in P1 were hydrolyzed extremely slowly. The spectrophotometric assay has been used to characterize the proteinase further with respect to pH dependence, ionic strength dependence, and the effect of competitive inhibitors of various types.

The proteinase (PR) that is responsible for catalyzing specific cleavages in the gag and gag-pol polyproteins of the human immunodeficiency virus is an attractive target for anti-viral chemotherapy. A virus that synthesizes a mutationally inactivated PR does not form infectious virions (1). The design of PR inhibitors would be facilitated by a detailed understanding of the architecture of the PR active site. This may be approached through a systematic definition of the substrate requirements of this important viral enzyme.

Oligopeptides containing the amino acid sequence spanning any one of the seven natural polyprotein cleavage sites may be used as substrates for PR cleavage in vitro (2). Such peptide substrates or derivatives thereof typically have Km values in the millimolar range near their limit of aqueous solubility. Proteinase cleavage of these substrates has been monitored by HPLC product resolution and quantitation. Such assays are time-consuming, discontinuous, and imprecise. In contrast, spectrophotometric assays are rapid, continuous, and more precisely quantitative. However, spectrophotometric techniques depend on the development of appropriate chromogenic peptide substrates. Nished et al. (3) described an HIV PR substrate with the reporter group 4-N02-phenylalanine (Nph) in the P1 position. Despite limited aqueous solubility and a Km in excess of 450 μM, the peptide illustrated the advantage of Nph-containing substrates in HIV PR assays.

This communication describes the hydrolysis of watersoluble chromogenic substrates based on Nph incorporation in the P1 position. In turn, these substrates facilitate further characterization of the HIV 1 PR.

EXPERIMENTAL PROCEDURES

The series of substrates, Peptides 1-11 (Table 1), based on the cleavage site spanning gag residues 359-367, Lys-Ala-Arg-Val-Leu-Ala-Glu-Ala-Met (Peptide 1), was synthesized by solid-phase methods utilizing an Applied Biosystems model 430A Synthesizer. In some peptides, the Met residue was replaced with isosteric Nle to avoid complications resulting from oxidation. A COOH-terminal Gly residue was added in the preparation of some peptide amides in an effort to improve the synthetic yield. However, recoveries of all products were excellent and were equivalent for peptides terminating either in Nle-NH2 or in Nle-Gly-NH2.

Homogeneous preparations of recombinant HIV-1 PR (single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were obtained by two different bacterial expression strategies (4, 5). The active concentration of individual preparations was determined by active site titration (6).

Stock solutions (~5 mM) of peptide substrates were prepared in distilled water. The hydrolysis of each peptide was characterized under a series of conditions at 30 or 37 °C with pH ranging from 4.1 to 9.0 and total ionic strength between 0.37 and 2.0 M. Specific conditions are described in each table legend. Assays were performed either by reverse-phase HPLC using a fast protein liquid chromatography instrument (Pharmacia) fitted with a PepRPC HR 5/5 column for quantitative analysis of cleavage products (7) or by spectrophotometric monitoring of the decrease in absorbance at 300 nm resulting from hydrolysis of the scissile peptide bond (8). In some assays, rates were determined by averaging the absorbance decrease over the range of 284-304 nm using a Hewlett-Packard diode array spectrophotometer.

RESULTS AND DISCUSSION

Previously published studies on HIV PR have concentrated largely on substrates that are based on the MA/CA junction in the gag polyprotein. To explore other determinants for proteinase recognition, this work has focused on the Leu*Ala cleavage site at one of the CA/NC junctions. Cleavage of Peptide 1 (Table 1) by HIV PR at pH 4.7 and an ionic strength of 1 M was followed by HPLC. A Km of 200 μM and a kcat of 17 s^-1 were derived using the procedure described previously (7).

Replacement of Ala in the P1 position with Phe or Nph

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(Peptides 11 and 2) yielded derivatives that were also hydrolyzed readily. Kinetic analysis using HPLC of the cleavage of Peptide 2, for example, by HIV PR at pH 4.7 and ionic strength = 1 M, generated a \( K_m \) of 60 \( \mu \)M and a \( k_{cat} \) of 28 s\(^{-1}\), i.e. similar values to those obtained for Peptide 1. The predicted cleavage products were verified by amino acid analysis, the N\(_2\)-terminal fragment having the composition Lys 0.9, Ala 0.9, Arg 1.1, Val 1.1, and Leu 1.0 with the COOH-terminal fragment containing Nph 0.7, Glu 1.4, Ala 1.1, and Met 0.7. The absence of additional product peaks in HPLC analysis confirmed that the -Leu*Nph- bond was a unique site of cleavage of Peptide 2. A control experiment, by observing the decrease in absorbance at 300 nm (see the legends to Tables II, III, and IV).

More detailed examination of the pH dependence of the hydrolysis of Peptide 2 was also carried out by observing changes in absorbance at 300 nm using HPLC of the cleavage of Peptide 2 at pH 4.7 and ionic strength = 1 M. Aliquots were removed at appropriate times and the reaction was stopped by the addition of 5% (v/v) trifluoroacetic acid for analysis by reverse-phase HPLC (for Peptides 1, 2, and 9-11). Alternatively, with Peptides 2-10, the cleavage reaction was monitored directly by following the decrease in absorbance at 300 nm (see the legends to Tables II, III, and IV).

**Table I**

| Peptide | Sequence |
|---------|----------|
| 1 | Lys-Ala-Arg-Val-Leu*Ala-Glu-Ala-Met |
| 2 | Lys-Ala-Arg-Val-Leu*Nph-Glu-Ala-Met |
| 3 | Lys-Ala-Arg-Val-Leu*Nph-Glu-Ala-Nle-Gly-NH\(_2\) |
| 4 | Lys-Ala-Arg-Val-Tyr*Nph-Glu-Ala-Nle-NH\(_2\) |
| 5 | Lys-Ala-Arg-Val-Met*Nph-Glu-Ala-Nle-NH\(_2\) |
| 6 | Lys-Ala-Arg-Val-Nle*Nph-Glu-Ala-Nle-NH\(_2\) |
| 7 | Lys-Ala-Arg-Val-Nle*Nph-Glu-Ala-Nle-Gly-NH\(_2\) |
| 8 | Lys-Ala-Arg-Val-Phe*Nph-Glu-Ala-Nle-Gly-NH\(_2\) |
| 9 | Lys-Ala-Arg-Val-Ile*Nph-Glu-Ala-Nle-NH\(_2\) |
| 10 | Lys-Ala-Arg-Val*Nph-Glu-Ala-Nle-NH\(_2\) |
| 11 | Lys-Ala-Arg-Val-Leu*Phe-Glu-Ala-Nle-Gly-NH\(_2\) |

The values obtained for \( k_{cat} \) (Table II) with Leu, Nle, Met, or Tyr in P\(_1\) differed by only about 2-fold at pH 4.7 and a similar uniformity in values was observed at pH 6.25. By contrast, peptides with Ile or Val (Peptides 9 or 10) were resistant to hydrolysis by HIV-1 PR (<20% cleavage in 16 h) suggesting that side chains branched at the \( \delta \)-carbon are sterically unacceptable in the P\(_1\) position.

The \( K_m \) values derived for Peptides 2-8 are presented in Table III. Replacement of the P\(_1\) residue had very little effect at either pH. The influence of replacement of the Nph group in the P\(_1\)' position by Phe (Peptide 11) was similarly examined. Peptide 11 has such a low intrinsic \( A_{300} \) value that it does not interfere with measurement of the absorbance change observed upon hydrolysis of the Nph-containing peptides. Its interaction with PR was thus determined by treating it as an apparent competitive inhibitor of the cleavage of Peptide 3. An apparent \( K_m \) value of 75 \( \mu \)M was obtained at pH 6.25 and 30°C. Using the construction of Blake et al. (10) which more correctly considers the second peptide to be an alternate substrate, an apparent \( K_m \) value of 60 \( \mu \)M was derived. Since the \( K_m \) (measured under identical conditions at pH 6.25) for the hydrolysis of Peptide 3 itself was 55 \( \mu \)M, it is apparent that the presence of the nitro group on the aromatic ring does not alter the substrate interaction significantly.

**Table II**

| Peptide | \( P_1 \) substituent | \( K_m \)
|---------|---------------------|
| 2 | Leu | 20 | 30
| 4 | Tyr | 20 | 25
| 5 | Met | 20 | 25
| 6 | Nle | 45 | 40
| 9 | Ile | ~0 | 
| 10 | Val | ~0 | 

**Table III**

| Peptide | \( P_1 \) substituent | \( K_m \)
|---------|---------------------|
| 2 | Leu | 22 | 60
| 3 | Leu | 6 | 40
| 6 | Nle | 15 | 90
| 7 | Nle | 12 | 115
| 8 | Tyr | 7 | 40
| 4 | Phe | 12 | 40
kinetic parameters was carried out for the hydrolysis of Peptides 2 and 6 (Fig. 1). The data points for both substrates fit well to a single line. In the upper panel (Fig. 1), a decrease in the parameter log \( k_{\text{cat}}/K_m \) defines an apparent \( pK_m \) of 5.7. This dissociation can arise from either the free enzyme or the free substrate. Peptides 2 and 6 both have a Glu residue in the \( P_1' \) position in a predominantly hydrophobic sequence. However, a similar \( pK \) dependence was observed for the hydrolysis of an unrelated peptide substrate that did not contain an acidic residue but which also included the sequence -Asp-Val-Tyr-*Nph-Val- around the scissile peptide bond (data not shown). On this preliminary basis, then, it would appear that the dissociation may arise from a group in the enzyme, e.g. Asp\(^{\alpha} \) or Asp\(^{\beta} \), known to be in the vicinity of the substrate binding cleft (11). A decrease in binding affinity at higher \( pK \) has been observed previously with inhibitors and other substrates (7). A more detailed rationalization should be obtained by synthesis of further analogs of Peptide 6 containing Gln or other amino acids in the \( P_1' \) position together with mutagenesis of the HIV PR.

A second apparent proton dissociation is observed in the upper panel of Fig. 1 at a \( pK \) of approximately 7.3. The change in molar extinction coefficient at 300 nm upon cleavage of Nph-containing peptides is too small at \( pK \) values of 7 or above to permit accurate measurement of rates of hydrolysis. Thus, the data above \( pK \) 7 (Fig. 1) were obtained by the HPLC method of analysis and hence are of lower precision. Once again, this ionization may arise from a side chain in the enzyme or from dissociation of a proton from a basic group in the NH\(_2\)-terminal segment of the substrate (e.g. the primary amino group at the NH\(_2\)-terminus of both substrates) which would remove a positive charge and facilitate binding to the hydrophobic cleft. Synthesis of further peptides, acylated at the NH\(_2\)-terminus and/or with replacement of the Lys and Arg residues will resolve this issue. For the hydrolysis of both substrates, the HIV-1 PR maintained similar values of \( k_{\text{cat}} \) to \( pK \) values as high as 9.0 (Fig. 1, middle panel). The absence of an inflection in this plot is in contrast to many previous investigations of archetypal aspartic proteinases (e.g. see Ref. 6) where the apparent loss of a proton (presumably from the catalytic aspartic acid residues) occurs at \( pK \) values between 5 and 6. Even with the renin branch of the aspartic proteinase family (12), activity declines rapidly at neutral \( pK \) and above. Thus, HIV PR would appear to be able to provide efficient catalysis at the \( pK \) values likely to exist within virally infected cells. The acid \( pK \) "optimum" for HIV-1 PR described previously would appear to be a reflection of the inability of substrates to interact strongly with this enzyme rather than a reduction in catalytic potency at higher \( pK \) values.

Previous investigations on other retroviral proteinases have shown that high ionic strength facilitates active site interaction with oligopeptide substrates (13). The effect of ionic strength on the activity of HIV PR was thus examined using the spectrophotometric assay. As the ionic strength was raised from 0.5 to 2.0 M, once again the \( k_{\text{cat}} \) parameter for the hydrolysis of Peptide 2 was unaffected, whereas the corresponding \( K_m \) values improved by approximately 4-fold (from 30 to 7 \( \mu \)M). Similarly, with Peptide 3, at \( pK \) 4.7 and 6.5, the \( K_m \) value consistently decreased with increasing ionic strength (Table IV). Since, this effect was observed for Peptide 2, terminating in a carboxylate group, and for Peptide 3, terminating in a carboxamide, it seems likely that hydrophobic interactions dominate binding of peptides to the active site cleft of HIV-1 PR.

It is clear that Peptide 2 and most of its derivatives have excellent kinetic properties over the range of conditions we have examined. The \( K_m \) values measured for these peptides are more than 20 fold lower than those previously reported for other HIV-1 PR substrates which may indicate tighter active site interactions. To emphasize the advantages of the spectrophotometric assay, inhibition constants were determined for several inhibitors (ranging in potency) of HIV PR. The \( K_i \) values thus obtained at \( pK \) 4.7 for H-261, a hydroxysterol isostere-containing renin inhibitor, and for acetyl-pepsatin were 5 and 90 \( \mu \)M, respectively, in excellent agreement with those reported previously using HPLC assays (7). The aromatic analogue of the statine dipeptide mimic, 4-amino-3-OH-phenylpentanoic acid (PheSta) was introduced into three pseudo-palindromic peptides.

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**Table IV**

The effect of ionic strength on the hydrolysis of Lys-Ala-Arg-Val-Leu* Nph-Glu-Ala-Nle-Gly-NH\(_2\) by HIV-1 proteinase at two \( pK \) values

| Ionic strength | pH 4.7 | pH 6.5 |
|---------------|--------|--------|
| M             | \( \mu M \) | \( \mu M \) |
| 0.5           | 13     | 255    |
| 0.65          | 11     |        |
| 0.67          |        | 130    |
| 0.90          | 6      |        |
| 1.07          | 100    |        |
| 1.15          | 5      |        |
| 1.57          | 37     |        |
| 1.65          | 6      |        |

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**Fig. 1.** pH dependence of the kinetic parameters (\( K_m \), \( k_{\text{cat}} \), and \( k_{\text{cat}}/K_m \)) for the hydrolysis of two chromogenic peptides by HIV-1 proteinase. The buffers used contained 4 mM EDTA, 5 mM mercaptoethanol, and NaCl to give a final ionic strength of 1 M and were sodium acetate (\( pK_4.1-5.2 \)), MES (\( pK_5.2-6.7 \)), HEPES (\( pK_6.7-7.0 \)), and Tris (\( pK_7.6-9.0 \)). Reactions at 37°C were initiated by the addition of HIV-1 proteinase (into a final volume of 800 \( \mu L \)) and monitored by following the decrease in the average absorbance over the range of 284-324 nm. Buffers were 100 mM sodium acetate, pH 4.7-5.2, MES, 20 mM, pH 5.2-6.7, or Tris, 20 mM, pH 6.5-8.0, containing 5 mM mercaptoethanol and 4 mM EDTA. NaCl was added to give the total ionic strength.

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The first two of these compounds were weak competitive inhibitors at pH 4.7 ($K_I = 440$ and 150 nM, respectively). The third was essentially ineffective at concentrations up to 19 nM.

It has been shown that -Tyr*Nph-, -Met*Nph-, or -Leu*-Nph- (as well as -Nle*Nph- or -Phe*Nph-) can all be substituted satisfactorily for the naturally occurring -Leu*Ala- scissile bond in a peptide that otherwise duplicates one of the CA/NC junctions (for terminology, see Ref. 14). In contrast, insertion of these three dipeptide pairs into sequences corresponding to the MA/CA, the other CA/NC, and (a derivative of) the RT/IN junctions in the HIV-1 polyprotein, to give, respectively,

-Arg-Ser-Gln-Asn-Tyr*Nph-Ile-Val-Gln
-Asn-Thr-Ala-Thr-Ile-Met*Nph-Gln-Arg-Gln-Arg-Gly-Asn
-Arg-Arg-Gln-Val-Leu*Nph-Leu-Glu-Lys-Arg

generated peptides which exhibited negligible rates of hydrolysis by HIV-1 PR at pH 4.7. Therefore, not only the relative size/nature of the residues contributing the scissile peptide bond but also the identities of those residues in the flanking positions have a considerable influence on the conformation that can be adopted by the substrate within the active site cleft.

Results in this communication establish a simple, rapid method to quantitatively evaluate active site interactions in HIV-1 PR and, potentially, in mutant forms of this enzyme. Utilizing synthetic peptide chemistry, further investigations are now in progress to attempt to capitalize on the newly-available three-dimensional structure of an inhibited-HIV-1 PR complex (11) to detail the structural demands of these other subsites within the active site cleft of this essential viral enzyme.

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REFERENCES
1. Kohl, N. E., Emini, K. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., and Sigal, I. S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4686-4690
2. Darke, P. L., Nutt, R. F., Brady, S. F., Garsky, V. M., Ciccarone, T. M., Leu, C.-T., Lumma, P. K., Friedinger, R. M., Veber, D. F., and Sigal, I. S. (1988) Biochem. Biophys. Res. Commun. 156, 297-303
3. Nasheed, N. T., Louis, J. M., Sayer, J. M., Wondrak, R. M., Mora, P. T., Orozslan, S., and Jerina, D. M. (1988) Biochem. Biophys. Res. Commun. 163, 1079-1085
4. Graves, M. C., Lim, J. J., Heimer, E. P., and Kramer, R. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9449-9453
5. Hirel, P.-H., Parker, F., Jung, G., Boizieu, J., Outerovitch, D., Dugue, A., Peltiers, C., Giulacci, C., Boulay, R., Lelièvre, Y., Cambou, B., Mayaux, J.-P., and Cartwright, T. (1990) Antiviral Chem. Chemother. 1, in press
6. Dunn, B. M., Valler, M. J., Rolph, C. F., Foundling, S. I., Jimenez, M., and Kay, J. (1987) Biochim. Biophys. Acta 913, 122-130
7. Richards, A. D., Roberts, R. F., Dunn, B. M., Graves, M. C., and Kay, J. (1989) FEBS Lett. 247, 113-117
8. Dunn, B. M., Jimenez, M., Porten, B. F., Valler, M. J., Rolph, C. E., and Kay, J. (1986) Biochem. J. 237, 899-906
9. Hofmann, K., Haas, W., Smithers, M. J., Wells, R. D., Wolman, Y., Yanaithara, N., and Zanetti, G. (1965) J. Am. Chem. Soc. 87, 620-631
10. Blake, R. C., Vassall, R. F., and Blake, D. A. (1989) Arch. Biochem. Biophys. 272, 102-108
11. Miller, M., Sathyaranayana, B. K., Wlodawer, A., Toth, M. V., Marshall, G. R., Clawson, L., Selk, L., Schneider, J., and Kent, S. B. H. (1989) Science 246, 1149-1152
12. Yamauchi, T., Nagahama, M., Hori, H., and Murakami, K. (1988) FEBS Lett. 230, 205-206
13. Koller, M., Dahnke, W., Katz, K. A., Leis, J., and Skaalka, A. M. (1989) J. Biol. Chem. 264, 3428-3435
14. Leis, J., Baltimore, D., Bishop, J., Coffine, J., Fleissner, E., Goff, S., Orozslan, S., Robinson, H., Skaalka, A., Temin, H., and Vojt, E. (1988) J. Virol. 62, 1808-1809
15. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162
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