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Effective blocking of HIV-1 proteinase activity by characteristic inhibitors of aspartic proteinases

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Inhibitory constants (K_i) between 5 and 35 nM were derived (under different conditions of pH and ionic strength) for the interaction of HIV-1 proteinase with acetyl-pepstatin and H-261, two characteristic inhibitors of aspartic proteinases. Thus this enzyme, essential for replication of the AIDS virus, may be classified unequivocally as belonging to this proteinase family.

HIV-1 proteinase; Tight-binding inhibitor; Acetyl-pepstatin; Buffer effect

1. INTRODUCTION

The proteinase encoded within the viral genome of HIV is required for processing of the viral gag proteins together with generation of other enzymatic activities (e.g. reverse transcriptase) essential for viral replication [1]. It has been postulated that this enzyme belongs to the aspartic proteinase family on the basis of (i) the presence of an Asp-Thr-Gly sequence in the predicted structure of the enzyme [2], (ii) mutation of this Asp to Ala [3] or Thr [4] with concomitant loss of processing activity and (iii) computer graphics modelling calculations which predict that the viral proteinase has a folding pattern for the central core of the molecule virtually identical to that for archetypal aspartic proteinases [5].

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Abbreviation: ψ[CHOH-CH_2], the hydroxy ethyl peptide bond replacement; see (1985) J. Biol. Chem. 260, 14–42

Aspartic proteinases include among their number such enzymes as pepsin, renin, cathepsin D and cathepsin E and, characteristically, the enzymes of this family are inhibited effectively by certain compounds of natural or synthetic origin e.g. pepstatin. This contains two residues of the unusual amino acid statine (4-amino-3-hydroxy-6-methylheptanoic acid), which acts as a transition state dipeptide analogue of the two residues contributing the scissile peptide bond in a substrate. However, in a number of previous reports (e.g. [4,6,7]), pepstatin has been demonstrated to be a rather poor inhibitor of HIV-1 proteinase. The simplest interpretation of this finding therefore is that the AIDS virus proteinase is not an aspartic proteinase. Pepstatin is most commonly described as the isovaleryl-derivative, viz. isovaleryl-Val-Val-Sta-Ala-Sta and is commercially available as such. However, other forms of the molecule e.g. acetyl-pepstatin = Ac-Val-Val-Sta-Ala-Sta and lactoyl-pepstatin = Lac-Val-Sta-Ala-Sta have also been reported [8,9] and characterised as inhibitors of classical aspartic proteinases [10]. The effect of these compounds on the viral proteinase was therefore examined in detail.

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2. MATERIALS AND METHODS

Acetyl-pepstatin (S-PI) and lactoyl-pepstatin were generous gifts from Professor S. Murao and Dr K. Oda, Osaka Prefecture University, Japan and Dr T. Aoyagi, Institute of Microbial Chemistry, Tokyo, Japan, respectively. The synthetic renin inhibitor, H-261, was kindly provided by Professor M. Szelke, Ferring Research Institute, Southampton, England.

HIV-1 proteinase was expressed in *E. coli* in two different constructs and was extracted and purified to an extent such that the enzyme represented about 15–30% of the total protein in the preparations used (full accounts of the details have been [11] or will be described elsewhere). Closely similar results were obtained with the two enzyme preparations. No activity whatsoever was observed in control cell extracts prepared in an identical manner but using vector without proteinase insert. The peptide substrate Tyr-Val-Ser-Gln-Asn-Phe*Pro-Ile-Val-Gln-Asn-Arg was synthesised by solid phase methods employing symmetric anhydride chemistry in an ABI model 430A synthesiser in the Protein Chemistry Core Facility of the University of Florida as described previously [12].

Incubation of this peptide with recombinant HIV-1 proteinase resulted in specific cleavage at the Phe-Pro bond. Initial rates of hydrolysis were determined by removal of samples at 3 different time points followed by analysis by reversed-phase HPLC/FPLC with quantitation of the cleavage product, Tyr-Val-Ser-Gln-Asn-Phe, generated by the proteinase action.

3. RESULTS AND DISCUSSION

Initial rates of cleavage of the synthetic peptide Tyr-Val-Ser-Gln-Asn-Phe*Pro-Ile-Val-Gln-Asn-Arg specifically at the Phe*Pro bond were measured at a variety of pH values (but at constant ionic strength, fig.1). Since substrate hydrolysis and inhibitor interaction with conventional aspartic proteinases is most effective at pH values below approx. 5.5 [12,13], more detailed investigations were carried out initially, therefore, in the region of pH 5 but at a higher ionic strength since preliminary investigations (not shown) had indicated that the enzyme was more active under such conditions. In a buffered solution consisting of 20 mM acetic acid/20 mM Mes/40 mM Tris and 800 mM NaCl, pH 5.0 (μ = 0.82 M), Michaelis-Menten kinetics were observed for the substrate hydrolysis and a mean K_m value of 140 ± 35 μM was obtained. With the ready availability of such a good substrate, it was then possible to examine the interaction of the HIV-1 proteinase with a number of classical inhibitors of aspartic proteinases. As discussed previously, isovaleryl-pepstatin was confirmed to be a rather poor inhibitor and lactoyl-pepstatin was even worse since concentrations in the micromolar range (0.4 μM and 6 μM respectively) were required for 50% inhibition. Indeed, it has very recently been reported that a number of synthetic statine-containing inhibitors were not effective unless concentrations verging on millimolar were used [14]. However, the acetyl-derivative of pepstatin was much more effective (table 1) with a mean K_i value of 35 nM measured for the competitive inhibition in this buffer at pH 5.0.

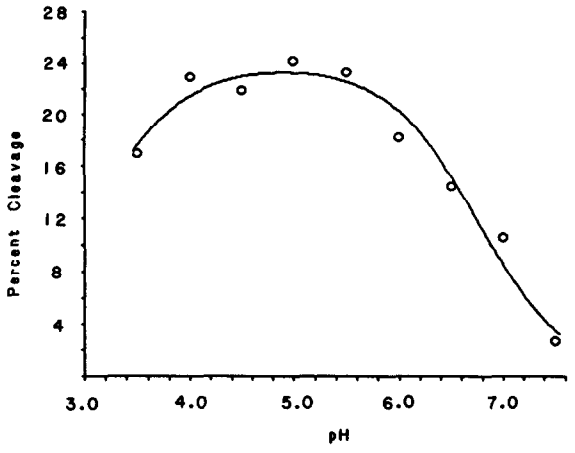

Fig.1. pH dependence of the hydrolysis of Tyr-Val-Ser-Gln-Asn-Phe*Pro-Ile-Val-Gln-Asn-Arg by HIV-1 proteinase. Substrate at a fixed concentration of 125 μM was incubated at 30°C with HIV-1 proteinase (approx. 0.1 μg) in buffers composed of 20 mM acetic acid, 20 mM Mes and 40 mM Tris at each of the indicated pH values but with a total ionic strength always at μ = 0.1 M [18]. At three appropriate times (normally all less than 10 min), aliquots were removed and the hydrolysis was quenched by the addition of 5% (v/v) trifluoroacetic acid before injection of the samples into a reversed-phase HPLC (Novapak, Waters) or a PepRP-FPLC (Pharmacia Ltd.) column. Elution was performed with a linear gradient of acetonitrile over 15–25 min. Peaks of (substrate and) generated products were quantitated by integration of the absorbances at 254 or 215 nm. Rates of reaction were calculated from computerised fits of each set of linear time course values thus derived. The solid line is a computer-generated fit of the points to a system with two acid dissociation groups (pKa_1 = 3.1 and pKa_2 = 6.7), assuming that enzymic activity is expressed only in the singly dissociated form.

Consequently these investigations were expanded to include the synthetic compound H-261 = *t*Boc-His-Pro-Phe-His-Leu Ψ[CHOH-CH_2]Val-Ile-His which contains the transition state analogue -CHOH-CH_2 instead of the CO-NH of the scissile

Table 1

The effect of HIV-1 proteinase of a naturally occurring and a synthetic inhibitor of aspartic proteinases

pH	K_i (nM)	
	Acetyl-pepstatin	H-261
4.7	20	5
5.0	35	15
7.0	>1000	25[a]

Reactions were carried out at 30°C in buffers consisting of 20 mM acetic acid, 20 mM Mes, 40 mM Tris, pH 5.0 (μ = 0.82 M); 0.1 M sodium acetate buffer, pH 4.7, containing 4 mM EDTA, 5 mM mercaptoethanol and 1000 mM NaCl (μ = 1.06 M); and 0.1 M Mes buffer, pH 7.0, containing 4 mM EDTA, 5 mM mercaptoethanol and 1000 mM NaCl but otherwise as described in the legends to figs 1 and 2. The estimated precision of the values obtained is in the range of ± 20% except at pH 7.0 where the value given ([a]) for H-261 is an IC_{50} determined at a substrate concentration of 84 μM (because of the linear dependence of v with varying [S] at this pH value)

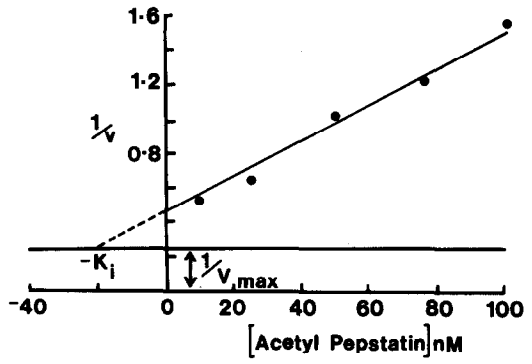

Fig.2. Inhibition of HIV-1 proteinase by acetyl-pepstatin. HIV-1 proteinase was incubated with various concentrations of acetyl-pepstatin at a fixed concentration (63 μM) of substrate peptide in 100 mM sodium acetate buffer, pH 4.7, containing 1 M NaCl, 4 mM EDTA, 5 mM mercaptoethanol at 30°C. Initial rates were determined from analysis of time courses of the reactions as described in the legend to fig.1. The horizontal line is included at the value of $1/V_{max}$ determined by varying [S] in the total absence of inhibitor.

peptide bond in a substrate. This has been shown to be a very potent inhibitor not only of human renin [15,16] but also of most other typical aspartic proteinases examined [17]. It was also found to be a competitive inhibitor of the HIV-proteinase with a mean K_i value of 15 nM in the pH 5.0 buffer (table 1). A further rationale behind the examination of these compounds was in an attempt to find an active site titrant to permit accurate quantitation of the amount of active HIV-1 proteinase in any solution. Currently, such measurements to define this essential parameter cannot be made experimentally, due to the absence of just such a titrant.

Consequently, buffer conditions under which the assays were performed were varied in order to try to improve yet further the proteinase-inhibitor interactions. It was found that by using a sodium acetate buffer at pH 4.7 containing 1 M NaCl (μ = 1.06 M), while Michaelis-Menten kinetics were still observed for the interaction of the synthetic peptide substrate, a (better) K_m value of 60 ± 8 μM was measured. On this basis, the interactions of acetyl-pepstatin and H-261 were re-determined under these buffer conditions at pH 4.7 (table 1). Competitive inhibition was observed in both cases and, while the acetyl-pepstatin interaction (a representative plot is shown in fig.2) was still too weak (mean K_i = 20 nM) for this compound to be of value as a titrant, the K_i for H-261 is approaching the level necessary (<1 nM) for stoichiometric binding. Since this compound was originally designed by Szelke and co-workers [15,16] to mimic the site in angiotensinogen cleaved by renin yet still displays effective inhibition of the viral enzyme (which has a totally different specificity), it would appear that a synthetic compound containing a peptide bond replacement and centred around an HIV cleavage site would be likely to provide an active site titrant.

At pH 7.0, the synthetic peptide substrate interacted much less favourably with the proteinase. Plots of v versus [substrate] were essentially linear up to concentrations that were limited by the supply of available material. K_m was estimated to exceed 1700 μM in 100 mM Mes buffer, pH 7.0, containing 1 M NaCl. Nevertheless, hydrolysis of the substrate was very readily measured at pH 7.0, even if saturation could not be achieved. The apparent reduction in activity observed at the higher pH values (fig.1) would thus appear to result from a diminished ability of the synthetic substrate to interact with the proteinase. Whether such an effect on binding would be observed with a polyprotein substrate (e.g. gag precursor) is clearly worthy of further investigation.

In contrast to its effect at the lower pH values, acetyl-pepstatin at a concentration as high as 1 μM was only able to inhibit the proteinase by approx. 35% at pH 7.0 (table 1). Such a diminution in the inhibitory potency of pepstatins at pH values above approx. 5.5 has been observed previously with typical aspartic proteinases (e.g. [13]) and indeed has been used to effect in developing affinity chromatographic procedures for the convenient purification of the enzymes [19]. H-261, however, remained an adequate inhibitor (table 1) with an IC_{50} value of 25 nM estimated at pH 7.0. Since no detailed structural information is yet available for retroviral proteinases, this different behaviour of the two inhibitors cannot be readily rationalised at present. H-261 is an excellent inhibitor of all aspartic proteinases due to the ability of the hydroxyl group of the peptide bond surrogate to interact with both active site aspartic acid residues [15]. Since acetyl-pepstatin also contains such a hydroxyl group, the different response in binding of these two compounds with changing pH implies that H-261 binding may involve some additional active site interactions, perhaps derived from the hydrophilic histidine residues, for example. Alternatively, the loss of binding observed for acetyl-pepstatin could be due to repulsion of its C-terminal carboxyl group by some group (e.g. a COOH) in the enzyme's active site that becomes negatively charged as the pH is raised from 5 to 7. This repulsion may not be manifested for H-261 due to the terminal His residue which could permit an altered binding conformation. Nevertheless, it seems that the proteinase which is so vital for the reproduction of the AIDS virus can now be classified with certainty as belonging to the aspartic proteinase family. The investigations described herein provide only a preliminary insight into structure-function relationships of this proteinase but do demonstrate that it is feasible to inhibit the HIV (aspartic) proteinase effectively in the pH range that is likely to exist within virally-infected cells. With the central role of this enzyme in retrovirus maturation, it would appear that these observations provide a substantial basis from which to launch further studies for the rational design of selective inhibitors of this strategically sensitive viral target enzyme.

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NOTE ADDED IN PROOF

Following submission of this manuscript, reports appeared subsequently describing the crystal structures of two retroviral proteinases at medium-high resolution (from Rous sarcoma virus [20] and HIV-1 itself [21] and demonstrating their structural homology with aspartic proteinases. In a third report on molecular modelling of possible substrate interactions in the HIV-1 proteinase active site [22], it was predicted 'that a smaller residue' (such as an acetyl moiety) 'would bind in subsite P_4, in agreement with the findings reported herein.