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REVIEW

The picornaviral 3C proteinases: Cysteine nucleophiles in serine proteinase folds

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Abstract

The 3C proteinases are a novel group of cysteine proteinases with a serine proteinase-like fold that are responsible for the bulk of polyprotein processing in the Picornaviridae. Because members of this viral family are to blame for several ongoing global pandemic problems (rhinovirus, hepatitis A virus) as well as sporadic outbreaks of more serious pathologies (poliovirus), there has been continuing interest over the last two decades in the development of antiviral therapies. The recent determination of the structure of two of the 3C proteinases by X-ray crystallography opens the door for the application of the latest advances in computer-assisted identification and design of anti-proteinase therapeutic/chemoprophylactic agents.

Keywords: antiviral; computer-assisted drug design; inhibitor; proteinase

Picornaviruses

The Picornaviridae are a family of small, closely related RNA viruses responsible for a variety of human and animal pathologies. The most famous member of the family is the well-studied poliovirus (HPV), the cause of poliomyelitis. In addition, the family includes rhinovirus (HRV), the etiologic agent for over 50% of common colds; hepatitis A virus (HAV), which produces a usually benign form of hepatitis that is endemic to many less-developed parts of the world; encephalomyocarditis virus (EMCV), responsible for a relatively rare inflammation of the myocardium; and foot and mouth disease virus (FMDV), a highly contagious livestock pathogen, to name but one example from each of the five genera. A tremendous number of studies have been published over the last 20 years to establish the details of picornavirus replication and proteolytic maturation. These have been thoroughly reviewed (Kräusslich & Wimmer, 1988; Lawson & Semler, 1990; Palmenberg, 1990; Dougherty & Semler, 1993) and will not be discussed here. It is sufficient to state that, although there are many subtle but profound differences between the genomes of the five genera of picornaviruses, they all nevertheless require the action of a 3C proteinase at some point in their maturation in order to generate new virions.

The life cycle of the Picornaviridae can be summarized quite briefly for the purposes of this discussion (for a detailed review see Kräusslich & Wimmer, 1988, and references therein). The virus attaches to and enters the cell via some form of endocytosis. The virus then uncoats, releasing its positive sense single-stranded RNA into the cytosol, where the latter functions as a messenger RNA to direct the synthesis of a single polyprotein of approximately 250 K_D (Fig. 1). This polyprotein undergoes a co-translational cleavage into a capsid (P1) and nonstructural protein (P2–P3) precursor. In the case of the entero- and rhinoviruses, this is mediated by the 2A proteinase (Toyoda et al., 1986; Sommergruber et al., 1989). In other Picornaviridae, how this cleavage is accomplished remains unclear. The 3C proteolytic activity then proceeds to release itself from the polyprotein. The remainder of the polyprotein is cleaved in *trans* into its component nonstructural gene products and capsid proteins, which then assemble into new virions. A final maturational cleavage occurs in the assembled virion by an unknown mechanism. Studies of chimeric and mutant picornaviruses have demonstrated that interruption of 3C proteolytic processing prevents the formation of new virions (Dewalt & Semler, 1987; Kean et al., 1988, 1990; Dewalt et al., 1989, 1990; Mirzayan et al., 1991).

3C proteinases

3C proteinases from all five genera have been expressed in a variety of systems including *Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian cells (Lawson & Semler, 1990; Ansardi et al., 1991; Aschauer et al., 1991; Gauss-Müller et al., 1991; Liebig et al., 1991; Windheuser et al., 1991; Dasmahapatra et al., 1992; Malcolm et al., 1992; McCall et al., 1994; Schultheiss et al.,

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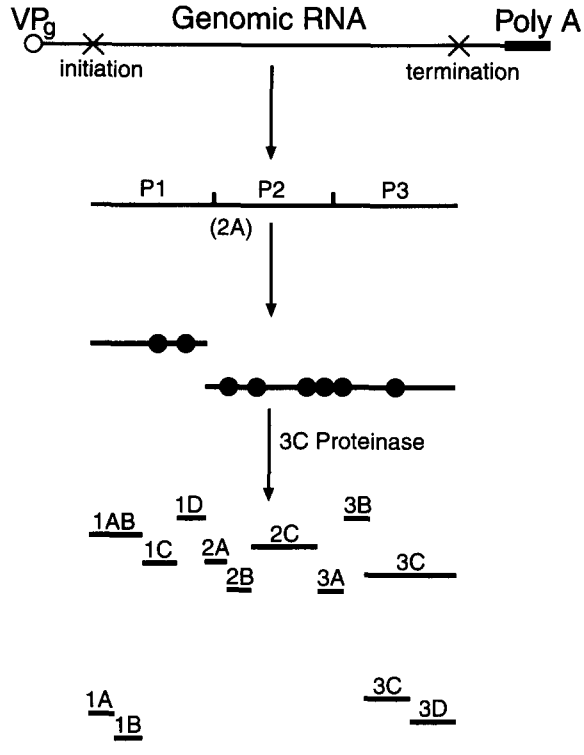

Fig. 1. Generalized schematic representation of polyprotein translation and cleavage in the Picornaviridae.

1994; Tesar et al., 1994). In addition, in vitro transcription-translation systems have been developed to express picornaviral RNA, further facilitating investigations into polyprotein processing in *trans* by recombinant 3C proteolytic activities (Parks et al., 1986; Vakharia et al., 1987; Ypma-Wong & Semler, 1987; Nicklin et al., 1988; Kusov et al., 1992; Schultheiss et al., 1994). Finally, the advancement in recent years of solid-phase peptide synthesis and the development of high throughput assays have permitted detailed comparisons of proteolytic behaviors of 3C proteinases from different picornaviruses (Cordingley et al., 1989, 1990; Long et al., 1989; Orr et al., 1989; Pallai et al., 1989; Petithory et al., 1991; Jewell et al., 1992). These approaches have generated a wealth of data concerning 3C proteinase specificity and mechanism for each system that are at times difficult to reconcile. In addition, differences between genera make it difficult to generalize from studies of any particular virus to other members of the family despite their relatively close evolutionary relationships (Kräusslich & Wimmer, 1988, and references therein).

For example, it has been shown that complete and efficient processing of the capsid portion of the poliovirus polyprotein is dependent on a larger precursor of the 3C proteinase, which includes the RNA-dependent RNA polymerase, the 3D gene product, referred to as the 3CD proteinase (Ypma-Wong & Semler, 1987; Jore et al., 1988; Ypma-Wong et al., 1988). Although the basic proteolytic machinery is still 3C, it has been suggested that the presence of the 3D polymerase "tail" facilitates interaction of the proteinase portion with the capsid precursor or somehow subtly alters the specificity of the proteinase to accelerate cleavage of these junctions (Ypma-Wong & Semler, 1987; Jore et al., 1988). Whether this is true for other picornaviruses is unclear (Vakharia et al., 1987; Clarke & Sangar, 1988; Parks et al., 1989; Jia et al., 1991; Harmon et al., 1992). From the point of view of therapeutic intervention, the issue (i.e., 3C versus 3CD) can be ignored for the time being because disruption of any stage in the proteolytic cleavage of the polyprotein, such as those probably accomplished in all Picornaviridae by the 3C enzyme alone, should be sufficient to halt the production of progeny virions. Furthermore, small-molecule inhibitors of 3C proteolytic activity will in all probability show activity against the 3CD enzyme as well. The phenomenon is nevertheless an intriguing one, but resolution of the mechanism of enhancement will have to await crystallographic studies of the 3CD fragment or, preferably, analysis of a 3CD proteinase-capsid precursor complex.

Overall fold and active site geometry

The crystal structure of the 3C proteinases from hepatitis A virus and rhinovirus 14 have recently been determined (Allaire et al., 1994; Matthews et al., 1994). Based on alignment and secondary structure predictions, it was suggested that these enzymes would adopt a two-domain β-barrel fold characteristic of the mammalian serine proteinases, such as chymotrypsin and trypsin (Gorbalenya et al., 1986, 1989; Bazan & Fletterick, 1988, 1990). This prediction has been borne out by the structure determinations. Prior to the elucidation of the three-dimensional structure, sequence alignment and secondary structure predictions (Gorbalenya et al., 1986, 1989; Bazan & Fletterick, 1988, 1990) and site-directed mutagenesis studies (Ivanoff et al., 1986; Cheah et al., 1990; Hämmerle et al., 1991; Kean et al., 1991; Malcolm et al., 1992) had strongly implicated cysteine 147 (poliovirus numbering) and histidine 40 as the nucleophile and general base, respectively, of the 3C proteinases. Again, the crystal structures confirm these assignments. Although the HAV-3C and HRV14-3C represent different subtypes of the 3C enzyme (long and short—215 and 182 residues, respectively), the critical active site geometry of the nucleophilic cysteine side chain (determined, in the rhinovirus structure [Matthews et al., 1994] and modeled [M. Allaire & M.N.G. James, unpubl. results] in the HAV mutant structure [Allaire et al., 1994]) as well as histidine general base are virtually superimposable with the equivalent residues, serine 195 and histidine 57 of chymotrypsin (Fig. 2).

Existence of a catalytic triad?

The existence and identity of the third member of a catalytic triad for the 3C proteinases have been and are still unclear. Sequence alignment had suggested that one of two residues, either aspartate 85 or glutamate 71 in poliovirus might function as the third member of a catalytic triad (Gorbalenya et al., 1986; Bazan & Fletterick, 1988) similar to the one established for chymotrypsin and trypsin (Blow et al., 1969; Sprang et al., 1987; Warshel et al., 1989). Site-directed mutagenesis studies were suggestive but inconclusive (Ivanoff et al., 1986; Cheah et al., 1990; Hämmerle et al., 1991; Kean et al., 1991). Speculation that a carboxylate third member was unnecessary to activate the more easily deprotonated thiol nucleophile, as generally accepted for the papain family of thiol proteinases (Drenth et al., 1976), has been

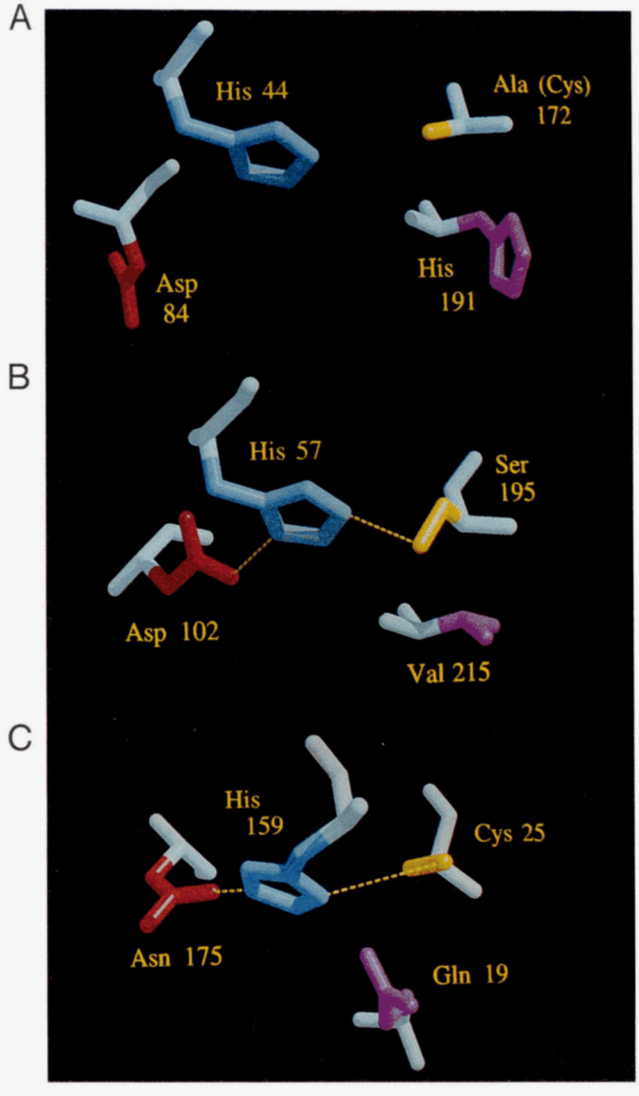

Fig. 2. Catalytic residues from the active sites of (**A**) HAV-3C proteinase, (**B**) α-chymotrypsin (Fujinaga et al., 1987), and (**C**) papain (Drenth et al., 1976) generated using RASTER3D (Bacon & Anderson, 1988). Side chains of catalytic residues are color coded: nucleophiles (cysteine and serine) in yellow, general bases (histidines) in blue. The third member or potential third members of the catalytic triad are shown in red. The histidine at the base of the P1 subsite in the HAV-3C proteinase is shown in purple as are the comparably located residues in chymotrypsin and papain. Coordinates for α-chymotrypsin (1CHO) and papain (1PPN) are from the Brookhaven Protein Data Bank.

offered as an alternative explanation for this apparent ambiguity (Allaire et al., 1994).

Unfortunately, the results of the structural studies are also somewhat paradoxical. The crystal structure of the HRV3C proteinase suggests that glutamate 71 is the third member of the catalytic triad. It is, however, in a rather unusual orientation (in which the anti lone pair of a carboxylate oxygen forms the necessary interaction with the histidine $N^{\delta 1}$). This leaves the previously obtained site-directed mutagenesis studies to be explained in some other manner (Matthews et al., 1994). The absence of any carboxylic acid, suitably positioned in the structure of the HAV-3C, suggests that either an alternative mechanism is at work in this particular 3C proteinase or that the thiolate-imidazolium system is, in general, sufficient for catalysis (Allaire et al., 1994). To cloud the issue further, site-directed mutagenesis studies, in which the active site cysteine of poliovirus and coxsackie 3C proteinase were replaced with a serine, showed some degree of autocatalytic activity in vitro (Lawson & Semler, 1990); although exactly what degree of enzymatic activity remains is again unclear. Studies in which the third member of the catalytic triad of chymotrypsin, aspartate 102, was replaced with asparagine also showed trace amounts of activity (Sprang et al., 1987). This suggests that even in the absence of a charge relay, the serine nucleophile shows some measurable activity, underscoring the need for more detailed kinetic evaluation of the cysteine to serine replacement in the polio and coxsackie 3C proteinases. Further kinetic and structural analysis will be required before definitive conclusions can be reached concerning the existence of a catalytic triad in any or all of the 3C enzymes.

Intramolecular versus intermolecular excision of 3C proteinases

The mechanism of release of the 3C proteinase from the polyprotein has been proposed to involve intramolecular (*cis*) cleavage based on a series of in vitro transcription–translation experiments with EMCV (Palmenberg & Rueckert, 1982). The observation that, at lower concentrations of polyprotein precursor the reaction appeared to be concentration independent, led to the suggestion that, at least early in infection, when translation had just begun and polyprotein concentration was extremely low, the 3C enzymes acted in *cis* upon their own N-terminal and C-terminal connections to the polyprotein to effect their own release (Palmenberg & Rueckert, 1982). The crystal structures of both the HAV and HRV 14 3C proteinases make it difficult to imagine how such a reaction would take place (Allaire et al., 1994; Matthews et al., 1994). In both structures, the N- and C-termini are located on the opposite side of the enzyme from the active site cleft. Even if a partial refolding is postulated to occur after proteolytic cleavage, only the N-terminal cleavage site is within reach of the active site (Matthews et al., 1994). As Palmenberg and Rueckert (1982) have pointed out, their results do not exclude the possibility of formation of a dimer or tight complex between two polyproteins that cleave each other in a reciprocal fashion. Again, further structural studies on larger precursors that include the 3C proteinase (or an inactive mutant) connected to the flanking 3B and 3D proteins will be necessary to clarify the issue of intramolecular versus intermolecular cleavage for release of 3C activity.

Understanding 3C proteinase specificity

From the standpoint of inhibitor design, it is essential to understand how these enzymes obtain their high degree of specificity. All 3C proteinases cleave after glutamine residues, although this is clearly not the only determinant of specificity (Dewalt et al., 1989; Kean et al., 1990; Mirzayan et al., 1991; Petithory et al., 1991; Jewell et al., 1992). In the cases of the enteroviruses and rhinoviruses, synthetic peptide studies have shown that the glutamine residue should be followed by a glycine and proline residue in the P_1' and P_2' subsites, respectively (notation of Schechter & Berger, 1967) to ensure efficient cleavage, suggest-

ing that a type II β turn may be an important structural recognition element (Cordingley et al., 1989, 1990; Long et al., 1989; Orr et al., 1989; Pallai et al., 1989). Studies on HAV-3C, on the other hand, show that it is, in general, less discriminating, effectively cleaving peptides in which any small amino acid (G, A, or S) is in the P_1' position and virtually any amino acid is in the P_2' position with the exception of proline and arginine, arguing against the turn motif for this 3C proteinase (Petithory et al., 1991). In addition to these determinants, peptide studies have also identified discrimination on the part of 3C enzymes for residues in the P_4 subsite: in the cases of poliovirus and rhinovirus, preferences for small side chains such as alanine are evident (Blair & Semler, 1991), whereas in the case of HAV-3C, large side chains such as the branched aliphatic or aromatic side chains of leucine, isoleucine, or tryptophan are preferred (Jewell et al., 1992).

Modeling of enzyme–substrate complexes (Matthews et al., 1994; M. Allaire, K. Bateman, B.A. Malcolm, & M.N.G. James, unpubl. results) help rationalize these preferences on the part of the 3C enzymes (Fig. 3). In the cases of both the HAV and HRV structures, the P_1 subsite is a shallow hydrophobic pocket formed, in the case of HAV-3C by residues glycine 167, leucine 168, proline 169, alanine 193, glycine 194, and leucine 188 and in the case of HRV14 is defined by comparable parts of the protein, βE_2, βF_2, and the loop connecting βC_2 to βD_2 (Matthews et al., 1994). At the base of this pocket resides a histidine residue (in HAV-3C, H191) in an appropriate position to form a hydrogen bond with the side chain of the glutamine of the substrate as was previously predicted (Gorbalenya et al., 1986, 1989; Bazan & Fletterick, 1989). Unfortunately, the resolution of the structures is insufficient to clarify whether it is the $N^{\epsilon 2}$ or the $N^{\delta 1}$ of the histidine that is involved in the interaction. Studies with peptide substrates containing glutamine analogs (B.A. Malcolm, J.P. Petithory, R.J. Simon, & D.A. Jewell, unpubl. results) suggest that the histidine at the base of the P_1 subsite most likely acts as a hydrogen bond donor to the carbonyl oxygen of the substrate's side chain. The inability of 3C enzymes to accept glutamate residues, however, in place of glutamine, may simply reflect the unfavorable effects of burying a charged side chain in an uncharged hydrophobic pocket.

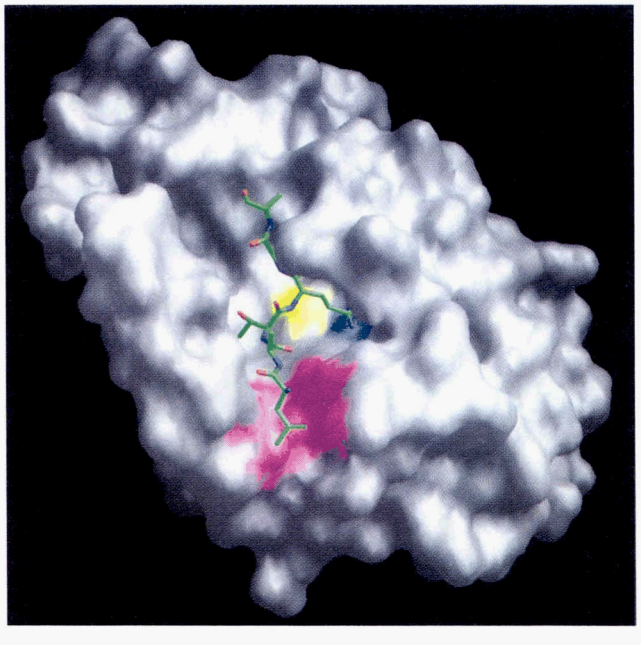

Fig. 3. GRASP (Nicholls et al., 1991) model showing a peptide substrate (LATQGA) modeled into the active site of HAV-3C proteinase. Modeling of the hexapeptide substrate was based on coordinates from the high-resolution structure of turkey ovomucoid–α-chymotrypsin complex (Fujinaga et al., 1987). Superposition of the HAV-3C proteinase and the chymotrypsin–ovomucoid complex yielded initial coordinates for the hexapeptide. Appropriate amino acid substitutions were made into the P_4–P_2' positions of the active site loop. The structure was subsequently refined using Discover (Biosym, San Diego, California). Yellow indicates the nucleophilic sulfur; blue, the histidine at the base of the P_1 subsite; pink, the postulated hydrophobic P_4 subsite. The oxyanion hole of the mutant HAV-3C was "repaired" using chymotrypsin prior to modeling of the peptide substrate.

Modeling studies on HAV-3C suggest that a small P_2 subsite may be formed between the side chains of histidine 145 and tyrosine 143. This may partially explain the preference shown in cleavage junction sequences for serine and threonine residues in the S_2 position. The HRV14 enzyme, in contrast, has a larger cavity in this area that is consistent with the demonstrated tolerance for larger side chains in S_2 (Cordingley et al., 1989, 1990; Orr et al., 1989; Long et al., 1989).

In both the HAV and HRV enzymes, the side chain of the S_3 residue appears to point away from the active site toward solvent, which would suggest that little or no specificity is obtained from this residue. This is consistent with studies that showed that susceptibility of a peptide substrate to proteolytic cleavage by HAV-3C was unaffected by replacement of an arginine residue in S_3 by alanine (Jewell et al., 1992).

The P_4 subsite in HAV-3C appears to be a rather open hydrophobic cleft, which easily rationalizes the enzyme's preference for a wide variety of large hydrophobic residues in S_4 (Fig. 4; Jewell et al., 1992). In contrast, the HRV14 enzyme appears to have a shallow pocket and consequently to prefer smaller residues such as valine and alanine (Fig. 4). In the case of HAV-3C, the P_4 subsite is defined by alanine 141, valines 144 and 200, glycine 195, and isoleucine 198. In HRV14, the P_4 pocket is comprised/filled in by the side chains of isoleucine 124, leucine 126, and phenylalanine 169 (rhinovirus numbering; Matthews et al., 1994).

The HAV-3C and HRV14-3C show dramatic differences in their specificity for S_1' and S_2' residues (Fig. 4). Rhinovirus, like poliovirus, has a virtually absolute requirement for glycine at S_1' and a strong preference for proline at S_2', suggesting a type II β turn or equally well-defined conformational motif. Matthews and colleagues suggest that this motif is essential for redirecting the substrate into a side canyon rather than avoiding any steric clashes between the substrate and the enzyme; however, the presence of phenyalanine 25 (rhinovirus numbering) in the P_1' subsite may disfavor even small side chains in S_1' as evidenced by the strong discrimination by HRV-3C against even the substitution of alanine for glycine (Cordingley et al., 1990).

In contrast, the HAV-3C enzyme shows considerable tolerance in S_1' substitution, preferring small residues such as serine, alanine, and glycine equally, but nevertheless tolerating large residues with only a few-fold drop in rate of proteolysis (Petithory et al., 1991). This may be the consequence of "the replacement" of the HRV14's phenyalanine 25 with the HAV-3C's valine 28 and methionine 29 (hepatitis virus numbering). In the P_2' position, HAV-3C is the obverse of rhinovirus 3C, tolerating virtu-

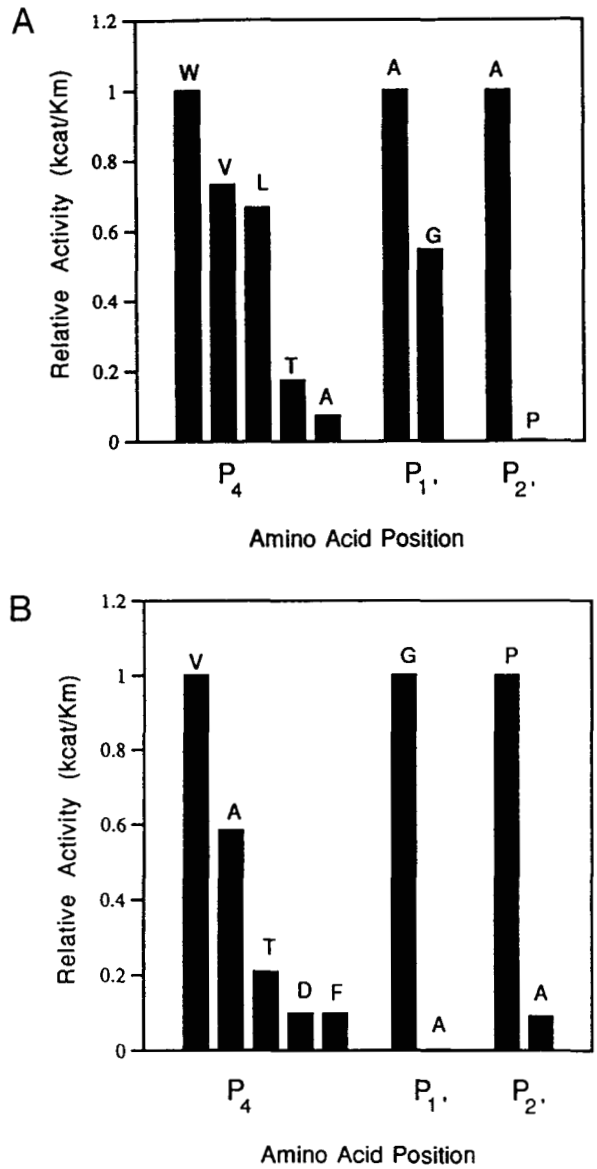

Fig. 4. Summary of amino acid preferences of HAV-3C (**A**) and HRV14-3C proteinases (**B**) in the P_4, P_1', and P_2' positions based on peptide studies (Orr et al., 1989; Cordingley et al., 1990; Petithory et al., 1991; Jewell et al., 1992).

ally all amino acids except proline (Petithory et al., 1991). The suggestion made by these data is that, in the case of HAV-3C, the substrate does not turn into a side canyon as proposed for HRV by Matthews et al. (1994). Despite efforts to model substrates into the active site, differing selectivities of the 3C enzymes in the P_1' and P_2' subsites will probably require detailed structural studies of enzyme-inhibitor complexes before a more definitive understanding can be obtained.

Prospects for inhibitor identification and design

In the interim, these preliminary structural and biochemical understandings should allow the application of computer-assisted identification and design of inhibitors tailored to each 3C enzyme (Ring et al., 1993; Li et al., 1994). Searches of chemical databases using coordinates from these initial structures via programs such as DOCK (Shoichet & Kuntz, 1993) and CAVEAT (Lauri & Bartlett, 1994) should greatly accelerate the identification of lead compounds. In addition, there is a wealth of information on the design and synthesis of peptide-based inhibitors, such as aldehydes and monofluoroketones, known to react with thiol enzymes, which, in spite of being of limited medicinal value, may nevertheless be useful for gaining insight into mechanistic and specificity issues for each particular 3C enzyme. The abundance of recombinant enzyme and synthetic peptides and peptide-based inhibitors, along with polyprotein substrates, should then allow the rapid acquisition of useful structure-activity relationships (SARs), which in turn should allow the expeditious development of the original lead compounds into highly specific and effective inhibitors for therapeutic and/or prophylactic administration. Because identity among 3C proteinases is modest (~30% identity), the Picornaviridae should provide several useful test cases for the application of comparative modeling to the generation of structures for the subsequent screening of small molecule databases. Testing of the identified compounds and the resultant SARs will help in the development of specific inhibitors for other members of the family, as well as closely related 3C-like enzymes, such as those from the poty and comovirus plant pathogens, in the absence of independent crystal structures (Ring et al., 1993).

To conclude, the 3C enzymes are a large and important group of related thiol proteinases that are unique in possessing a serine proteinase-like fold. On the basis of biochemical studies, they appear to be a hybrid between the serine and thiol enzymes, although the mechanistic details are far from resolved. The recent determination of the atomic structure of two of these enzymes, rather than clarifying mechanistic details, raises further questions that can only be resolved by further structural and biochemical studies. Nevertheless, these structures, together with the wealth of knowledge concerning inhibition of proteinases, should facilitate identification and development of medicinally useful inhibitors.

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