Hepatitis A Virus 3C Proteinase Substrate Specificity†

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ABSTRACT: Hepatitis A virus (HAV) 3C proteinase is responsible for processing the viral precursor polyprotein into mature proteins. The substrate specificity of recombinant hepatitis A 3C proteinase was investigated using a series of synthetic peptides representing putative polyprotein junction sequences. Two peptides, corresponding to the viral polyprotein 2B/2C and 2C/3A junctions, were determined to be cleaved most efficiently by the viral 3C proteinase. The k_{cat}/K_m values determined for the hydrolysis of a further series of 2B/2C peptides, in which C-terminal and N-terminal amino acids were systematically removed, revealed that P_4 through P_2' amino acids were necessary for efficient substrate cleavage. The substitution of Ala for amino acids in P_1 and P_4 positions decreased the rate of peptide hydrolysis by 100- and 10-fold, respectively, indicating that the side chains of Gln in P_1 and Leu in P_4 are important determinants of substrate specificity. Rates of hydrolysis measured for other P_1- and P_4-substituted peptides indicate that S_1 is very specific for the Gln side chain whereas S_4 requires only that the amino acid in P_4 be hydrophobic. A continuous fluorescence quench assay was developed, allowing the determination of k_{cat}/K_m dependence on pH. The pH rate profile suggests that catalyzed peptide hydrolysis is dependent on deprotonation of a reactive group having a pK_a of 6.2 (±0.2). The results of tests with several proteinase inhibitors indicate that this cysteine proteinase, like other picornaviral 3C proteinases, is not a member of the papain family.

Hepatitis A virus (HAV) is a member of the family Picornaviridae. These positive-strand RNA viruses include the enteroviruses (e.g., coxsackievirus, poliovirus), rhinoviruses, cardioviruses (e.g., encephalomyocarditis virus, mengovirus), and apthoviruses (e.g., foot-and-mouth disease virus). Picornaviral replication requires processing of a polyprotein (250 kDa) coded by the RNA genome. These viruses have evolved highly specific proteinases for processing this precursor, uncoupling virus maturation from dependence on host enzymes [for review see Kräusslich and Wimmer (1988)]. While this may represent a selective advantage, it exposes viral proteinases as targets for antiviral therapy (Kay & Dunn, 1990; Kohl et al., 1988; Peng et al., 1989; Meek et al., 1990).

The mechanism of cleavage site recognition and proteolysis within the polyprotein to yield mature proteins is poorly understood. Like other picornaviruses, the HAV genome encodes a major processing proteinase, designated 3C. HAV 3C proteinase has been shown to be a member of the cysteine proteinase family (Coulepis et al., 1982; Argos et al., 1984; Malcolm et al., 1992). By analogy with other picornaviral enzymes, the HAV 3C proteinase is postulated to produce most of the mature virual proteins by cleaving eight processing sites (see Table I) (Kräusslich & Wimmer, 1988; Linemeyer et al., 1985). Comparative structure analysis of viral 3C proteinases suggests that a catalytic triad similar to that in serine proteinases has been conserved (Gorbalenya et al., 1989; Bazan & Fletterick, 1989). This similarity of picornavirus 3C proteinases to serine proteinases has recently been further supported by site-directed mutagenesis (Kean et al., 1991; Hammerle et al., 1991; Dessens & Lomonossoff, 1991). It is, therefore, likely that the chemical mechanism functioning in picornaviral proteinases involves a thiolate–imidazolium ion pair and the formation of an acylenzyme intermediate during catalysis, similar to the mechanism outlined for papain (Polgar, 1974; Lewis et al., 1976, 1981). However, to date, no structural information has been obtained for the 3C proteinases that might help to corroborate this proposed mechanism.

It has recently been shown that the picornavirus 3C proteinases of human rhinovirus 14 (HRV 14) and poliovirus have substrate sequence requirements flanking the scissile bond (Orr et al., 1989; Long et al., 1989; Pallai et al., 1989; Cordingley et al., 1990; Blair & Semler, 1991; Weidner & Dunn, 1991). Orr et al. (1989) demonstrated that HRV 14 3C proteinase exhibited a strong specificity for the -Gln*Gly- scissile peptide bond. Expanding on the work of Orr and colleagues, Long et al. (1989) determined that HRV 14 3C proteinase most efficiently cleaved peptides with the minimum sequence of P_5-Val/Thr-P_3-P_2-Gln-Gly-Pro.[1] The studies of Cordingley et al. (1990) indicated that HRV 14 3C proteinase has peptidase activity which is sensitive to amino acid substitutions at P_4, P_1, P_1', and P_2' positions. Pallai et al. (1989) found that the P_4 position of a poliovirus 3C proteinase substrate was sensitive to the replacement of a Thr with an Ala, with the latter derivative resulting in 40-fold faster cleavage of the corresponding peptide. Conversely, Blair and Semler (1991) provided evidence that the poliovirus 3CD protein functions as a proteinase with substrate specificity differing from poliovirus 3C proteinase in that it prefers Thr

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[1] Abbreviations: t-Boc, *N-tert*-butoxycarbonyl; TFA, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; pNO_2F, *p*-nitrophenylalanine; DNS-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; HF, hydrogen fluoride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BICINE, *N,N*-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid. The designation of residues in substrates (P_2, P_1, P_1', P_2', ...) is according to Schechter and Berger (1967).

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Table I: Peptides Representing HAV Junction Sequences and Having Homology with Polio 3C Proteinase Processing Sites and the Relative Amount of Peptide Cleaved by HAV 3C

polyprotein junction	sequence	relative amount cleaved
	P_5 P_4 P_3 P_2 P_1 P_1' P_2' P_3'	
1B/1C	Y G L P T L S T Q * Mx Mx R N E F R	0.55[a]
1C/1D	Y A Mx D V T T Q * V G D D S G G F	0.60[a]
2A/2B	Y Q E I K E Q * G V G L I A R	0.10
2B/2C	R Mx E L R T Q * S F S N W L R	1.00[a,b]
2C/3A	Y Mx E L W S Q * G I D D D R	1.00[a]
3A/3B	Y E P I P A E * G V Y H G V K	<0.02
3B/3C	Y D P V E S Q * S T L E I A G I K	0.25
3C/3D	Y K K I E S Q * R I Mx K V E F	0.15[a]

[a] Mx, methionine in the polyprotein junction sequence was substituted with the isosteric and more stable amino acid norleucine. [b] k_{cat}/K_m determined by HPLC analysis: 2100 ± 120 M^{-1} s^{-1} (substrate concentration was 0.311 mM; enzyme concentration was 0.54 μM).

over Ala in P_4. Weidner and Dunn (1991) have reported that the poliovirus 3C proteinase requires a residue larger than Gly in the P_3 position for efficient cleavage. Petithory et al. (1991) used Edman degradation methods to resolve HAV 3C peptide cleavage rates from peptide mixtures and determined HAV 3C substrate specificity for P_1' and P_2' amino acids. The amount of information gained from each of these studies has fallen short of describing the enzymatic mechanism used by 3C proteinases to translate specific sequences into efficient peptide cleavage. Identification of the types of substrate–enzyme interactions playing pivotal roles in catalysis will enhance efforts to design and develop effective inhibitor molecules to be used in antiviral chemotherapy.

In this study, we employ HPLC analysis or trinitrobenzenesulfonate (TNBS) derivatization to detect cleavage products of several series of peptides designed to identify HAV 3C substrate specificity. We have also synthesized a fluorescent substrate which was used in a continuous assay to construct a pH rate profile and assess the efficacy of commercially available inhibitors.

MATERIALS AND METHODS

Proteinases. Recombinant HAV 3C protein was expressed in *Escherichia coli* and purified as reported by Malcolm et al. (1992). Induced cells were pelleted and lysed by freeze/thaw in the presence of lysozyme and lysis buffer (50 mM Tris-HCl, pH 8.5, and 2.5 mM EDTA, 2 mM DTT). Cell lysate was cleared by centrifugation and absorbed with an equal volume of DEAE-Sephadex equilibrated in lysis buffer. The supernatant was decanted and applied to a CM-Sepharose column previously equilibrated in the same buffer. A linear salt gradient (0–1.0 M NaCl) was used to elute the protein from the column, and fractions, assessed by SDS–PAGE analysis to contain enzyme, were pooled. The protein samples were dialyzed and concentrated into a storage buffer (50 mM potassium phosphate, pH 7.5, 0.25 mM EDTA, 5 mM 2-mercaptoethanol) to a final protein concentration of 1 mg/mL and stored at −80 °C. Purity of the enzyme samples was greater than 80% as determined by SDS–PAGE analysis. Proteinase concentrations were determined using the BCA protein assay reagent (Pierce) with bovine serum albumin as a standard. Variation in the enzyme activity was corrected for by normalizing peptidase activity with respect to the control peptide Ac-ELRTQSFS-NH_2.

Peptide Substrates. The peptides were synthesized using solid phase chemistry by either the Fmoc (Atherton & Sheppard, 1989) or t-Boc methods (Stewart & Young, 1984), and the N-termini were blocked using acetic anhydride. Peptides with C-terminal amides were generated using Rink resin (Rink, 1987) for the Fmoc procedure or methyl-BHA resin for the t-Boc procedure. The fluorescent substrate was prepared by t-Boc synthesis on an Applied Biosystems Model 430A instrument. Following completion of the assembly of the peptide LRTQS(*p*NO_2F)S-NH_2 on a 0.1-mmol scale, the N-terminal amino group was labeled by reaction with 5-(dimethylamino)-1-naphthalenesulfonyl chloride (DNS-Cl). Modification was performed on the peptide prior to cleavage from the resin using a variation of a published procedure of Consalvo et al. (1989). Briefly, the N-terminus of the peptide was deprotected with 50% TFA and neutralized with DIEA. A 1.7-fold molar excess of DNS-Cl (0.17 mmol in CH_2Cl_2/DMF, 5:1) was added to the peptide–resin along with 0.19 mmol of DIEA. The mixture was stirred for 1 h in the dark at room temperature. An additional 0.19 mmol of DIEA was added, and the reaction was continued for an additional hour. The resin was washed with dichloromethane, dried, and deprotected with HF using standard methods of Gross et al. (1981), yielding DNS-LRTQS(*p*NO_2F)S-NH_2. All nonfluorescent peptides were purified by reversed-phase HPLC (C-18, 5 × 25 cm, Vydac, 2%/min linear gradient of 0.1% TFA/water adding 0.1% TFA/acetonitrile). The fluorescent substrate was purified by reversed-phase HPLC (C-18, 0.8 × 10 cm, Waters, 0.78%/min linear gradient of 0.1% TFA/water adding 0.1% TFA/acetonitrile). Composition and concentration of the substrate peptides were determined by amino acid analysis.

HPLC-Based Analysis of Proteolysis. Reversed-phase HPLC analysis of reaction mixtures was used to establish cleavage, isolate products, and determine initial rates of hydrolysis for HAV junction peptides. The HAV 3C proteinase and substrate were incubated in reaction buffer (94 mM potassium phosphate, pH 7.5, containing 6 mM sodium citrate, 2 mM EDTA) at 37 °C. Peptide cleavage was quenched with 0.05 volumes of 100% TFA, and the extent of hydrolysis was determined from the decrease in the area of the substrate peak (monitored at either 214 or 254 nm). The reaction products were separated by reversed-phase HPLC (C-18, 0.25 × 25 cm, Vydac, 1%/min linear gradient of 0.1% TFA/water adding 0.1% TFA/acetonitrile or C-18, 0.8 × 10 cm, Waters, 0.78%/min linear gradient of 0.1% TFA/water adding 0.1% TFA/acetonitrile in the case of junction sequence peptides). The area of the substrate peak was converted to concentration

by comparing areas obtained upon injection of standard amounts of substrate. The progressive hydrolysis of substrate was observed to be first-order with respect to time, and the apparent rate constant was calculated when data were fitted by nonlinear regression (Enzfitter, Elsevier Biosoft, U.K.) to

$$S = S_0 e^{-kt} \quad (1)$$

where S represents the concentration of peptide at time t, S_0 is the initial substrate concentration, and k is the pseudo-first-order rate constant.

Similarly, initial rates of peptide cleavage were measured by quenching aliquots taken from the reaction mixture at timed intervals and determining the disappearance of substrate with respect to time. The time course of the reaction was determined by regression analysis to be linear over 40 min and a 10-fold range of substrate concentration.

Steady-State Kinetics. k_{cat}/K_m values were determined by measuring the dependence of initial rates on substrate concentrations using a modification of the method described by Billich and Winkler (1990). Proteinase and substrate were incubated in reaction buffer at 25 °C. Four to ten 12-μL aliquots were removed from the reaction mixture at timed intervals, and peptide lysis was quenched with 50 μL of 0.25 M sodium borate, pH 10. A total of 12 μL of freshly prepared 0.14 M TNBS in 0.25 M sodium borate solution was added to the quenched reaction mixture and the solution was incubated for 10 min at room temperature. The color was stabilized by adding 225 μL of 3 mM Na_2SO_3, 0.2 M KH_2PO_4. The concentration of free amine generated during peptide lysis was determined by measuring the absorbance at 405 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA) and ΔSoft software (BioMetallics, Princeton, NJ).

Standard curves were determined using the HPLC-purified C-terminal fragment of the cleavage products, NH_3^+-SFS-NH_2. The rate of cleavage was determined to be linear with respect to enzyme concentration below 0.8 μM. All initial rate determinations were carried out at enzyme concentrations within the range of 0.2–0.4 μM. The initial rates of cleavage for each peptide concentration, determined from time point measurements representing <30% of the total substrate cleaved, were linear by regression analysis (KaleidaGraph; Abelbeck, Reading, PA). The k_{cat}/K_m values were calculated from nonlinear least-squares fits of initial rates as a function of substrate concentration as related by the Michaelis–Menten equation (EnzymeKinetics; Trinity Software, Campton, NH). The standard errors in k_{cat}/K_m were under 15%. In several cases, k_{cat}/K_m values were also determined from HPLC analysis at several initial substrate concentrations.

Fluorescence Quench Assay and pH Rate Profile. The time-dependent increase in fluorescence of the dansylated substrate (see above) resulting from enzyme-catalyzed cleavage between Gln and Ser was monitored on a SLM 4800C spectrofluorometer with the excitation monochromator set at 346 nm and the emission monochromator set at 555 nm. The pseudo-first-order rate constant was calculated by fitting the following equation to experimental data by nonlinear regression (Enzfitter, Elsevier Biosoft, U.K.):

$$F = F_0 + (F_\infty - F_0)(1 - e^{-kt}) \quad (2)$$

where F is fluorescence at time t, F_0 is initial fluorescence, F_∞ is fluorescence after complete hydrolysis, and k is the apparent first-order rate constant.

Activity was monitored using the fluorescent assay described above from pH 5.5 to 8.5 (MES, pH 5.5–6.5; HEPES, pH 7.0–7.5; and BICINE, pH 8.0–8.5). Measurements of k_{cat}/K_m were performed at buffer concentrations of 25, 50, and 100 mM. The values obtained were fitted to a straight line and extrapolated to zero buffer concentration. The pK_a of the reactive group giving rise to pH-dependent enzyme activity was calculated from nonlinear regression fitting (Enzfitter; Elsevier Biosoft, U.K.) of the following equation to experimental data:

$$k_{cat}/K_m = \frac{k_{cat}/K_m(\text{min}) + k_{cat}/K_m(\text{max}) \times 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (3)$$

where k_{cat}/K_m(min) and k_{cat}/K_m(max) are estimates of lower and upper limits of k_{cat}/K_m.

Commercial Inhibitors. TPCK, TLCK, E-64, chicken egg white cystatin, iodoacetic acid, leupeptin (1/2 sulfate salt), soybean trypsin inhibitor (type S-I), isovalerylpepstatin, calpain inhibitor peptide, and PTPS-NH_2 were purchased from Sigma Chemical Co. (St. Louis, MO). Eglin c, α_1-antichymotrypsin, and human plasma cystatin C were from Calbiochem Co. (San Diego, CA). Tyrostatin and acetylpepstatin were a generous gift from Dr. Kohei Oda (Osaka Prefecture University).

Inhibition Studies. Inhibition of enzyme activity by commercially available proteinase inhibitors was measured using the fluorescence quench assay described above. The enzyme (0.2 μM) was incubated with the inhibitor in the reaction mixture for 15 min at 37 °C. After this time, the fluorescent substrate DNS-LRTQ*S(pNO_2F)S-NH_2 was added and the k_{cat}/K_m was calculated as described above. The percentage of enzyme inhibition was calculated from the ratio of k_{cat}/K_m values determined in the presence and absence of inhibitor.

RESULTS

Junction Sequences. The relative k_{cat}/K_m values of peptides representative of each of the eight putative junction sequences are shown in Table I. The efficiency of peptide cleavage may be ranked as follows: 2B/2C and 2C/3A > 1B/1C and 1C/1D > 2A/2B, 3B/3C, and 3C/3D > 3A/3B. One of the most efficiently cleaved peptide sequences, 2B/2C (Table I), was used for further study, by the construction of a variety of derivatives.

Rates of catalyzed cleavage of peptide substrates were measured by two methods: discontinuous detection of free amine, using TNBS reaction to detect product, and a continuous fluorescence quench assay. The validity of these kinetic assays was verified by more standardized HPLC methods (peptide **2**, Table II).

Truncation Peptides. The k_{cat}/K_m values determined for a series of peptides in which amino acids are truncated from either the amino or carboxy terminus of the 2B/2C octapeptide are presented in Table II. Removal of the N-terminal glutamic acid residue from the octamer caused less than a 2-fold increase in the bimolecular rate constant (peptide **2** vs **1**, Table II). Dramatically, no significant cleavage was detected for peptides with the P_4 amino acid removed (peptides **4**–**7**, Table II).

Peptides with deletions in P_3' and P_2' of C-terminal residues were less efficiently cleaved (compare peptides **8** with **10** and **9** with **11**, Table II). The k_{cat}/K_m of peptide **10**, with the C-terminus having a free carboxyl group, was notably decreased relative to the capped homologue (peptide **9**, Table II). The sequence Ac-ELRTQSFS-NH_2 (peptide **1**, Table II) was used to further investigate HAV 3C substrate specificity at the amino acid level.

Table II: Steady-State Kinetic Parameters for Truncated Peptides of the 2B/2C Peptide

	peptide	k_{cat}/K_m (M^{-1} s^{-1})[a]	K_i (mM)
1	Ac-ELRTQSFS-NH2	840 (±100)	
2	Ac-LRTQSFS-NH2	1400 (±100)	
		1600 (±190)[b]	
3	LRTQSFS-NH2	600 (±40)[b]	
4	Ac-RTQSFS-NH2	NC[c]	>20
5	RTQSFS-NH2	NC[b]	>20[b]
6	Ac-TQSFS-NH2	NC	
7	Ac-QSFS-NH2	NC	
8	Ac-ELRTQSFS-CO2	1100 (±100)	
9	Ac-ELRTQSF-NH2	1300 (±200)	
10	Ac-ELRTQSF-CO2	210 (±30)	
11	Ac-ELRTQS-NH2	450 (±40)	
12	Ac-ELRTQS-CO2	370 (±40)	

[a] Determined in 94 mM sodium phosphate, 6 mM sodium citrate, 0.2 mM EDTA, pH 7.5, at 25 °C as described under Materials and Methods where k_{cat}/K_m are approximate values calculated on the basis that 85% of the protein is active. [b] Determined by HPLC methods. [c] NC, no detectable cleavage.

Table III: Steady-State Kinetic Parameters for Substituted Peptide Substrates Determined for HAV 3C Proteinase

	peptide	k_{cat}/K_m[a] (M^{-1} s^{-1})	v_i[b] (μmol/(mg·h))
1	Ac-ELRTQSFS-NH2	800 (±100)	240
13	Ac-**A**LRTQSFS-NH2	630 (±70)	
14	Ac-**E**ARTQSFS-NH2	84 (±8)	
15	Ac-ELA**T**QSFS-NH2	800 (±100)	
16	Ac-ELR**A**QSFS-NH2	800 (±100)	
17	Ac-ELRT**A**SFS-NH2	NC[c]	
18	Ac-ELRTQ**A**FS-NH2	900 (±100)	
19	Ac-ELRTQS**A**S-NH2	800 (±100)	
20	Ac-ELRTQSF**A**-NH2	800 (±100)	
21	Ac-ELA**A**QSFS-NH2	360 (±40)	
22	Ac-ELRT**H**SFS-NH2		13[d]
23	Ac-ELRT**E**SFS-NH2		5[d]
24	Ac-ELRT**N**SFS-NH2		2.5[d]
25	Ac-ELRT**O**SFS-NH2		0.8[d]
26	Ac-E**W**RTQSFS-NH2	1220 (±80)	
27	Ac-E**V**RTQSFS-NH2	900 (±80)	
28	Ac-E**I**RTQSFS-NH2	720 (±80)	
29	Ac-E**B**RTQSFS-NH2	950 (±80)[e]	
30	Ac-E**H**RTQSFS-NH2	NC[f]	
31	Ac-E**E**RTQSFS-NH2	NC	
32	Ac-E**U**RTQSFS-NH2	410 (±30)[e]	
33	Ac-E**T**RTQSFS-NH2	210 (±40)[e]	
34	Ac-E**X**RTQSFS-NH2	220 (±20)[e]	
35	Ac-EIRKQNMT-NH2	NC	
36	Ac-EVGKQRLK-NH2	NC	
37	Ac-RVVTQGPE-NH2	NC	
38	Ac-YRPVVVQGPNTEF-NH2	NC	

[a] Determined in 94 mM sodium phosphate, 6 mM sodium citrate, 0.2 mM EDTA, pH 7.5, at 25 °C as described under Materials and Methods where k_{cat}/K_m are approximate values calculated on the basis that 80% of the protein is active enzyme. [b] Peptide and enzyme concentrations were 1 mM and 0.09 mg/mL, respectively. [c] NC, no detectable cleavage. [d] These low rates of hydrolysis were determined by extending the time course out to over 2 h and taking larger aliquots of the reaction for the TNBS-based assay. The errors associated with these measurements were as low as 60% for the His-containing peptide and as high as 100% for the Asn- and Orn-containing peptides. [e] X, B, O, and U indicate norleucine, D-Leu, ornithine, and norvaline, respectively. [f] The peptide was tested at pH 6.0, 7.5, and 8.3 with 100 mM MES, MOPS, and BICINE, respectively. Bold characters indicate the changes made relative to peptide **1**.

Alanine Scan of the Truncated Substrate. The second-order rate constants determined for Ala replacement peptides are presented in Table III. Cleavage of the P_1-altered peptide substrate, in which Ala was substituted for Gln, was undetectable (peptide **17**, Table III). The substitution of Ala for Glu in the P_5 position had no significant effect on k_{cat}/K_m, while replacement of Leu by Ala in the P_4 position caused k_{cat}/K_m to decrease 10-fold, from 800 to 84 M^{-1} s^{-1} (peptides **13** and **14**, respectively; Table III). Replacement of each amino acid at P_3, P_2, P_1', P_2', or P_3' positions with Ala resulted in undetectable changes in k_{cat}/K_m values (peptides **15**, **16**, and **18–20**, Table III). The combined substitutions of Ala for both P_3 and P_2 amino acids resulted in a modest 2-fold decrease in k_{cat}/K_m (peptide **21**, Table III).

HAV 3C P_1 Specificity. The initial velocities measured for the P_1-substituted peptides of Ac-ELRTQSFS-NH_2 are compared in Table III. Three amino acids substituted into P_1, His, Glu, and Asn (peptides **22**, **23**, and **24**, respectively; Table III), were chosen because of their structural similarities with Gln. In each case, the rate of peptide cleavage was decreased greater than 10-fold. This large decrease in cleavage rate was also seen when Orn was substituted into P_1 of the 2B/2C peptide (peptide **25**, Table III).

HAV 3C P_4 Specificity. The k_{cat}/K_m values determined for P_4-substituted peptides are presented in Table III. All 2B/2C-like peptides synthesized with hydrophobic amino acids (Leu, Trp, Val, Ile, D-Leu, nor-Val, and nor-Leu, peptides **1**, **26–29**, **32**, and **34** (Table III), respectively) in the P_4 position were readily cleaved by HAV 3C proteinase. Cleavage of peptides with His or Glu in P_4 was undetectable (peptides **30** and **31**, Table III). The k_{cat}/K_m for the octamer peptide increased significantly to 1220 M^{-1} s^{-1} when Trp was placed in the P_4 position (peptide **26**, Table III). k_{cat}/K_m remained unchanged at 950 M^{-1} s^{-1} when D-Leu was substituted into this position (peptide **29**, Table III). k_{cat}/K_m decreased 2-fold when nor-Val was placed in P_4 (peptide **32**, Table III) and 4-fold when either Thr or nor-Leu were placed in P_4 (peptides **33** and **34** (Table III), respectively). Table III also presents the lack of cleavage observed when HAV 3C was incubated with four additional peptides. Peptides **35** and **36** are based on two additional putative cleavage sites in the HAV polyprotein sequence (see discussion). Peptides **37** and **38**, previously studied by Long et al. (1989) as substrates of HRV 14 3C proteinase, are based on the HRV 14 3B/3C junction. Although peptides **37** and **38** were cleaved very efficiently by HRV 14 3C, they are not cleaved by HAV 3C proteinase.

Product Inhibition. The three peptides Ac-ELRTQ-CO_2, Ac-SFS-NH_2, and Ac-ELRTQ-NH_2, representing product fragments, were assayed as simple competitive inhibitors. The pentamer and trimer peptides, Ac-ELRTQ-CO_2 and Ac-SFS-NH_2, did not show inhibition up to 20 mM peptide (data not shown). The amide capped peptide, Ac-ELRTQ-NH_2, showed very weak inhibition with an estimated K_i of 10 mM.

Dependence of k_{cat}/K_m on pH. The heptamer corresponding to the 2B/2C junction was converted to a fluorescent substrate by replacing Phe in P_2' with *p*-NO_2F and labeling the amino terminus with DNS-Cl. Due to low solubility in water and intermolecular quenching at concentrations above 25 μM, further measurements using this substrate were focused on the determination of k_{cat}/K_m at concentrations of substrate not higher than 10 μM. The rate of cleavage for the heptamer was identical as determined by the rate of fluorescence change (k_{cat}/K_m = 1600 ± 140 M^{-1} s^{-1}, Figure 1A) or by HPLC product analysis (k_{cat}/K_m = 1600 ± 210 M^{-1} s^{-1}, Figure 1B) and was 76% of the value for the 14-mer corresponding to the 2B/2C junction sequence (k_{cat}/K_m = 2100 ± 120 M^{-1} s^{-1}, Table I). The reaction between HAV 3C proteinase and the fluorescent substrate was determined to be first-order with enzyme concentration (data not shown). At pH 7.5, cleavage of the fluorescence substrate was relatively insensitive to NaCl concentrations of up to 1 M (data not shown).

Incubation of the enzyme under acidic conditions (15 min, in 50 mM sodium citrate, pH 4.8) prior to the measurement

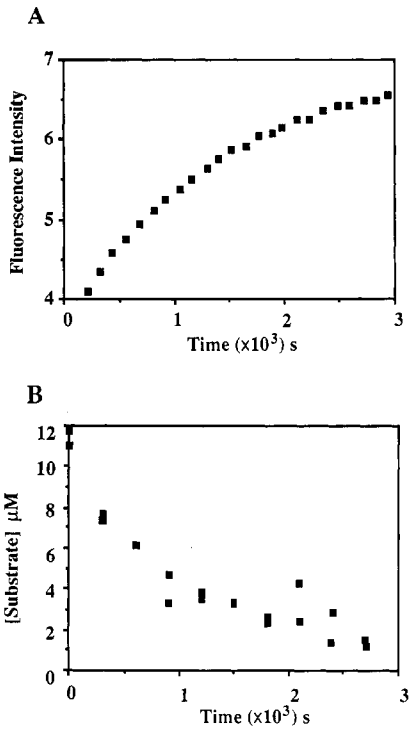

FIGURE 1: Fluorescent assay (panel A) compared with HPLC assay (panel B). The products and the substrate quantitation for the HPLC assay and the fluorescence measurements for the fluorescent assay were performed as described under Materials and Methods. Concentrations of enzyme and substrate were 0.54 μM and 9.73 μM, respectively. The k_{cat}/K_m values calculated from these progress curves were (A) 1600 ± 140 M^{-1} s^{-1} and (B) 1600 ± 210 M^{-1} s^{-1}.

of peptide hydrolysis at the readjusted pH of 7.5 did not affect activity. An analogous experiment with preincubation at pH 10.4 (47.5 mM CAPS) followed by assay at pH 7.5 indicated that only 70% of the catalytic activity was recovered. The drop in activity did not appear to be caused by dimerization of the enzyme as judged by a nonreducing SDS–PAGE analysis (data not shown). Consequently, the pH rate profile was restricted to measurements between pH 5 and 8.5 and revealed an optimum in enzyme activity between pH 7 and 8.5. There was a pH dependence for k_{cat}/K_m in the lower pH range that can be described as enhanced by the deprotonated form of a single ionizable group with a pK_a = 6.2 (±0.2). Phosphate was found to increase k_{cat}/K_m values in the pH range of 6.5–7.5 relative to values determined with MES, HEPES, or BICINE as buffers (Figure 2).

Inhibition by Commercially Available Inhibitors. In accord with previous studies indicating picornaviral 3C proteinases to be cysteine proteinases, the enzyme was completely inhibited by reagents, such as *N*-ethylmaleimide and iodoacetic acid, which are known to covalently react with cysteine sulfhydryl groups (Table IV). Inhibitors of aspartic proteinases (acetyl- or isovalerylpepstatin and tyrostatin) showed no inhibitory activity against the HAV 3C enzyme (Table IV). All assays were carried out in the presence of EDTA, indicating that metal ions are not essential for activity. Hence, it is unlikely that this enzyme would fall in the metalloproteinase family. Many classical protein inhibitors of serine proteinases did not inhibit the hepatitis enzyme (Table IV).

Curiously, two standard cysteine proteinase inhibitors, E-64 and egg white cystatin, showed little or no inhibition of this viral enzyme. Again, this is similar to results reported with other picornaviral processing proteinases. While elastatinal, a common inhibitor of serine proteinases, especially elastase, shows demonstrable inhibition at a massive concentration (5.6 mM, Table IV), this is considered to be less significant than the nearly total inhibition exhibited by TPCK or TLCK at moderate concentrations (Table IV).

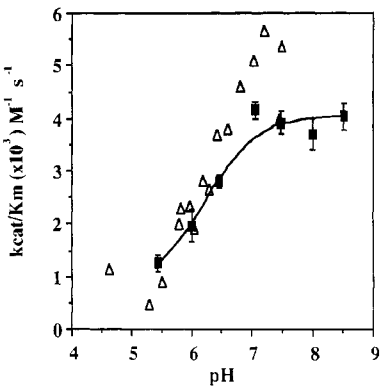

FIGURE 2: pH profile of HAV 3C protease. k_{cat}/K_m values were extrapolated to buffer concentration equal to zero as described under Materials and Methods. A nonlinear regression analysis of the data using the Enzfitter program gave a pK_a value of 6.2 (±0.2) (see under Materials and Methods for details). Symbols: ■, data extrapolated to buffer concentration equal to zero; Δ, data obtained in the presence of 50 mM potassium phosphate.

Table IV: Partial Inhibition Studies of HAV 3C Protease

inhibitor	concn (μM)	% inhibition
NEM	4.3	100
iodoacetic acid	43	100
E-64	4.3	15
egg white cystatin	4.3	0
human plasma cystatin C	1.0	0
	4.2	0
leupeptin	45	0
calpain inhibitor peptide[a]	22	0
antipain	11100	18
elastatinal	5600	50
TPCK	43	100
TLCK	43	83
eglin c	4.3	0
	17.3	0
α_1-antichymotrypsin	1.05	0
soybean trypsin inhibitor	48	0
isovalerylpepstatin	8.4	0
tyrostatin	15	0
Ac-pepstatin	22	0
P-T-P-S-NH_2	3600	0

[a] The enzyme was incubated for 15 min with the inhibitor at 37 °C and then assayed using the fluorescent substrate DNS-LRTQ*S-(pNO_2F)S-NH_2 as described under Materials and Methods. The reaction mixture contained 0.21 mM EDTA. The concentrations of enzyme and the substrate were 0.2–0.3 μM and 2.8 or 10 μM, respectively. [a] DPMSSTYIEELGKRGVTIPPKYRELLA (Maki et al., 1989).

DISCUSSION

Amino acid analysis of cleavage products indicates that the peptides having sequences corresponding to the polyprotein junction sequences are hydrolyzed at the predicted scissile bond. It is therefore correct to assume that the productive mode of enzyme–peptide binding is the only kinetically significant binding mode. Because of substrate insolubility, concentrations sufficiently high (>3 mM) to yield saturation kinetics could not be reached with many of the peptides studied, prohibiting the calculation of individual kinetic parameters (k_{cat} and K_m) essential for discussion of effects that specific amino acids have on a general cleavage mechanism.

Minimal Peptide Substrate and Product Inhibition. The large decrease in peptide hydrolysis upon removal of the Leu in P_4 indicates that there is a major contribution to substrate

cleavage from this residue (see peptide **4**, Table II, and further discussion below). The possibility that removal of the P_4 Leu causes the hexamer Ac-RTQSFS-NH_2 to bind in an unproductive mode is not supported by its very high K_i value (peptides **4** and **5**, Table II). The k_{cat}/K_m value in Table II for the peptide series in which C-terminal amino acids have been removed indicates that the P_2' amino acid contributes to substrate recognition (compare peptides **9** and **11**, Table II). The decrease in the apparent K_i of the peptide Ac-ELRTQ-NH_2, relative to the product Ac-ELRTQ-COO^-, suggests an active site intolerant of negative charges. It is possible that the energy gained from solvent interaction with a free carboxyl group has evolved as a part of the mechanism to help expel cleavage products from a presumably hydrophobic active site.

Alanine Scan of Substrate. In order to probe peptide specificity at the amino acid level, the minimal substrate, Ac-ELRTQSFS-NH_2 (peptide **1**, Table II), was used as a template sequence for amino acid replacement studies. Alanine was chosen for initial substitution because it represents the removal of most of the amino acid side chain and yet, due to the methyl group, retains some conformational restriction (ϕ, ψ angles) on either side of the α-carbon. With the exceptions of the P_1 and P_4 Ala substitutions, the replacement of amino acids with Ala had little effect on k_{cat}/K_m (peptides **13–20**, Table III).

P_1 Specificity. The data in Table III show that HAV 3C can cleave substrates containing Gln in P_1 with much greater efficiency than peptides with other amino acids at this position. The greater than 10-fold decrease in the rate of hydrolysis of His-, Glu-, and Orn-substituted peptides (peptides **22**, **23**, and **25**, Table III) suggests either that the mechanism of peptides cleavage does not tolerate a charged amino acid at S_1 or that efficient cleavage of the peptide substrate is favored when P_1 is occupied by an uncharged amino acid. Consequently, the very low rate of peptide hydrolysis of the 3A/3B peptide (Table I) can in part be accounted for by the Glu residue at P_1. When Ala or Asn (peptide **17** or peptide **24**, respectively; Table III) were substituted at P_1, peptide cleavage was also significantly decreased. This shows S_1 has a degree of specificity which requires not only an uncharged amino acid but also the presentation of an amido group in a geometry unique to the Gln side chain.

Enzyme systems have been described to utilize cosolutes in mechanisms which compensate for removal of specific functionalities originally present in either the protein (Carter & Wells, 1987; Tu et al., 1990) or the substrate (Inagami, 1965). The apparent need of HAV 3C, during substrate recognition, to have an amido group at the P_1 position could not be met by including urea (up to 2 M) or methylacetamide (at 150 mM) in the reaction mixture with P_1-Ala-substituted peptide (data not shown). Although urea and methylacetamide were observed not to decrease hydrolysis of the good substrate, peptide **1** (Table II), it is most likely that they cannot interact with the active site and the P_1-Ala-substituted peptide in a "rescue"-type mechanism.

P_4 Specificity. The 10-fold decrease in cleavage efficiency resulting from substituting Ala for Leu in P_4 of the 2B/2C peptide (peptide **14**, Table III) and lack of peptide cleavage of the P_4-truncated peptide (peptide **4**, Table II) suggests an essential determinant recognized by HAV 3C in this position. HAV 3C proteinase cleaved peptides with increased (Trp, peptide **26**, Table III) or similar (Val, Ile, and D-Leu, peptides **27**, **28**, and **29**, respectively; Table III) efficiency relative to the 2B/2C octamer when hydrophobic amino acids were substituted into the P_4 position. It is interesting to note that a consensus sequence constructed from the junction sequences in Table I would not have predicted a large hydrophobic amino acid to be beneficial in peptide cleavage. A similar improvement in cleavage rates of peptides substituted in P_4 with hydrophobic groups was also observed for HRV 14 3C proteinase substrates but was limited to the relatively small hydrophobic amino acids (Cordingley et al., 1990). This, together with the 10- and 4-fold decrease in k_{cat}/K_m values determined for the Ala (peptide **14**, Table III) and Thr (peptide **33**, Table III) P_4-substituted peptides, suggests that HAV 3C can utilize specific hydrophobic interactions between P_4 and S_4 to increase the efficiency of the catalytic process.

When the hydrophilic amino acids, Glu and His (peptides **31** and **30**, respectively; Table III), were incorporated into the P_4 position, the peptides were not cleaved by HAV 3C proteinase. It is unclear why the His-substituted peptide (**30**) continued to show no cleavage at pH values above 8 where it would be expected that a high percentage of the side chain would normally be uncharged ($pK_a \sim 6.8$). It is possible that within the context of the peptide the imidazole ring has a pK_a significantly higher due to ionic interactions with the Glu in P_5 and/or conformational restrictions caused by such a salt bridge. It is also possible that the uncharged imidazole side chain may continue to be hydrophilic enough to favor solvation over S_4 interaction. This apparent exclusion of substrates based on charged or hydrophilic amino acids in P_4 is a feature shared with HRV 14 3C (Cordingley et al., 1990).

Dependence of k_{cat}/K_m on pH. Values of k_{cat}/K_m at different pH were fit with a simple titration curve yielding a calculated pK_a of 6.2 (±0.2). This pK_a value may correspond to that of a His or Cys group in the active site. The optimal activity is observed in the region of pH 7.5 to 8.5. The pH optimum for the poliovirus 3C proteinase was similarly observed to lie between 7 and 8 (Weidner, 1989). The pH optimum for papain, a member of a different class of cysteine proteinases but using a thiolate–imidazolium pair, was determined to be between 5 and 8 (Lowe, 1976). The pH dependence reported here (Figure 2) supports, but does not prove, a papain-like mechanism for HAV 3C proteinase. Also presented in Figure 2 are data obtained in the presence of 50 mM phosphate buffer. The increase in k_{cat}/K_m seen may reflect a general base assistance to the reaction similar to the imidazole- or phosphate-promoted CO_2 hydration reaction catalyzed by wild-type human carbonic anhydrases (Tu et al., 1990; Paranawithana et al., 1990).

Studies with Commercial Proteinase Inhibitors. Inhibition studies of poliovirus and rhinovirus 14 3C proteases (Weidner & Dunn, 1991; Orr et al., 1989) suggested that they are cysteine proteinases although with active sites different from members of the papain family (Hanada et al., 1978). Inhibition studies of the HAV 3C proteinase indicate a similar pattern. The lack of inhibition by chicken egg white and human plasma C cystatins, and only a weak inhibition by E-64, suggest that HAV 3C, like other picornaviral proteinases, has a unique active site specificity. The inhibition by TPCK and TLCK, characteristic inhibitors of serine proteases, is somewhat unexpected. The chloromethyl moieties of TPCK and TLCK may react with the active site cysteine or histidine or with a residue close to the active site, thereby inactivating the proteinase. The inactivation of the enzyme by TPCK is time dependent (data not shown) which may indicate a mechanism consistent with affinity label-type derivatization. However, since neither TPCK nor TLCK would be expected to fit the P_1 specificity of HAV 3C proteinase (containing Phe vs Lys, respectively), the similar levels of

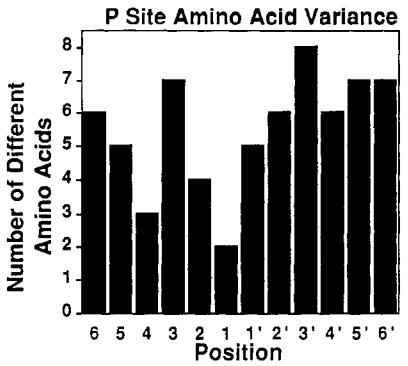

FIGURE 3: Graphic representation of the number of different amino acids found in the cleavage sites recognized by HAV 3C proteinase. The sequences used to construct this graph are listed in Table I.

inhibition by TPCK and TLCK suggest that the reaction is most likely proceeding through a relatively nonspecific covalent complex.

Junction Sequence Analysis. Tabulation of the number of different amino acids found at each position in the eight junction sequences (Table I and Figure 3) reveals that positions P_1 and P_4 have the most limited variation, suggesting specific amino acid requirements in these two positions. In general, P_4, P_2, and P_1' seem to show a type of amino acid conservation related to the chemical character of each side chain. Following these observations, the most significant structural feature of the cleavage sites recognized by HAV 3C appears to be the conserved glutamine in the P_1 position. However, other positions appear to affect the HAV 3C specificity (Petithory et al., 1991). The most efficiently cleaved peptides listed in Table I correspond to the junction sequences 2B/2C and 2C/3A. In addition to Gln in the P_1 position, these two peptides contain the same amino acids in three other positions (P_6, P_5, and P_4): Met (nor-Leu), Glu, and Leu. The essentially identical k_{cat}/K_m values may indicate that the amino acid variations in P_3 and P′ side amino acids do not present changes which can significantly affect the rate of peptide cleavage. This conclusion is borne out by the number of different amino acids found in these positions (see Figure 3). The next best cleaved peptides correspond to 1B/1C and 1C/1D junctions. Their sequences show multiple changes relative to 2B/2C and 2C/3A, making additional trends unclear. The low cleavage activity determined for some of the putative junction sequences listed in Table I suggest that HAV may need a mechanism which can alter 3C substrate specificity similar to that reported by Blair and Semler (1991) in poliovirus 3CD and 3C proteinase.

Similar to the analysis of HIV-1 proteinase cleavage sites by Pettit et al. (1991), we have used the hydrophilicity, size, and polarity scales reported by Hellberg et al. (1991) to describe the physicochemical character of amino acids found in P_5–P_3' in substrates of HAV 3C. Preference for hydrophilic amino acids is suggested for the P_3–P_1 positions while for the P_4 position hydrophobic amino acids are preferred. Hydrophobicity biases for the P_5, P_1', P_2', and P_3' positions are less clear and are therefore not assigned. In a similar fashion, the size of the amino acid side chains seems to indicate that small amino acids are preferred in P_4 and large amino acids in P_5. The other positions, with the exception of the highly conserved P_1 position, allow a full range of sizes. With regard to the polarity of the amino acids, only P_4 and P_1 show conservation.

The lack of effect for some of the Ala-substituted peptides may be similarly rationalized in terms of conserved physicochemical character of the cleavage sites. On the basis of the scales described by Hellberg et al. (1991), Ala substitutions would not significantly alter the conserved hydrophilic preferences for the amino acids found in P_3, P_2, P_1', P_2', and P_3' positions but would change the conserved size and hydrophobicity preferences found in the P_5 and P_4 positions, respectively. However, it was determined that together P_3 and P_2 Ala substitutions did significantly alter the cleavage rate of the peptide (compare peptides **1**, **15**, **16**, and **21**, Table III). This indicates that synergy can occur between P_3 and P_2 positions. Although these qualitative correlations are not the result of an exhaustive investigation (requiring consideration of over 500 different physicochemically designed peptide sequences), they do support the view that these physical–chemical scales can be used in predicting peptide activity. The observation that hydrophobicity should correlate with activity in a viral proteinase system is also supported by independent experimental observations reported by Pettit et al. (1991) with the aspartic proteinase from HIV-1.

Using the physical–chemical conservation described above, the HAV polyprotein sequence was reconsidered for potential cleavage sites. A computer program was written to search for sequences within the polyprotein (translated from the open reading frame of the HAV genome reported in GenBank) which shared conservation of the physicochemical characteristics described in the junction sequences of Table I. Two additional sequences were identified as potential 3C substrate sequences since they would contain small hydrophobic amino acids in P_4, Gln in P_1, and hydrophilic amino acids in P_3 and P_2 positions: Ac-EIRKQNMT-NH_2 and Ac-EVGKQRLK-NH_2 (peptides **35** and **36**, respectively; Table III). These two peptides were synthesized and tested as substrates of HAV 3C. Neither peptide was cleaved by the enzyme. With the exception of Lys in P_2 and Arg in P_1' of the second peptide, these sequences contain multiple amino acid changes which do not translate into drastically altered physicochemical character seen to be preferred in the junction sequences (Table I). Therefore, the charge on Lys in P_2, not previously considered, may in large part be responsible for the low cleavage activity toward these peptides (also compare 2A/2B and 2B/2C activities, Table I).

The inability of HAV 3C to cleave peptides readily cleaved by HRV 14 3C proteinase indicates that there is very little cross-recognition of substrates by HRV 14 and HAV 3C proteinases (peptides **37** and **38**, Table III). These results may have been predicted from the observations of Petithory et al. (1991) where HAV 3C was shown to cleave peptides with Pro in the P_2' position inefficiently. Similarly, it was also found that HRV 14 3C proteinase is unable to cleave the HAV 3C peptide substrate **1** rapidly (data not shown).

The studies described in this report have established several important parameters of the reaction catalyzed by HAV 3C proteinase, including the minimum substrate size, the sensitivity (or insensitivity) to substitution at various positions along the substrate sequence, the pH optimum, and the inhibitor sensitivity.

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