Avian Retroviral Protease and Cellular Aspartic Proteases Are Distinguished by Activities on Peptide Substrates*

Moshe Kotler‡, Waleed Danhoş, Richard A. Katz‡, Jonathan Leis**, and Anna Marie Skalka‡‡

From the Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, Pennsylvania 19111

(Received for publication, September 20, 1988)

The avian sarcoma/leukemia virus protease (PR), purified from avian myeloblastosis virus has a native molecular mass of 26 kDa, suggesting a dimeric structure. The enzymatic activity of PR has been characterized using synthetic peptide substrates. PR is most active at pH 5.5, 35 °C and 2–3 M NaCl. Under these conditions PR cleaves decapeptides which are resistant in low ionic strength. This high, nonphysiological, salt concentration also increases the proteolytic activity of a cellular aspartic protease, pepsin. PR and pepsin show additional similarities: they both cleave a synthetic decapeptide at the same Tyr-Pro bond in low and high salt, while the cleavage site preferences of human renin and cathepsin-D in this substrate are altered at high salt concentrations. In addition, iodination of the tyrosine residue in this decapeptide causes an increase in the rates of hydrolysis by both PR and pepsin. However, \( K_a \) values are too high to be estimated accurately for PR using Tyr-Pro and Tyr(1)-Pro decapeptides as substrates. Comparison of the digestion products of two additional decapeptides, altered in a single amino acid residue, shows that PR cleaves at fewer sites than all three cellular enzymes. Furthermore, pepstatin, a strong inhibitor of pepsin, renin, and cathepsin-D has little effect on PR.

The proteases encoded by retroviruses are related to the family of cellular aspartic proteases also called acidic or carboxypeptidases (1–7). However, significant differences between the two groups exist. The cellular aspartic proteases contain more than 300 residues, whereas the maximum length of retroviral proteases (PR) is 130. The cellular enzymes contain two domains. Each domain includes the conserved residues Asp-Thr-Gly (e.g., aspartic acid residues are in positions 32 and 215 in pepsin), juxtaposed to form the catalytic site along a perfect 2-fold axis of symmetry. The retroviral PRs contain only a single Asp-Thr(or Ser)-Gly sequence. Similarities in sequence and structure in the two domains of the aspartic proteases and the single domain in viral enzymes have been noted, leading to the hypothesis that the cellular enzymes evolved from molecules with one domain by a process of gene duplication and fusion (1). Viral PR may therefore represent a monomeric form of the two active domains of the cellular aspartic proteases, and both presumably evolved from a common ancestor. A model suggesting that the viral enzyme acts as a dimer has recently been proposed (1). The predicted tertiary structure for such a dimer would form an active site with a 2-fold axis of symmetry, as in the cellular aspartic proteases (1).

PR cleaves retroviral precursor proteins at particular sites to yield mature structural proteins, PR itself, reverse transcriptase, and a protein required for DNA integration (8–23). Other proteases, such as bovine serum albumin, are poor substrates in both native and denatured states (12, 24). This high specificity for their natural substrates distinguishes PRs from many other members of the aspartic protease family. It also makes these enzymes difficult to study. However, we (25) and others (6, 26) have shown that this difficulty can be circumvented by using a synthetic peptide, corresponding to a sequence in the natural substrate that contains a target peptide bond (25). Such peptides are cleaved precisely by PR and this activity can be measured by high performance liquid chromatography (HPLC) and thin layer electrophoresis (TLE) (25). Since proteolytic processing via PR is required for viral infectivity (15, 19, 27), an inhibitor of its activity could potentially be useful for therapy of retroviral diseases (28).

However, cell-encoded aspartic proteases are active intra- or extracellularly, fulfilling essential physiological functions (29). Therefore, drugs of therapeutic value must be specific for the viral PR and should have a minimal effect on cellular acid proteases. A comparison of the biochemical properties of the viral PR, and other cellular aspartic proteases is an important first step toward delineating significant differences. Here we report: (i) that PR purified from virus is a dimer; (ii) the optimal conditions for monitoring the avian sarcoma leukemia virus (ASLV) PR activity; (iii) substrate requirements and specificity of the cleavage sites; and (iv) the effect of pepstatin, an inhibitor of aspartic proteases. These properties of viral PR are compared to three cellular aspartic proteases, pepsin, cathepsin-D, and renin.

**EXPERIMENTAL PROCEDURES**

*Materials*

Thin layer electrophoresis plates, plastic cellulose sheets Art. 5577, 20 × 20 cm, were from E. M. Science, W. Germany. Triethylamine
(TEA) is from Pierce, Sephadex G-75 from Sigma, fluorescamine, from Hoffmann-La Roche, and pepsinogen (porcine stomach), cathespin-D (grade 1-5, bovine spleen), and peptatin-A from Sigma. Renin (human) was from Calbiochem. Viral protease (PR) was purified from AMV particles as described previously (30).

Methods

Decapeptide Synthesis—Decapeptides were synthesized by solid-phase methods (31). Deprotection and cleavage from the resin were achieved by treatment with anhydrous hydrogen fluoride according to the procedure of Tam et al. (32). The peptides were purified by preparative HPLC using a Bondapak C18 column. The purity of the peptides was confirmed by analytical HPLC, amino acid analysis, and microsequencing.

Decapeptides containing Tyr-Pro were labeled with 125I-(Amer sham), using Iodo Beads (Pierce), as recommended by the producer. The labeled peptide was mixed with a Tyr(1)-Pro peptide (Table I, sham), using Iodo Beads (Pierce), as recommended by the producer.

Fig. 1. Gel filtration of the AMV PR. Purified preparations of AMV PR (1.6–1.8 A280 units) were subjected to gel filtration through a 0.5-mL Sephadex column (1.1 × 20 cm) at 4 °C in the presence of 10 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol with 0.1 M NaCl or 1 M NaCl and absorbance at 280 nm was monitored. Ovalbumin and cytochrome c (Δ) and soybean trypsin inhibitor (▴) were used to standardize the column in separate runs. The elution profile of the standards was not affected by the ionic conditions of the eluant.

TABLE I

The proteolysis of synthetic peptides by PR

<table>
<thead>
<tr>
<th>Decapeptide</th>
<th>-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>+5</th>
<th>Low salt</th>
<th>High salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NH2-Thr Phe Gln Ala Tyr Pro Leu Arg Glu Ala-COOH</td>
<td>+ (1)</td>
<td>++ (10.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NH2-Thr Phe Gln Ala Ala Pro Leu Arg Glu Ala-COOH</td>
<td>-</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NH2-Thr Phe Gln Ala Phe Pro Leu Arg Glu Ala-COOH</td>
<td>±</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NH2-Thr Phe Gln Ala His Pro Leu Arg Glu Ala-COOH</td>
<td>-</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NH2-Thr Phe Gln Ala Tyr Gly Leu Arg Glu Ala-COOH</td>
<td>±</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NH2-Thr Phe Gln Ala Tyr Arg Leu Glu Ala-COOH</td>
<td>±</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NH2-Thr Phe Gln Ala Tyr (I) Pro Leu Arg Glu Ala-COOH</td>
<td>++ (4)</td>
<td>+++ (39)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>NH2-Thr Phe Gln Ala Tyr (I)</td>
<td>Pro Leu Arg Glu Ala-COOH</td>
<td>++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Low salt is no NaCl addition. The reaction mixture contains 0.1 M sodium citrate buffer, pH 5.5.

High salt is 2.5 M NaCl.
was the sole target peptide bond for both proteases as verified by TLE (Fig. 2A, lanes 1 and 8) and HPLC analysis (not shown). The following describes results of a comparison of the biochemical activities of both enzymes using Tyr(I)-Pro and Tyr-Pro-containing decapeptides as substrates.

Fig. 3A shows that PR is quite active on the Tyr(I)-Pro decapeptide over a range of pH from 4.5 to 6.5 with an optimum at 5.5. A similar optimum was observed previously, using as substrates a retroviral gag precursor protein or bovine serum albumin denatured by boiling in 0.6% sodium dodecyl sulfate as substrates (11, 24, 37). This similarity with different substrates suggests that the pH effect is on the enzyme and not on the substrate. Pepsin is active on the decapeptide at a lower range, with an optimum at pH 3.5 and no measurable activity at pH 6.0 (Fig. 3A). As shown in Figure 3B, incubation of both enzymes at 35 °C for 30 min yields the highest amount of product.

To address the possibility that PR activity requires intrachain disulfide bonds or metals, additional experiments were performed using the Tyr(I)-Pro substrate. Assays (data not included) showed that β-mercaptoethanol has little effect on either PR or pepsin, with only 25–30% reduction in activities at concentrations as high as 25–200 mM. EDTA (up to 250 mM) also had very little effect on PR activity, in agreement with the previous results of others (6, 11).

Fig. 4 shows the effect of salt on PR and pepsin cleavage of the Tyr(I)-Pro decapeptide at pH 5.5 and 3.5, respectively. Both enzymes exhibit increased activity at high NaCl concentrations (Fig. 4A). PR is most active from 2 to 3 M; pepsin shows an optimum at 2 M. Preincubation of PR in high salt solutions (for 10 min) followed by dilution into low salt for assay of activity gave results identical to those in which the enzyme was not preincubated. High concentrations of (NH₄)₂SO₄, like NaCl, also increased the proteolytic activities of both enzymes. The highest rates were observed at 1 M, an ionic concentration similar to that at optimal NaCl concentrations (Fig. 4B). High ionic concentration did not alter the substrate specificity of these enzymes; the Tyr-Pro or Tyr(I)-Pro peptide bond was the sole target detected in the corresponding decapeptides (Fig. 2 and see below). To address whether the effect of salt was due to dehydration (or molecular crowding), reactions were carried out under low salt conditions in the presence of ethylene glycol. Ethylene glycol at concentrations as high as 50% caused a slight inhibition in PR activity, while pepsin activity was reduced by 85% in 25% ethylene glycol.

The Hydrolysis Rates at Low and High Salt Concentrations—The time course for hydrolysis of the Tyr-Pro and Tyr(I)-Pro containing decapeptides by PR and pepsin is shown in Fig. 5. The reactions were carried out in low (without NaCl) and high salt (2 M NaCl) at the pH which was optimal for each enzyme. The results show that the Tyr(I)-Pro decapeptide is cleaved more efficiently than the Tyr-Pro decapeptide by both enzymes at both salt concentrations (Fig. 5, A–C). The turnover numbers calculated from these reactions (Table I) show a 10-fold increase for both the iodinated and the noniodinated decapeptides at high as compared with low salt concentration. The rates of hydrolysis of both decape-
Activities of Avian Retroviral and Cellular Aspartic Proteases

Fig. 4. The effect of sodium chloride (A) and ammonium sulfate (B) concentrations on PR and pepsin activities. Reactions were carried out with 7.7 nmol of the Tyr-Pro (PE) cleavage products of reactions run at increasing salt concentrations. We had determined by HPLC separation and amino acid analysis of products (data not included) that two sites in this dipeptide are recognized by these enzymes at low salt concentrations; one is between the glutamine and alanine residues at positions 3 and 2 and the other at Tyr-Pro. As expected, two radioactive products can be seen with both enzymes at low salt concentrations. However, with increasing salt the faster migrating product, formed by cleavage of the Gln-Ala bond, is lost and there is a concomitant increase in the amount of product formed by cleavage of the Tyr-Pro bond. Thus, unlike PR and pepsin, the specificities of these two enzymes for target sites on the Tyr-Pro dipeptide is altered by high ionic strength.

Fig. 5. The kinetics of hydrolysis of decapeptides containing Tyr-Pro (Table I, decapeptide 1) and Tyr-Pro (Table I, decapeptide 8) by PR and pepsin (PE) in low and high salt. Reactions were carried with 77 nmol of substrates in 25 μl. Samples were taken at the indicated times, boiled for 3 min, and analyzed by HPLC as described under "Methods." A, Tyr-Pro (●) and Tyr-Pro (■) decapeptides cleaved by PR (3.2 pmol) in 0.1 M sodium citrate buffer (pH 5.5); B, Tyr-Pro (○) and Tyr-Pro (□) decapeptides cleaved by pepsin (0.1 unit) in 0.1 M sodium citrate buffer (pH 3.5); C, cleavage of Tyr-Pro and Tyr-Pro decapeptides by PR and pepsin in 2 M NaCl. Symbols are as in A and B.
concentrations of $10^{-7}$-$10^{-6}$ M. The PR was not affected by the pepstatin at low salt concentration (Fig. 3C). In high salt, where PR activity was increased, $0.5$-$10$ mM pepstatin reduced PR activity to the level observed at low salt. It is of interest that low concentrations of pepstatin completely inhibit the enzymatic activities of renin and cathepsin-D, while PR remains active even at very high concentration of the inhibitor. Thus, PR and the cellular proteases are clearly distinguished by the effect of pepstatin (6, 39).

**DISCUSSION**

The analyses reported here extend previous results obtained with AMV PR using peptide substrates (25). We have determined the optimal conditions for the assay and have compared PR activity with that of three cellular aspartic proteases. The AMV PR as purified from virions has a native mass, estimated by gel filtration, of 26 kDa suggesting that the enzyme may function as a dimer. This is consistent with crystallographic structural studies of the virtually identical RSV PR which have shown that there are two enzyme molecules in the asymmetric unit of the PR crystals (40). As a dimer, the native structure of PR could resemble that of the cellular proteases which contain two domains each of which may be analogous to a PR monomer.

We find that although there are many other similarities between the viral and cellular aspartic proteases, their activities can be differentiated in several ways. For example, comparison of the cleavage products resulting from hydrolysis of decapeptides demonstrate significant similarities between pepsin and AMV PR. Both enzymes cleave Tyr-Pro and Phe-Pro substrates at the same sites, both react more efficiently with Tyr-Pro than with Tyr-Pro, and both activities are increased at high salt concentration. However pepsin differs from PR in the optimum pH for activity and by the specificity of cleavage of particular substrates: pepsin hydrolyzes the Tyr-Gly product at four sites, while both activities are increased at high salt concentration. However pepsin differs from PR in the optimum pH for activity and by the specificity of cleavage of particular substrates: pepsin hydrolyzes the Tyr-Gly peptide (Table I, decapeptides 3 and 4) at locations other than the X-Pro site (results not shown), and PR does not cleave these peptides at all in low salt. In addition, pepstatin which inhibits pepsin, renin, and cathepsin-D at concentrations of $10^{-7}$-$10^{-6}$ M only partially inhibits PR at concentration of $10^{-3}$ M.

It is known that processing of the precursor polypeptides does not occur until at or near the time when the virus is budding from the cell surface. Physiological conditions (pH, ionic concentration) within the infected cell or viral particle could possibly influence this programming. The relative stability of the viral precursor in cells may be enhanced by the pH of the cytoplasm which at 7.2 is far from the optimum for AMV PR activity. We find that the activities of both PR and pepsin can be stimulated by severalfold if the reaction is run in high ionic concentrations. In the case of PR, this salt effect is significant since it reveals activity with a decapeptide (Ala-Pro) that was not previously recognized as either a substrate or inhibitor and thus assumed not to bind to the enzyme. The Ile-Pro decapeptide has an inhibitory activity at low ionic concentration (25).

Several mechanisms could account for the stimulatory effect of salt. (i) Salt could cause dehydration or molecular crowding, thus driving the reaction. Although not excluded, this seems unlikely since the dehydrating agent, ethylene glycol, does not stimulate PR and it actually inhibits pepsin activity. (ii) Salt could influence the conformation of the peptide substrate, making it more susceptible to cleavage. Since Vogt et al. (11) have shown that cleavage of the RSV
TABLE II
Activities of avian retroviral and cellular aspartic proteases on decapeptides 3 and 5

Part A, the hydrolysis products of decapeptide Phe-Pro (no. 3) and Tyr-Gly (no. 5) treated with aspartic proteases. The decapeptides, 20 mM each, were subjected to the proteases as described in Fig. 2, and the major reaction products were purified by HPLC and subjected to amino acid analysis as described under "Experimental Procedures." Only amino acids with a molar ratio of 0.75 to 1.25 in the same fraction were included in the composition of the peptides. Part B, the cleavage sites for each enzyme were deduced from the results summarized in Part A.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrate (no. 3) Products</th>
<th>Thr</th>
<th>Phe</th>
<th>Gln</th>
<th>Ala</th>
<th>Phe</th>
<th>Pro</th>
<th>Leu</th>
<th>Arg</th>
<th>Glu</th>
<th>Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Pepsin</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Cathepsin-D</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Human renin</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Phe</td>
<td>Pro</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrate (no. 5) Products</th>
<th>Thr</th>
<th>Phe</th>
<th>Gln</th>
<th>Ala</th>
<th>Tyr</th>
<th>Gly</th>
<th>Leu</th>
<th>Arg</th>
<th>Glu</th>
<th>Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Pepsin</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Cathepsin-D</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td>Human renin</td>
<td>1</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Thr</td>
<td>Phe</td>
<td>Gln</td>
<td>Ala</td>
<td>Tyr</td>
<td>Gly</td>
<td>Leu</td>
<td>Arg</td>
<td>Glu</td>
<td>Ala</td>
</tr>
</tbody>
</table>

B. Summary*

PR's natural precursor protein substrate, Pr76, is also stimulated by salt this phenomenon is not restricted to reactions with peptide substrates. (iii) Salt may promote dimerization of PR which may be required for its activity (1). However, salt also stimulates pepsin (Figs. 4 and 5), which contains an active site composed of two domains, and thus acts as a monomeric enzyme (29). (iv) The salt may provide an ionic environment at the active site which enhances the reaction with the substrates or which alters the conformation of the enzymes in a favorable way. This interpretation would be consistent with the fact that the effect is not ion-specific; (NH₄)₂SO₄ and NaCl stimulate the enzyme activity similarly. The low turnover numbers, observed at both high and low ionic strength, and the fact that AMV PR could not be saturated by the synthetic peptides may suggest that the binding of the substrate to the enzyme is a slow process while the release of products is very fast. This explanation, which obviously is not the only one, is supported by results which suggest that enhanced binding of substrate to enzyme, is correlated with increased enzymatic activity. These include (a) the stimulating effect of salt, (b) the fact that Tyr(1)-Pro is a better substrate than the Tyr-Pro peptide, and (c) the fact that the Ala-Pro peptide (alanine is neutral or weakly hydrophilic) is cleaved at high salt concentration whereas at low salt it is neither cleaved, nor otherwise recognized by the enzyme (25).

It is not yet clear why substitution of the tyrosine with iodinated tyrosine in the Tyr-Pro peptide creates a better substrate for AMV PR and pepsin. It could be that the iodine increases the hydrophobicity at the target site, a feature which appears to be important (1, 17, 25). Alternatively, the iodine may affect the Tyr-Pro peptide bond facilitating the creation

---

*CA = cathepsin-D; RE = renin; PE = pepsin.
would create a better substrate or, alternatively, a more potent tide. It would be of interest to determine whether introduction of a hydrogen bond between the substrate and enzyme. Substitution of the iodine with negatively charged groups or other electron withdrawing groups may clarify this point. Unpublished results obtained in our laboratory indicate that synthetic peptides containing four amino acids from the amino side and two amino acids from the carboxyl side of the cleavage sites inhibit the cleavage of the Tyr(I)-Pro decapeptide. It would be of interest to determine whether introduction of iodine or a negatively charged group into such a hexamer would create a better substrate or, alternatively, a more potent inhibitor. Such information would be useful in the design of potential inhibitors of retroviral PRs.

Recently there has been a resurgence of interest in retroviral proteases (1, 4), in part because they represent an attractive target for antiviral therapy in diseases such as AIDS (28). Although the AIDS virus (HIV) PR, at 99 amino acids, is shorter than the 124-amino-acid AMV PR, various lines of evidence suggest these viral enzymes have very similar mechanisms of action. There is significant amino acid conservation among all of the retroviral PRs and apparently significant cross-specificity as well (21, 25, 37, 41, 42). We show here that the specificity of the cellular aspartic proteases cathepsin-D and renin are quite different from that of AMV PR. Among the aspartic proteases tested, the AMV PR seemed most similar to pepsin in substrate recognition properties; however, even here important differences could be identified. Thus it should be possible to design an inhibitor that can distinguish between the viral enzymes and these cell-derived proteases.

Acknowledgments—We thank Dr. L. Cohen for his advice with the HPLC analysis and for additional helpful suggestions. Pat Roat provided expert technical assistance with HPLC, and Anthony A Pamenti performed the amino acid analyses. We also thank I. Rose, K. Jones, J. Kulkosky, and D. Soltis for their critical review of this manuscript.

Note Added in Proof—The three-dimensional structure of the Rous sarcoma-virus PR confirms that the enzyme is a dimer and is related to the aspartic protease family (Miller, M., Jankolski, M., Rao, J.K.M., Leis, J., and Wlodawer, A., manuscript submitted for publication).

REFERENCES
Activities of Avian Retroviral and Cellular Aspartic Proteases