Mammalian Chymotrypsin-like Enzymes. Comparative Reactivities of Rat Mast Cell Proteases, Human and Dog Skin Chymases, and Human Cathepsin G with Peptide 4-Nitroanilide Substrates and with Peptide Chloromethyl Ketone and Sulfonyl Fluoride Inhibitors†

James C. Powers,* Takumi Tanaka, J. Wade Harper, Yoshihiro Minematsu, Larry Barker, Danforth Lincoln,‡ and Katherine V. Crumley

School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

Jorma E. Fraki

Department of Dermatology, University of Kuopio, SF-70211 Kuopio 21, Finland

Norman M. Schechter and Gerald G. Lazarus

Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Kiichiro Nakajima and Katsuhiro Nakashino

Faculty of Science, Kwansei Gakuin University, Nishinomiya, Japan

Hans Neurath and Richard G. Woodbury

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Received August 7, 1984

ABSTRACT: The extended substrate binding sites of several chymotrypsin-like serine proteases, including rat mast cell proteases I and II (RMCP I and II, respectively) and human and dog skin chymases, have been investigated by using peptide 4-nitroanilide substrates. In general, these enzymes preferred a P_1 Phe residue and hydrophobic amino acid residues in P_2 and P_3. A P_2 Pro residue was also found to be quite acceptable. The S_4 subsites of these enzymes are less restrictive than the other subsites investigated. The substrate specificity of these enzymes was also investigated by using substrates which contain model desmosine residues and peptides with amino acid sequences of the physiologically important substrates angiotensin I and angiotensinogen and α_1-antichymotrypsin, the major plasma inhibitor for chymotrypsin-like enzymes. These substrates were less reactive than the most reactive tripeptide reported here, Suc-Val-Pro-Phe-NA. The thiobenzyl ester Suc-Val-Pro-Phe-SBzl was found to be an extremely reactive substrate for the enzymes tested and was 6–171-fold more reactive than the 4-nitroanilide substrate. The four chymotrypsin-like enzymes were inhibited by chymostatin and N-substituted saccharin derivatives which had K_I values in the micromolar range. In addition, several potent peptide chloromethyl ketone and substituted benzenesulfonyl fluoride irreversible inhibitors for these enzymes were discovered. The most potent sulfonyl fluoride inhibitor for RMCP I, RMCP II, and human skin chymase, 2-(Z-NHCH$_2$CONH)C$_6$H$_4$SO$_2$F, had $k_{obsd}/[I]$ values of 2500, 270, and 1800 M^{-1} s^{-1}, respectively. The substrates and inhibitors reported here should be extremely useful in elucidating the physiological roles of these proteases.

A variety of mammalian tissues and cell types have been shown to contain serine proteases. These enzymes are most active in the physiologic range and have been implicated in such processes as chemotaxis, endocytosis, exocytosis, protein turnover, tumorigenesis, and fertilization (Woodbury & Neurath, 1980). Imbalances in the levels or regulation of tissue or cellular proteases are thought to manifest themselves in various disease states including inflammatory disorders, arthritis, and certain skin disorders (Fraki et al., 1982).

Two chymotrypsin-like enzymes have been isolated and purified, respectively, from typical peritoneal and atypical (mucosal) rat mast cells (Woodbury et al., 1981). These two enzymes, which we will refer to as rat mast cell protease I (RMCP I)[1] and rat mast cell protease II (RMCP II), are now frequently called rat chymase I and rat chymase II (Barrett & McDonald, 1980).[2] In earlier literature, these enzymes have been referred to as the rat skeletal muscle (group specific) protease or rat intestinal muscle (group specific) protease. This cumbersome nomenclature developed over the years due to a lack of understanding of the source and function of these mast

† This research was supported by a grant from the Council for Tobacco Research (to J.C.P.) to the Georgia Institute of Technology and by National Institutes of Health Grant AM 32070 (to N.M.S. and G.G.L.) to the University of Pennsylvania.

‡ 1981 National Science Foundation Undergraduate Research Participant.

[1] Abbreviations: NA, 4-nitroanilide; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; Suc, succinyl; Boc, *tert*-butyloxycarbonyl; Me_2SO, dimethyl sulfoxide; Lys(Pic), ε-(2-picolinoyl)lysine; SBzl, $-SCH_2C_6H_5$; Z, benzyloxycarbonyl; DFP, diisopropyl fluorophosphate; HL, human leukocyte; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

[2] Most cellular proteases have been named cathepsin regardless of their mechanistic class or pH optimum. However, many tissue chymotrypsin-like enzymes are frequently referred to in the literature as chymases (the name chymase was once applied to chymosin, but this usage is now completely obsolete).

0006-2960/85/0424-2048$01.50/0 © 1985 American Chemical Society

cell proteases. Recently, two chymases have been isolated from human and dog skin by using a 2 M KCl extraction, and the levels of the human chymase have been shown to be elevated in patients with cutaneous mastocytosis (Schechter et al., 1983). Preliminary studies using the immunocytochemical technique suggest that the enzyme is located in mast cell granules (N. Schechter, J. Choi, and G. G. Lazarus, unpublished results). We will refer to these enzymes as dog skin chymase and human skin chymase, respectively.

The four chymases have many properties in common. They are extractable and are more active in high salt. They are inhibited by typical serine protease inhibitors such as DFP and cleave typical chymotrypsin substrates such as Bz-Tyr-OEt and Ac-Tyr-OEt. The complete amino acid sequence of RMCP II has been reported, and the sequence of RMCP I has been partially determined (Woodbury et al., 1978a,b). Both enzymes show considerable homology with each other, with bovine pancreatic chymotrypsin, and with that portion of the N-terminus of human leukocyte cathepsin G which has been sequenced.

In a previous paper, we studied the substrate specificity of RMCP I and RMCP II with a limited set of peptide 4-nitroanilide substrates and developed a fairly reactive peptide chloromethyl ketone inhibitor, Suc-Pro-Leu-Phe-CH_2Cl, for RMCP I (Yoshida et al., 1980). In this paper, we report a systematic mapping of the extended substrate binding site of RMCP I, RMCP II, and the human skin chymase, and more limited mapping of the dog skin chymase. We have utilized peptide 4-nitroanilide substrates and have varied the P_4 through P_1 residues of the substrate to include many of the amino acid residues commonly found in proteins.[3] In addition, we have investigated 4-nitroanilides containing model desmosine residues, several unusual amino acid residues such as *N*-methylvaline, and substrates with sequences related to the chymase cleavage site in human angiotensinogen and angiotensin I, and to the reactive site of human α_1-antichymotrypsin. We have prepared a reactive peptide thiobenzyl ester with the sequence of one of the most reactive 4-nitroanilide substrates and have found it to be more reactive. We have developed some extremely potent inhibitors for most of the enzymes studied and report the inhibition of the enzymes by chymostatin, *N*-acyl and aryl saccharin derivatives, and substituted sulfonyl fluorides. Our kinetic studies have allowed us to compare the relative reactivities of the chymases with other chymotrypsin-like enzymes such as leukocyte cathepsin G and bovine pancreatic chymotrypsin. The substrates and inhibitors which we have reported in this paper should be extremely useful in studies of the physiologic role of various mast cell chymases.

Materials and Methods

RMCP I and II were prepared as previously described (Everitt & Neurath, 1979; Woodbury & Neurath, 1978; Woodbury et al., 1981). Human and dog skin chymases were purified from the high-salt extract of skin by protamine precipitation followed by gel filtration chromatography (Schechter et al., 1983). At this stage of purification, they do not appear to be contaminated by other proteases. Human leukocyte cathepsin G was a gift from Dr. J. Travis and his research group at the University of Georgia. Bovine chymotrypsin A_α, *N*-benzoyl-L-tyrosine ethyl ester, and chymostatin were purchased from Sigma Chemical Co., St. Louis, MO. The syntheses of the 4-nitroanilide substrates and Suc-Val-Pro-Phe-SBzl are reported in the supplementary material of our companion paper [see Tanaka et al. (1985)]. Peptide chloromethyl ketone inhibitors and most of the sulfonyl fluoride inhibitors have been reported previously (Kurachi et al., 1973; Yoshida et al., 1980; Powers et al., 1977; Yoshimura et al., 1982) as has the synthesis of Suc-Ala-Ala-Pro-Phe-SBzl (Harper et al., 1981). The saccharin derivatives were a generous gift of Dr. Morris Zimmerman of Merck (Zimmerman et al., 1980; Ashe et al., 1981).

Substrate Kinetics. The rates of hydrolysis of the 4-nitroanilide substrates were measured by adding 0.050 mL of the appropriate enzyme solution to 2.0 mL of a substrate solution (the buffers are listed in the tables). The increase in absorbance at 410 nm was measured, and the slopes were used to calculate initial velocities using an extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961). Kinetic constants were determined from the hydrolysis rates at five separate substrate concentrations by using Lineweaver–Burk plots. Correlation coefficients were typically greater than 0.99 and never lower than 0.97. Thiobenzyl ester hydrolysis rates were measured as previously described (Harper et al., 1981). All kinetic measurements were performed on a Beckman 35 spectrophotometer.

The concentration of RMCP I was determined by using Bz-Tyr-OEt (specific activity 58.3 units/mg) (Kobayashi & Katsumuma, 1978) with 26 000 for the molecular weight (Everitt & Neurath, 1979). The RMCP II concentration was determined by using $E^{1\%}_{280nm}$ = 11.0 and by titration with 4-nitrophenyl 4-guanidinobenzoate (Yoshida et al., 1980). The concentrations of the dog skin chymase were determined by using radiolabeled diisopropyl phosphorofluoridate (Schechter et al., 1983). The concentration of the human skin chymase was determined by using its molar specific activity (7700 units/μmol of enzyme) measured with 0.5 mM Bz-Tyr-OEt as substrate where 1 unit is defined as the micromoles of enzyme that will hydrolyze 1 μmol of substrate per minute (Schechter et al., 1983). The concentration of cathepsin G was determined as described in the preceding paper (Tanaka et al., 1985), and the concentration of chymotrypsin A_α was determined by active-site titration using Ac-Ala-Ala-Aleu-4-nitrophenyl ester [Aleu = $NHN[CH_2CH(CH_3)_2]CO$; Gupton et al., 1984].

Inhibitor Kinetics. Rates of inactivation by peptide chloromethyl ketones (Yoshida et al., 1980) and sulfonyl fluorides (Yoshimura et al., 1982) were measured and calculated as previously reported, except that residual activity was measured by using either Suc-Val-Pro-Phe-NA or Suc-Ala-Ala-Pro-Phe-SBzl (Harper et al., 1982). Correlation coefficients were greater than 0.99 for the first-order inactivation plots.

Synthesis. Benzoylglycine, 2-aminobenzenesulfonyl fluoride, and benzylamine were obtained from Aldrich Chemical Co., Milwaukee, WI. 2-(Fluorosulfonyl)phenyl isocyanate was prepared as previously described (Lively & Powers, 1978). All structures were consistent with their NMR spectra, which were recorded on a Varian T-60 spectrometer, and elemental analyses, which were performed by Atlantic Microlabs, Atlanta, GA.

2-[(N-Benzoylglycyl)amino]benzenesulfonyl Fluoride [*2-*($C_6H_5CONHCH_2CONH)C_6H_4SO_2F$]. Benzoylglycine (5.4 g, 30 mmol) was added to PCl_5 (6.2 g, 30 mmol) in carbon tetrachloride (100 mL) in a stoppered vessel and stirred for 16 h. The mixture was rapidly filtered and the solid washed with carbon tetrachloride followed by petroleum ether. The solid was then refluxed with 2-aminobenzenesulfonyl fluoride

[3] The nomenclature used for the individual amino acid residues (P_1, P_2, etc.) of a substrate and the subsites (S_1, S_2, etc.) of the enzyme is that of Schechter & Berger (1967).

Table I: Effect of the P_1 Residue on the Specificity and Reactivity of Mammalian Chymotrypsin-like Enzymes

substrate P_4 P_3 P_2 P_1	kinetic constant[a]	RMCP I[b]	RMCP II[c]	human skin chymase[d]	dog skin chymase[d]
Suc-Val-Pro-Phe-NA	K_M	0.12	0.73 (3.0)	0.093	0.14
	k_{cat}	57	26 (32)	75	54
	k_{cat}/K_M	4.8×10^5	3.6×10^4 (1.1×10^4)	8.1×10^5	3.9×10^5
Suc-Val-Pro-Tyr-NA	K_M	0.084	0.19	0.17	0.074
	k_{cat}	5.5	0.70	19	4.4
	k_{cat}/K_M	6.5×10^4	3.7×10^3	1.1×10^5	5.9×10^4
Suc-Val-Pro-Trp-NA	K_M	0.24	0.11	1.6	
	k_{cat}	5.6	0.042	8.4	
	k_{cat}/K_M	2.3×10^4	4.0×10^2	5.3×10^3	
Suc-Val-Pro-Leu-NA	K_M	1.1	1.6	0.88	
	k_{cat}	13	2.7	21	
	k_{cat}/K_M	1.2×10^4	1.7×10^3	2.4×10^4	
Suc-Val-Pro-Met-NA	K_M	0.53	2.2	1.2	
	k_{cat}	5.0	2.9	8.7	
	k_{cat}/K_M	9.4×10^3	1.3×10^3	7.3×10^3	

[a] The units of K_M, k_{cat}, and k_{cat}/K_M are respectively mM, s^{-1}, and M^{-1} s^{-1}. [b] Conditions: pH 8.0, 50 mM phosphate and 10% Me_2SO at 25 °C. [c] Conditions: pH 6.6, 50 mM phosphate and 10% Me_2SO at 25 °C; data at pH 8.0 listed in parentheses. [d] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C.

(1.75 g, 10 mmol) in methylene chloride (100 mL) for 2 h, excess acid chloride removed by filtration, and the solvent removed in vacuo. The product was recrystallized in ethanol/water, yielding light orange crystals; mp 158–159 °C, 1.1 g (31% yield). Anal. Calcd for $C_{15}H_{13}N_2O_4SF$: C, 53.41; N, 8.31; H, 3.89. Found: C, 53.51; N, 8.29; H, 3.90.

N-Benzyl-N'-[2-(fluorosulfonyl)phenyl]urea [*2-($C_6H_5CH_2NHCONH$)$C_6H_4SO_2F$*]. 2-(Fluorosulfonyl)phenyl isocyanate (0.32 g, 1.6 mmol) was dissolved in dry tetrahydrofuran (75 mL) and benzylamine (0.17 g, 1.6 mmol) added dropwise. The mixture was stirred for 16 h at 25 °C followed by 2 h at 65 °C. After the solvent was removed in vacuo, the residue was recrystallized from Me_2SO to give white crystals; mp 148–150 °C, 0.30 g (61.2% yield). Anal. Calcd for $C_{14}H_{13}N_2O_3SF$: C, 54.55; N, 9.09; H, 4.22. Found: C, 54.60; N, 4.25; H, 3.90.

Results

Effect of the P_1 Residue. A series of tripeptides of the general sequence Suc-Val-Pro-AA-NA, where AA = Phe, Tyr, Trp, Leu, and Met, has been used to map the S_1 subsite of RMCP I, RMCP II, and human skin chymase (Table I). The P_1 Phe substrate, Suc-Val-Pro-Phe-NA, was the most reactive toward all the enzymes tested, while substrates containing P_1 Tyr, Trp, and Leu residues were intermediate in reactivity. The P_1 Met substrate was generally the most unreactive substrate in this series and had k_{cat}/K_M values 27–120 times lower than those for the Phe substrate.

Effect of the P_2 Residue. The S_2 subsite of RMCP I, RMCP II, and human skin chymase has been mapped extensively by using a series of 10 tripeptide 4-nitroanilides of the general structure Suc-Phe-AA-Phe-NA where AA represents at least one amino acid from every class commonly found in proteins (Table II). In general, changes in the P_2 residue had a relatively small effect (16–24-fold) on k_{cat}/K_M. Substrates with hydrophobic amino acids (Leu, Val) or Pro in P_2 were 1.1–23 times more reactive with RMCP I and II than those which contained charged or polar amino acids in P_2 and had k_{cat}/K_M values from 1.2×10^4 to 3.1×10^5 M^{-1} s^{-1}. The human skin chymase was the most insensitive to changes in P_2 with k_{cat}/K_M values varying only by a factor of 7.3 with every substrate except Suc-Phe-Glu-Phe-NA, which was 16 times less reactive than Suc-Phe-Pro-Phe-NA. The k_{cat}/K_M values reported here for Suc-Phe-Pro-Phe-NA and Suc-Phe-Leu-Phe-NA with RMCP I and II are 2–3 times lower than those reported earlier (Yoshida et al., 1980). These differences probably arise from the fact that kinetics were measured by using different batches of enzyme.

Effect of the P_3 Residue. As summarized in Table III, the effect of the P_3 residue on specificity and reactivity was examined by using Suc-AA-Pro-Phe-NA, where AA represents any one of 10 different amino acids. The peptide with Val in P_3 was the most reactive tripeptide nitroanilide substrate found for all the enzymes studied. Substitution of Met, Phe, Thr, Leu, or Gln for Val resulted in relatively small decreases in k_{cat}/K_M with RMCP I, RMCP II, and human skin chymase while substrates containing Ser, Glu, and Ala were 7–10 times less reactive with RMCP I and II. With human skin chymase, however, the substrate with Glu in P_3 was only 2 times less reactive than Suc-Val-Pro-Phe-NA. Suc-Lys-Pro-Phe-NA was the least reactive substrate in this series among all the enzymes investigated and was 17–60 times less reactive than Suc-Val-Pro-Phe-NA.

Effects of Unusual Amino Acids in P_3. Several studies have shown that the placement of D-amino acids in P_3 of a peptide substrate can result in enhanced specificity and reactivity toward some serine proteases (Morossy et al., 1980; Lottenberg et al., 1981; Kettner & Shaw, 1981). Therefore, we investigated some derivatives of Suc-Val-Pro-Phe-NA which contained several unusual amino acids, including D-Val and *N*-methyl-D-Val, in P_3. As shown in Table IV, these substrates were considerably less reactive than Suc-Val-Pro-Phe-NA. Cathepsin G did not cleave either of the substrates with D-amino acids in P_3, while chymotrypsin A_α cleaved them slowly (*N*-methyl-D-Val) or not at all (D-Val). RMCP I hydrolyzed all of these substrates quite effectively and had k_{cat}/K_M values between 430 and 5100 M^{-1} s^{-1}. The *N*-methyl-L-Val substrate was also hydrolyzed quite effectively by the rat mast cell proteases and chymotrypsin A_α but was 5–19 times less reactive toward cathepsin G.

Effect of the P_4 Residue. Here we report the hydrolysis of several tetrapeptides of the general sequence Suc-AA-Val-Pro-Phe-NA by rat mast cell proteases I and II, human skin chymase, and dog skin chymase (Table V). Hydrophobic and charged amino acids in P_4 were investigated. With RMCP I and II, k_{cat}/K_M values varied by a factor of 6–10, with the Met substrate being the most reactive and the Lys peptide being the least reactive. With human skin chymase, however, there was only a 4-fold difference between the most and least reactive substrate. In contrast to the rat mast cell proteases, the Glu peptide is 3 times more reactive than the Lys peptide with human skin chymase.

Table II: Effect of the P_2 Residue on the Specificity and Reactivity of Mammalian Chymotrypsin-like Enzymes

substrate P_4 P_3 P_2 P_1	kinetic constant[a]	RMCP I[b]	RMCP II[c]	human skin chymase[d]
Suc-Phe-Leu-Phe-NA	K_M	0.054	0.16 (1.7)	0.091
	k_{cat}	17	3.0 (10)	7.5
	k_{cat}/K_M	3.1×10^5	1.9×10^4 (6.1×10^3)	8.2×10^4
Suc-Phe-Pro-Phe-NA	K_M	0.12	1.1	0.29
	k_{cat}	17	17	28
	k_{cat}/K_M	1.4×10^5	1.5×10^4	9.7×10^4
Suc-Phe-Val-Phe-NA	K_M	0.15	0.55	0.071
	k_{cat}	15	6.8	4.0
	k_{cat}/K_M	1.0×10^5	1.2×10^4	5.6×10^4
Suc-Phe-Lys-Phe-NA	K_M	0.20	1.5	
	k_{cat}	18	0.61	
	k_{cat}/K_M	9.0×10^4	4.1×10^2	
Suc-Phe-Thr-Phe-NA	K_M	0.14	2.1	0.44
	k_{cat}	9.3	8.1	10
	k_{cat}/K_M	6.6×10^4	3.9×10^3	2.3×10^4
Suc-Phe-Ala-Phe-NA	K_M	0.7	0.38	0.24
	k_{cat}	7.1	0.99	10
	k_{cat}/K_M	4.2×10^4	2.6×10^3	4.2×10^4
Suc-Phe-Met-Phe-NA	K_M	0.22	0.45	0.29
	k_{cat}	8.2	1.6	5.9
	k_{cat}/K_M	3.7×10^4	3.6×10^3	2.0×10^4
Suc-Phe-Gln-Phe-NA	K_M	0.83	0.22	0.21
	k_{cat}	25	1.3	3.9
	k_{cat}/K_M	3.0×10^4	6.0×10^3	1.9×10^4
Suc-Phe-Ser-Phe-NA	K_M	0.36	0.60	0.49
	k_{cat}	5.4	0.66	6.2
	k_{cat}/K_M	1.5×10^4	1.1×10^3	1.3×10^4
Suc-Phe-Glu-Phe-NA	K_M	0.072	1.1	1.4
	k_{cat}	0.90	0.92	8.4
	k_{cat}/K_M	1.3×10^4	8.4×10^2	6×10^3

[a] The units of K_M, k_{cat}, and k_{cat}/K_M are respectively mM, s^{-1}, and M^{-1} s^{-1}. [b] Conditions: pH 8.0, 50 mM phosphate and 10% Me_2SO at 25 °C. [c] Conditions: pH 6.6, 50 mM phosphate and 10% Me_2SO at 25 °C; data at pH 8.0 listed in parentheses. [d] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C.

Table III: Effect of the P_3 Residue on the Specificity and Reactivity of Mammalian Chymotrypsin-like Enzymes

substrate P_4 P_3 P_2 P_1	kinetic constant[a]	RMCP I[b]	RMCP II[c]	human skin chymase[d]	dog skin chymase[d]
Suc-Val-Pro-Phe-NA	K_M	0.12	0.73 (3.0)	0.093	0.14
	k_{cat}	57	26 (32)	75	54
	k_{cat}/K_M	4.8×10^5	3.6×10^4 (1.1×10^4)	8.1×10^5	3.9×10^5
Suc-Met-Pro-Phe-NA	K_M	0.11	1.3 (2.7)	0.44	
	k_{cat}	25	25 (18)	100	
	k_{cat}/K_M	2.3×10^5	1.9×10^4 (6.7×10^3)	2.3×10^5	
Suc-Phe-Pro-Phe-NA	K_M	0.12	1.1 (2.5)	0.29	
	k_{cat}	17	17 (13)	28	
	k_{cat}/K_M	1.4×10^5	1.5×10^4 (5.2×10^3)	9.7×10^4	
Suc-Thr-Pro-Phe-NA	K_M	0.26	1.1 (4.6)	0.25	0.28
	k_{cat}	38	18 (21)	120	65
	k_{cat}/K_M	1.5×10^5	1.6×10^4 (4.7×10^3)	4.8×10^5	2.3×10^5
Suc-Leu-Pro-Phe-NA	K_M	0.31	1.2 (5.2)	0.82	
	k_{cat}	37	25 (33)	77	
	k_{cat}/K_M	1.2×10^5	2.3×10^4 (6.3×10^3)	9.4×10^4	
Suc-Gln-Pro-Phe-NA	K_M	0.46	2.7	0.88	
	k_{cat}	56	19	86	
	k_{cat}/K_M	1.2×10^5	7.0×10^3	9.8×10^4	
Suc-Ser-Pro-Phe-NA	K_M	0.29	3.0	0.51	
	k_{cat}	20	13	38	
	k_{cat}/K_M	6.9×10^4	4.3×10^3	7.5×10^4	
Suc-Glu-Pro-Phe-NA	K_M	0.14	2.1	0.32	
	k_{cat}	9.2	6.5	61	
	k_{cat}/K_M	6.6×10^4	3.1×10^3	1.9×10^5	
Suc-Ala-Pro-Phe-NA	K_M	0.84	0.94	0.67	
	k_{cat}	38	4.1	51	
	k_{cat}/K_M	4.5×10^4	4.4×10^3	7.6×10^4	
Suc-Lys-Pro-Phe-NA	K_M	4.0	17	0.65	
	k_{cat}	32	36	14	
	k_{cat}/K_M	8.0×10^3	2.1×10^3	2.2×10^4	

[a] The units of K_M, k_{cat}, and k_{cat}/K_M are respectively mM, s^{-1}, and M^{-1} s^{-1}. [b] Conditions: pH 8.0, 50 mM phosphate and 10% Me_2SO at 25 °C. [c] Conditions: pH 6.6, 50 mM phosphate and 10% Me_2SO at 25 °C; data at pH 8.0 listed in parentheses. [d] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C.

Hydrolysis of Peptides Containing Model Desmosine Residues. The finding that HL elastase shows a preference for substrates containing desmosine-like amino acid residues (Yasutake & Powers, 1983) led us to investigate the hydrolysis

Table IV: Kinetic Constants for Hydrolysis of Nitroanilide Substrates by Cathepsin G (Cat G), Chymotrypsin A_α (ChyT), Rat Mast Cell Protease I, and Rat Mast Cell Protease II[a]

substrate	enzyme	[S] range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Suc-Val-Pro-Phe-NA	Cat G	0.17–1.1	1.4	9.6	6900
	ChyT	0.01–0.3	0.065	20	308000
	RMCP I	0.16–1.1	0.12	57	480000
	RMCP II	0.31–2.4	0.73	26	36000
Suc-D-Val-Pro-Phe-NA	Cat G	2.2			NR[c]
	ChyT	1.1			NR
	RMCP I	0.22–1.1	3.6	1.5	430
Suc-MeVal-Pro-Phe-NA	Cat G	0.33–2.2	0.69	0.19	270
	ChyT[b]	0.27–1.1			2600
	RMCP I	0.11–0.27	1.2	6.2	5100
	RMCP II	0.22–1.1	2.9	3.9	1300
Suc-D-MeVal-Pro-Phe-NA	Cat G	2.2			NR
	ChyT[b]	0.28–1.1			345
	RMCP I	0.28–1.1	2.0	2.2	1100
	RMCP II	0.28–1.1	3.9	1.8	450

[a] Conditions were pH 7.50, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C. [b] Lineweaver–Burk (double-reciprocal) plot extends through the origin and only the k_{cat}/K_M ratio was determined. [c] NR, no reaction.

Table V: Effect of the P_4 Residue on the Specificity and Reactivity of Mammalian Chymotrypsin-like Enzymes

substrate P_5 P_4 P_3 P_2 P_1	kinetic constant[a]	RMCP I[b]	RMCP II[c]	human skin chymase[d]	dog skin chymase[d]
Suc-Met-Val-Pro-Phe-NA	K_M	0.060	2.8	0.055	0.063
	k_{cat}	25	18	53	30
	k_{cat}/K_M	4.2×10^5	6.4×10^3	9.6×10^5	4.8×10^5
Suc-Ala-Val-Pro-Phe-NA	K_M	0.062	0.91		
	k_{cat}	17	5.1		
	k_{cat}/K_M	2.7×10^5	5.6×10^3		
Suc-Phe-Val-Pro-Phe-NA	K_M	0.17	0.67	0.056	0.069
	k_{cat}	24	1.9	56	33
	k_{cat}/K_M	1.4×10^5	2.8×10^3	1.0×10^6	4.8×10^5
Suc-Leu-Val-Pro-Phe-NA	K_M	0.15	4.6	0.13	
	k_{cat}	19	7.4	79	
	k_{cat}/K_M	1.3×10^5	1.6×10^3	6.1×10^5	
Suc-Glu-Val-Pro-Phe-NA	K_M	0.90	1.9	0.36	
	k_{cat}	53	13	68	
	k_{cat}/K_M	5.9×10^4	6.8×10^3	1.9×10^5	
Suc-Lys-Val-Pro-Phe-NA	K_M	1.5	6.3	0.078	
	k_{cat}	66	9.4	58	
	k_{cat}/K_M	4.4×10^4	1.5×10^3	7.4×10^5	

[a] The units of K_M, k_{cat}, and k_{cat}/K_M are respectively mM, s^{-1}, and M^{-1} s^{-1}. [b] Conditions: pH 8.0, 50 mM phosphate and 10% Me_2SO at 25 °C. [c] Conditions: pH 6.6, 50 mM phosphate and 10% Me_2SO at 25 °C. [d] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C.

Table VI: Kinetic Constants for Hydrolysis of Peptide 4-Nitroanilide Substrates Containing Model Desmosine Residues by Chymotrypsin-like Enzymes

substrate	enzyme	substrate concn range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M, Lys derivative (M^{-1} s^{-1})	k_{cat}/K_M ratio for Lys(Pic)/Lys
Suc-Phe-Lys(Pic)-Phe-NA	Cat G[a]	0.04–0.21			3400	260	13
	chymase[a,b]	0.04–0.22			37000		
	ChyT[a]	0.04–0.22	0.94	270	290000		
	RMCP I[c]	0.05–0.52	0.20	23	120000	90000	1.3
	RMCP II[c]	0.10–0.52			670	410[d]	1.6
Suc-Lys(Pic)-Pro-Phe-NA	Cat G[a]	0.28			NR[f]	230	small
	chymase[a,b]	0.07–0.19			14000	22000[e]	0.64
	ChyT[a]	0.03–0.14	0.23	13	57000		
	RMCP I[c]	0.04–0.21	2.7	4.7	1700	8000	0.21
	RMCP II[c]	0.04–0.21			49	2100	0.023
Suc-Lys(Pic)-Val-Pro-Phe-NA	Cat G[a]	0.40–0.79	0.63	5.1	8100	2800	2.9
	chymase[a,b]	0.21–1.04	1.0	480	480000	740000[e]	0.65
	ChyT[a]	0.06–0.35	0.35	4.5	13000		
	RMCP I[c]	0.26–1.28	2.3	86	37000	44000	0.84
	RMCP II[c]	0.04–0.19			580	1500[d]	0.39

[a] Conditions: 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO, pH 7.5 at 25 °C. [b] Human skin chymase. [c] Conditions: 50 mM phosphate and 10% Me_2SO, pH 8.0 at 25 °C. [d] Conditions: 50 mM phosphate and 10% Me_2SO, pH 6.6 at 25 °C. [e] Conditions: 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO, pH 8.0 at 25 °C. [f] NR, no reaction.

of three 4-nitroanilide substrates with P_1 Phe which contained an ϵ-picolinoyllysine residue at P_2, P_3, and P_4 by several chymotrypsin-like enzymes. Human skin chymase, RMCP I, and chymotrypsin A_α cleaved these substrates more effectively than cathepsin G and RMCP II (Table VI). The substrate with ϵ-picolinoyllysine in P_2 was 13 times more re-

Table VII: Kinetic Constants for Hydrolysis by Mammalian Chymotrypsin-like Enzymes of 4-Nitroanilide Substrates with Sequences of Physiologically Important Substrates and Inhibitors

substrate P_4 P_3 P_2 P_1	kinetic constant[a]	RMCP I[b]	RMCP II[c]	human skin chymase[d]	chymotrypsin A_α[b]
		Human Angiotensinogen			
-Ile-His-Pro-Phe-					
Suc-His-Pro-Phe-NA	K_M	0.17	0.76	0.093	
	k_{cat}	2.6	0.65	12	
	k_{cat}/K_M	1.5×10^4	8.6×10^2	1.3×10^5	
Suc-Ile-His-Pro-Phe-NA	K_M	0.28		0.53	
	k_{cat}	8.0		54	
	k_{cat}/K_M	2.9×10^4	5.3×10^e	1.0×10^5	
		Human α_1-Antichymotrypsin			
Ile-Thr-Leu-Leu-Ser					
Suc-Ile-Thr-Leu-Leu-Ser-NA	K_M	0.031			
	k_{cat}	0.032			
	k_{cat}/K_M	1.0×10^3	15		
Suc-Ile-Thr-Leu-Leu-Phe-NA	K_M	0.034	0.036		
	k_{cat}	20	1.1		
	k_{cat}/K_M	5.9×10^5	3.1×10^4		
Suc-Ile-Thr-Leu-Leu-NA	K_M	0.20	0.33[b]		0.47
	k_{cat}	2.5	0.0051		0.03
	k_{cat}/K_M	1.3×10^4	15	1.4[b,e]	64

[a] The units of K_M, k_{cat}, and k_{cat}/K_M are respectively mM, s^{-1}, and M^{-1} s^{-1}. [b] Conditions: pH 8.0, 4 mM phosphate and 10% Me_2SO at 25 °C. [c] Conditions: pH 6.6, 5 mM phosphate and 10% Me_2SO at 25 °C. [d] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C. [e] The Lineweaver–Burk plot went through the origin, and only k_{cat}/K_M could be determined.

active than the lysine derivative toward cathepsin G but was only 1.3 and 1.6 times more reactive than the lysine derivative toward RMCP I and II, respectively. In general, peptides with the ε-picolinoyllysine derivative in P_3 or P_4 were significantly poorer substrates than the corresponding lysine peptide. The only exception was cathepsin G with Suc-Lys(Pic)-Val-Pro-Phe-NA which was 3 times more reactive than the Lys peptide.

Hydrolysis of 4-Nitroanilide Substrates with Sequences of Physiologically Important Substrates and Inhibitors. The reaction of several chymotrypsin-like enzymes with synthetic substrates which contained a portion of the human angiotensin I sequence was studied. As shown in Table VII, tri-and tetrapeptide nitroanilides which contain the sequences His-Pro-Phe and Ile-His-Pro-Phe are cleaved quite effectively by RMCP I and human skin chymase while RMCP II is significantly less reactive. RMCP I and human skin chymase are 1.7–8 times less reactive toward Suc-His-Pro-Phe-NA and Suc-Ile-His-Pro-Phe-NA than toward Suc-Val-Pro-Phe-NA, the most reactive tripeptide NA studied here, while RMCP II is 42–697 times less reactive than with Suc-Val-Pro-Phe-NA.

The finding that HL elastase effectively hydrolyzes peptides with sequences related to the reactive site of α_1-protease inhibitor (Nakajima et al., 1978; McRae et al., 1980) led us to investigate the reaction of several chymotrypsin-like enzymes with sequences related to the reactive site of α_1-antichymotrypsin, the plasma protease inhibitor which is primarily responsible for controlling cathepsin G in vivo (Morii & Travis, 1983). As shown in Table VII, tetra- and pentapeptide 4-nitroanilides related to the reactive site of α_1-antichymotrypsin such as Suc-Ile-Thr-Leu-Leu-NA are cleaved to varying degrees by RMCP I, RMCP II, human skin chymase, and chymotrypsin A_α. The pentapeptide Suc-Ile-Thr-Leu-Leu-Ser-NA, which contains a sequence out of phase by one amino acid (Ser), is cleaved slowly by RMCP I (k_{cat}/K_M = 1.0×10^3 M^{-1} s^{-1}) and more slowly by RMCP II (k_{cat}/K_M = 15 M^{-1} s^{-1}). It is not known if the Leu–Ser bond of this substrate is cleaved by these enzymes in addition to the Ser–NA bond. As expected, substitution of Phe for the Ser residue results in a dramatic increase in k_{cat}/K_M values (590–2000 times). The tetrapeptide Suc-Ile-Thr-Leu-Leu-NA, with the correct α_1-antichymotrypsin sequence, was quite reactive toward RMCP I but was hydrolyzed slowly by RMCP II, human skin chymase, and chymotrypsin A_α.

Table VIII: Kinetic Constants for Hydrolysis of Suc-Val-Pro-Pro-Phe-SBzl by Chymotrypsin-like Enzymes

enzyme	substrate concn range (μM)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\times 10^{-6}$ M^{-1} s^{-1})
cathepsin G	90.3–14.1[a]	19	22	1.2
human skin chymase	65.2–15.1[a]	48	400	8.3
chymotrypsin A_α	90.3–5.0[a]	21	39	1.9
RMCP I	65.2–5.0[b]	25	130	5.2
RMCP II	90.3–5.0[b]	26	13	0.50

[a] Conditions: 0.1 M Hepes, 0.5 M NaCl, and 10% v/v Me_2SO, pH 7.5, 25 °C. [b] Conditions: 50 mM phosphate and 10% v/v Me_2SO, pH 8.0, 25 °C.

Hydrolysis of Suc-Val-Pro-Phe-SBzl. Since peptide thio ester substrates have been shown to be more sensitive than the corresponding 4-nitroanilide substrates (Castillo et al., 1979; Harper et al., 1981), we prepared the thiobenzyl ester derivatives of the most reactive tripeptide 4-nitroanilide, Suc-Val-Pro-Phe-NA. As shown in Table VIII, Suc-Val-Pro-Phe-SBzl is significantly more reactive than the corresponding 4-nitroanilide substrate with all the enzymes tested. With human skin chymase, RMCP I, and RMCP II, the thio ester is 6–14 times more reactive but is 171 times more reactive with cathepsin G.

Inhibition by Chymostatin and Substituted Saccharin Derivatives. *N*-Furoyl- and *N*-(2,4-dicyanophenyl)saccharin, which have been shown to be extremely potent inhibitors of HL elastase and chymotrypsin (Zimmerman et al., 1980; Ashe et al., 1981), were investigated as inhibitors of RMCP I and II and of the human skin chymase. In addition, inhibition by the fermentation product chymostatin was studied. As shown in Table IX, these enzymes were effectively inhibited by chymostatin and the substituted saccharin derivatives. Chymostatin was the most potent reversible inhibitor tested and gave K_I values from 0.02 to 0.6 μM. *N*-Furoylsaccharin was 110–120 times less effective with cathepsin G and RMCP II than with RMCP I and human skin chymase while the 2,4-

Table IX: Inhibition of Mammalian Chymotrypsin-like Enzymes by Chymostatin and Saccharin Derivatives

enzyme	K_I[a] (μM) chymostatin	*N*-furoyl-saccharin	*N*-(2,4-dicyanophenyl)-saccharin
cathepsin G[b]	0.28	120	6.2
RMCP I[c]	0.026	110	0.58
RMCP II[d]	0.56	1.1	0.79
human skin chymase[e]	0.017	1.0	0.64
chymotrypsin A_α	0.25[f]	0.07[g]	1.74

[a] K_I values were determined by using Dixon plots with Suc-Phe-Pro-Phe-NA as a substrate. [b] Conditions: pH 7.5, 0.1 M Hepes, 0.5 M NaCl, and 10% Me_2SO at 25 °C. [c] Conditions: pH 8.0, 50 mM phosphate and 10% Me_2SO at 25 °C. [d] Conditions: pH 6.6, 50 mM phoophate and 10% Me_2SO at 25 °C. [e] Conditions: pH 8.0, 50 mM phosphate, 1.5 M KCl, and 10% Me_2SO at 25 °C. [f] IC_{50}; data of Umezawa & Aoyagi (1977). [g] IC_{50}; data of Zimmerman et al. (1980).

dicyanophenyl derivative showed little or no selectivity (K_I values from 0.6 to 6.2 μM).

Inhibition by Peptide Chloromethyl Ketone and Sulfonyl Fluoride Inhibitors. The reaction of several chymotrypsin-like enzymes, including cathepsin G and human skin chymase, with peptide chloromethyl ketones and substituted benzenesulfonyl fluorides was studied (Table X). In general, the most effective chloromethyl ketone inhibitors for each enzyme were tripeptides with Phe in P_1, a hydrophobic P_2 residue, and a Boc group in P_4. MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, a potent elastase inhibitor, did not inhibit RMCP II, human skin chymase, or dog skin chymase at concentrations as high as 2.0 mM.

The most reactive substituted benzenesulfonyl fluoride for RMCP I, RMCP II, human skin chymase, and chymotrypsin A_α was 2-(Z-GlyNH)$C_6H_4SO_2F$ while cathepsin G was inhibited most rapidly by 2-($C_6H_5CH_2NHCONH$)$C_6H_4SO_2F$. The general serine protease inhibitor 4-$CH_3C_6H_4SO_2F$ did not inhibit human skin chymase or RMCP II (Yoshida et al., 1980). Other sulfonyl fluoride derivatives tested were usually 1–2 orders of magnitude less reactive than the most reactive inhibitors studied.

Discussion

RMCP I, RMCP II, human skin chymase, and dog skin chymase are chymotrypsin-like serine proteases whose primary physiological functions are still not well understood. The rat mast cell proteases have been implicated in a number of processes including mast cell degranulation (Woodbury & Neurath, 1980) and increasing the epithelial permeability during immune-mediated expulsion of intestinal parasites (Miller et al., 1983). The human skin chymase, when incubated with isolated skin sections, has been shown to cause epidermal–dermal separation along the zone of the dermal–epidermal junction, suggesting a pathological role for it in blister formation (Briggaman et al., 1984; Fraki et al., 1983). Since human plasma contains a major inhibitor for chymotrypsin-like enzymes (α_1-antichymotrypsin), it is likely that additional functions and disease states associated with these enzymes remain to be described.

The substrate specificities of RMCP I and II have been investigated previously with a limited number of peptide 4-nitroanilide substrates and peptide chloromethyl ketone inhibitors (Yoshida et al., 1980), and in addition, a number of peptide thiobenzyl ester substrates have been reported for both the rat mast cell enzymes and the skin chymases (Harper et al., 1981, 1984). The studies indicate that the enzymes are quite different from one another in many respects including their extended substrate binding regions and susceptibility to inhibitors. In order to gain more insight into the possible physiological roles of these enzymes, we investigated systematically their primary and extended substrate binding regions using peptide 4-nitroanilide substrates and using inhibitors including peptide chloromethyl ketones, substituted sulfonyl fluorides, and substituted saccharin derivatives.

Substrate Reactivity and Specificity. RMCP I and human skin chymase are generally as much as 10 times more reactive toward the peptide 4-nitroanilide substrates than RMCP II. The low reactivity of RMCP II toward these substrates is most often due to relatively high K_M values. While the dog skin chymase is also highly reactive toward many of the substrates investigated, it is as much as 2-fold less reactive than the human skin enzyme.

RMCP I and II preferred Phe in P_1 and showed similar trends in reactivity toward substrates with varying P_2 residues. The k_{cat}/K_M values were less sensitive to amino acid substitution in P_2 than in P_1. One exception to the trends was Suc-Phe-Lys-Phe-NA, which was the least reactive tripeptide toward RMCP II but was quite reactive toward RMCP I. Previous studies with tetrapeptide 4-nitroanilide substrates (Yoshida et al., 1980) indicate that RMCP II prefers Thr in P_2 over Pro or Leu. With the tripeptides studied here, however, P_2 Leu and Pro are more effective than the Thr substrate. The

Table X: Inhibition of Mammalian Chymotrypsin-like Enzymes by Peptide Chloromethyl Ketones and Sulfonyl Fluorides

compound	$k_{obsd}/[I]$ (M^{-1} s^{-1}) RMCP I	RMCP II	human skin chymase[a]	dog skin chymase[a]	cathepsin G[a]	chymotrypsin A_α[a]
Z-Leu-Phe-CH_2Cl		1.6[b]	5.2	4.4	4.7	
Ac-Ala-Gly-Phe-CH_2Cl		0.9[b]	1.6	4.1		
Boc-Gly-Leu-Phe-CH_2Cl	8.9[c]	1.6[b]	173	43	15	61
Suc-Pro-Leu-Phe-CH_2Cl	37[b]	0.4[b]	8.8	2.8	0.7[b]	3.6[b]
MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl		NI[b,f]	NI	NI		
$C_6H_5CH_2SO_5F$			19	21		
4-$CH_3C_6H_4SO_2F$		NI[b]	NI	1.3	1.5[d]	8.6[d]
3-$CF_3C_6H_4SO_2F$			0.57	0.89	5.9[e]	67[e]
3-$C_6H_5CONHC_6H_4SO_2F$			0.55	2.3	3.1[e]	58[e]
2-($C_6H_5CONHCH_2CONH$)$C_6H_4SO_2F$	77[c]	4.7[c]			33	176
2-($C_6H_5CH_2NHCONH$)$C_6H_4SO_2F$	147[c]	3.4[c]			37	81
2-(Z-$NHCH_2CONH$)$C_6H_4SO_2F$	2500[c]	270[c]	1800		19[e]	3300[e]
2-[Z-$NH(CH)_2CONH$]$C_6H_4SO_2F$	14[c]	1.5[c]	97		7.7[e]	82[e]
2-[Z-$NH(CH_2)_5CONH$]$C_6H_4SO_2F$	41[c]	36[c]	600		3.6[e]	47[e]

[a] Conditions: 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 10% Me_2SO, 28 °C. [b] Data from Yoshida et al. (1980). Conditions: 50 mM phosphate buffer, pH 8.0, and 10% Me_2SO, 30 °C. [c] Conditions: 50 mM phosphate buffer, pH 8.0, and 10% Me_2SO, 30 °C. [d] Data from Lively & Powers (1978). Conditions: 0.1 M Hepes, 1.0 M NaCl, and 9% methanol, pH 7.5, 30 °C. [e] Yoshimura et al. (1982). Conditions: 0.1 M Hepes, 0.5 M NaCl, pH 7.5, and 10% Me_2SO, 30 °C. [f] NI, no inhibition.

effects are both in k_{cat} and in K_M. Unlike RCMP II (and human skin chymase), RMCP I can readily accommodate charged residues such as Lys or Glu in P_2. Indeed, the P_2 Lys substrate was the fourth most reactive substrate in this series with RMCP I. RMCP I and II show similar trends in reactivity toward the P_3-substituted substrates. As the the P_2 series, the k_{cat}/K_M values were fairly insensitive to changes in the amino acid residue. In general, hydrophobic or bulky amino acid residues gave higher k_{cat}/K_M values. These results are consistent with earlier studies (Yoshida et al., 1980) which indicate that a hydrophobic S_3 binding site is present in RMCP II. The best P_4 residue was found to be Met. Substitution of bulky and charged amino acids in P_4 resulted in only small changes in reactivity toward RMCP I and II. Indeed, Suc-Glu-Val-Pro-Phe-NA was the most reactive tetrapeptide 4-nitroanilide toward RMCP II, while the least reactive was the P_4 Lys peptide. This is consistent with a specific S_4–P_4 interaction between the carboxylate side chain of P_4 and a cationic group (probably His-200) near the S_4 binding site (Yoshida et al., 1980). The observation that increasing substrate length from a tripeptide to a tetrapeptide results in negligible changes in the magnitude of k_{cat}/K_M values indicates that substrate hydrolysis is affected only slightly by interactions at the S_4 subsite.

The human and dog skin chymases and rat mast cell proteases show similar primary subsite specificities, but the skin chymases were less discriminating in their secondary subsite specificities. While both the rat mast cell enzymes and the human chymase preferred bulky residues or Pro in P_2, the human skin chymase was less sensitive to substitution of polar residues in P_2. For instance, substitution of Ser for Leu in Suc-Phe-Leu-Phe-NA results in a 20-fold decrease in k_{cat}/K_M with RMCP I but only a 6-fold decrease with the human skin enzyme. As with the S_2 subsite of the human skin chymase, the S_3 subsite shows less specificity than with RMCP I and II. For instance, the substrate Suc-Glu-Pro-Phe-NA was the fourth best substrate for the human skin chymase but was one of the poorest for RMCP I and II. As with the rat mast cell proteases, increasing the substrate length from a tripeptide to a tetrapeptide results in small increases in k_{cat}/K_M, again indicating that P_4–S_4 interactions are of minor importance.

Previous reports indicate that enhanced selectivity or reactivity of substrates toward proteases can be obtained by the proper placement of D-amino acid residues in peptide substrates. For instance, Boc-D-Phe-Ala-Nle-NA was shown to have an extremely low K_M value (4.2 μM) with human leukocyte elastase (Morossy et al., 1980), and Suc-D-Phe-Pro-Ala-NA was shown to bind very tightly to porcine pancreatic elastase (Szabo et al., 1980). More recently, peptide nitroanilide substrates (Lottenberg et al., 1981) and peptide chloromethyl ketones (Kettner & Shaw, 1981) with P_3 D-amino acids, particularly D-Phe, and Arg in P_1 have been shown to be relatively specific for thrombin. Therefore, in an attempt to increase specificity and reactivity, we investigated the hydrolysis of Suc-AA-Pro-Phe-NA with several unusual amino acids including D-Val and *N*-methyl-D-Val in P_3.

While these substrates were generally unreactive toward the enzymes investigated, considerable specificity was achieved toward RMCP I. The substrate Suc-D-Val-Pro-Phe-NA was hydrolyzed at a moderate rate by RMCP I (k_{cat}/K_M = 430 M^{-1} s^{-1}) but was not hydrolyzed by chymotrypsin or cathepsin G. Interestingly, Suc-D-MeVal-Pro-Phe-NA was cleaved by chymotrypsin, RMCP I, and RMCP II but was not a substrate for cathepsin G. The lower k_{cat}/K_M values for these substrates, as compared to Suc-Val-Pro-Phe-NA, were due to both increases in K_M and decreases in k_{cat}. The *N*-methyl group in Suc-MeVal-Pro-Phe-NA and Suc-D-MeVal-Pro-Phe-NA possibly disrupts hydrogen bonding between the enzyme backbone and the P_4–P_3 amide bond of the substrate which results in decreased binding and reactivity. These results indicate that the accommodation of a D-amino acid in P_3 of a substrate is not a general feature of all serine proteases and therefore could be utilized in the design of more specific serine protease substrates and inhibitors, particularly for RMCP I.

Several peptide thiobenzyl ester substrates have been reported for chymotrypsin-like enzymes. The thio ester substrate investigated here, Suc-Val-Pro-Phe-SBzl, is 1.2 times more reactive toward cathepsin G than the most reactive thio ester substrate yet reported, Boc-Ala-Ala-Phe-SBzl, but is 4–9 times less reactive than this substrate with chymotrypsin A, RMCP I, and RMCP II (Harper et al., 1984). The best thio ester substrate for human skin chymase, Suc-Ala-Ala-Pro-Phe-SBzl, is 1.7 times more reactive than Suc-Val-Pro-Phe-SBzl (Harper et al., 1981).

It is generally true that for the serine protease catalyzed hydrolysis of esters and thio esters, deacylation is rate limiting, while with amides, acylation is rate limiting. However, evidence has recently been presented which indicates that some of the best 4-nitroanilide substrates of human leukocyte elastase, such as MeOSuc-Ala-Ala-Pro-Val-NA, are hydrolyzed with rate-limiting deacylation (Stein et al., 1984). It is not clear at present whether this is generally true with other mammalian serine proteases. Comparison of the k_{cat} values for Suc-Val-Pro-Phe-NA (Tables I and IV) and the thiobenzyl ester derivative Suc-Val-Pro-Phe-SBzl shows that with cathepsin G, chymotrypsin A_α, RMCP I, and RMCP II, these values are within a factor of 2.2 of each other. This may indicate that with this nitroanilide substrate and these enzymes, deacylation may be at least partially rate limiting. This is not the case with human skin chymase which has a 5 times higher k_{cat} value with the thiobenzyl ester derivative. The rate-limiting step of nitroanilide hydrolysis by these chymotrypsin-like enzymes can only be determined by careful investigation of a series of peptide nitroanilides, thiobenzyl ester derivatives, and ester substrates under extremely controlled conditions. However, our limited results indicate that future investigation of the hydrolysis mechanism of mammalian serine proteases would be warranted.

Model Substrates. Cathepsin G is thought to act synergistically with human leukocyte elastase in the degradation of mature cross-linked elastin which occurs in chronic emphysema (Boudier et al., 1981). The presence of the pyridinium ring containing the amino acid desmosine is a unique feature of mature elastin. We recently reported that human leukocyte elastase and bovine pancreatic elastase show a preference for tetrapeptide 4-nitroanilides substrates which contain Val in P_1 and model desmosine residues, such as ϵ-benzimidoyl- and ϵ-picolinoyl-substituted lysine, in P_3 and P_4 (Yasutake & Powers, 1983). It was also shown that cathepsin G cleaved these substrates slowly or not at all.

Since chymotrypsin-like enzymes such as cathepsin G would not be expected to rapidly hydrolyze substrates with P_1 Val, we prepared and tested the corresponding P_1 Phe substrates containing ϵ-picolinoyllysine (Lys-Pic) in P_2, P_3, and P_4. RMCP I, human skin chymase, and chymotrypsin were found to be significantly more reactive with these substrates than RMCP II and cathepsin G. While RMCP I and II, human skin chymase, and chymotrypsin were generally less reactive with the Lys(Pic) substrates than with the Lys substrate, cathepsin G was 13- and 2.9-fold more reactive with the P_2

and P_4 Lys(Pic) substrates, respectively. The P_2 Lys(Pic) substrate, however, was not cleaved by cathepsin G. Therefore, portions of cross-linked elastin which contain structural features similar to the model desmosine substrates reported here may be cleaved more effectively by cathepsin G than uncross-linked regions. It was noted previously that the rat mast cell proteases prefer bulky hydrophobic amino acid residues in P_3 and P_4. However, they did not react well with the model desmosine substrates, probably because the hydrophobic picolinoyl moiety occupies a completely different spacial orientation and requires a larger binding subsite than amino acid residues such as phenylalanine.

The possible physiological roles of cathepsin G and human skin chymase have recently been expanded to include hydrolysis of angiotensinogen and angiotensin I to form the potent vasoconstrictor angiotensin II (Reilly et al., 1982; Klickstein et al., 1982; Wintroub et al., 1984). Therefore, we examined peptide 4-nitroanilide substrates which contained a portion of the angiotensin I sequence. While Suc-His-Pro-Phe-NA and Suc-Ile-His-Pro-Phe-NA are somewhat less reactive than Suc-Val-Pro-Phe-NA toward human skin chymase and the rat mast cell proteases, they are still readily hydrolyzed by RMCP I and human skin chymase. RMCP II is, however, quite unreactive toward these substrates. These results are consistent with the recent findings that chymotrypsin-like enzymes of neutrophil or mast cell origin can effectively convert angiotensinogen or angiotensinogen I to angiotensin II (Reilly et al., 1982; Klickstein et al., 1982; Wintroub et al., 1984). Human skin chymase, which is a more potent converting enzyme than angiotensin converting enzyme itself (Reilly et al., 1982), cleaves these model peptides with k_{cat}/K_M values only 1.2–1.4 times lower than the k_{cat}/K_M value of angiotensin converting enzyme with angiotensin I ($k_{cat}/K_M = 1.5 \times 10^5$ M^{-1} s^{-1}) (Bunning et al., 1983). The fact that some hypertensive individuals are not affected by the angiotensin converting enzyme inhibitor captopril (Antonaccio & Cushman, 1981) indicates there are multiple mechanisms which lead to hypertension. Some of these may involve angiotensin II formation by these and other chymotrypsin-like enzymes.

Previously, we reported that substrates related to the reactive site sequence of α_1-protease inhibitor are hydrolyzed effectively by human leukocyte elastase and cathepsin G (Nakajima et al., 1978; McRae et al., 1980). In many cases, these model substrates had low K_M and k_{cat} values, which would be preferred for a good inhibitor sequence. This led us to investigate peptide 4-nitroanilide substrates which contain a portion of the reactive site of α_1-antichymotrypsin, the principal inhibitor of cathepsin G and other chymotrypsin-like serine proteases released into plasma. Inhibition of serine proteases by α_1-antichymotrypsin is due to interactions of the enzyme with the Leu–Ser bond at the reactive site (Ile-Thr-Leu-Leu-Ser) (Morii & Travis, 1983). The model reactive site 4-nitroanilide substrates are generally much less reactive than the most reactive tripeptide 4-nitroanilides such as Suc-Val-Pro-Phe-NA. The finding that the P_1 Ser substrate, Suc-Ile-Thr-Leu-Leu-Ser-NA, is hydrolyzed by both RMCP I and RMCP II is consistent with previous studies which showed that these enzymes cleave Boc-Ala-Ala-Ser-Bzl with k_{cat}/K_M values of 210000 and 30000 M^{-1} s^{-1}, respectively (Harper et al., 1984). Interestingly, porcine pancreatic elastase, which is not inhibited by α_1-antichymotrypsin, cleaves at the reactive site to release a carboxy-terminal Ser residue, indicating cleavage after the Leu-Ser sequence (Morii & Travis, 1983). Although the mechanism of inhibition of plasma proteases by plasma protease inhibitors is not known, the most preferable protease inhibitor reactive site sequences would be expected to bind tightly and to be hydrolyzed slowly. The model reactive site substrates studied here are turned over slowly by the rat mast cell proteases and also have fairly low K_M values (less than 0.47 mM). A conformationally restricted structure, such as that in native α_1-antichymotrypsin, would bind much more tightly and be turned over much more slowly than the model sequences investigated here.

Inhibitors. We have reported the inactivation of RMCP I, RMCP II, and cathepsin G by several peptide chloromethyl ketones (Yoshida et al., 1980), and we have developed a highly reactive series of substituted benzenesulfonyl fluoride inhibitors for serine proteases (Yoshimura et al., 1982). In the present work, we have expanded both types of inhibitors to enzymes not previously studied. The inhibition of several chymotrypsin-like enzymes by the peptide aldehyde chymostatin (Umezawa & Aoyagi, 1977) and the acylating agents *N*-furoylsaccharin and *N*-(2,4-dicyanophenyl)saccharin (Zimmerman et al., 1980; Ashe et al., 1981) was also investigated.

The peptide chloromethyl ketone inhibitors were generally more reactive with the human and dog skin chymases than with other enzymes studied. The selective RMCP I inhibitor Suc-Pro-Leu-Phe-CH_2Cl (Yoshida et al., 1980) is 4–13 times less reactive with human and dog skin chymases than with RMCP I.

The most reactive substituted benzenesulfonyl fluorides were from 10- to 179-fold more reactive than the most reactive chloromethyl ketones. In fact, the more reactive sulfonyl fluorides are some of the most potent irreversible inhibitors for many of the enzymes tested. Inhibitors which contained hydrophobic substituents in the ortho position were much more potent than the meta derivatives, and the distance of the aromatic ring in the substituent to the sulfonyl fluoride moiety also affected the inhibitory reactivity significantly. In all cases except cathepsin G, the most effective inhibitor was 2-(Z-$NHCH_2CONH)C_6H_4SO_2F$, in which the aromatic ring of the substituent is seven atoms away from the benzenesulfonyl fluoride moiety. Increasing or decreasing this distance resulted in a significant decrease in $k_{obsd}/[I]$ values. On the other hand, cathepsin G is inhibited most effectively by compounds which contain relatively short spacers such as 2-(C_6H_5CONH-$CH_2CONH)C_6H_4SO_2F$ and 2-($C_6H_5CH_2NHCONH$)-$C_6H_4SO_2F$. These results indicate the existence of a hydrophobic binding site in the chymases different from that in cathepsin G. This structural feature, which is interacting with the hydrophobic side chain of the substituted sulfonyl fluoride, could possibly be used to design more reactive or selective inhibitors.

In most cases, K_I values for inhibition of RMCP I, RMCP II, human skin chymase, and chymotrypsin A by chymostatin and the substituted saccharin derivatives were 1 μM or lower. Cathepsin G gave the highest K_I values with the saccharin derivatives which were generally an order of magnitude higher than with the other enzymes tested. Both RMCP I and human skin chymase were inhibited most effectively by chymostatin and had K_I values of 26 and 17 nM, respectively.

Summary. The most reactive protease investigated here was generally human skin chymase, except when P_3 was Phe, in which case RMCP I was significantly more reactive than the skin chymase. RMCP I and the dog skin chymase were intermediate in reactivity toward the tri- and tetrapeptide nitroanilide substrates. RMCP II was the least reactive enzyme studied here but was still found to be significantly more reactive than human leukocyte cathepsin G toward these substrates (Tanaka et al., 1985). RMCP I was more tolerant of

D-amino acids in P_3 than the other enzymes tested and was also more effective at cleaving substrates containing model desmosine residues, except in the case of Suc-Lys(Pic)-Val-Pro-Phe-NA, where human skin enzyme was significantly more reactive. The enzymes studied here cleave substrates which contain portions of the sequence of angiotensin I and the reactive site of α_1-antichymotrypsin. The human skin chymase is the most reactive enzyme toward angiotensin I like substrates while RMCP I cleaves substrates containing the reactive site of α_1-antichymotrypsin most effectively. The thiobenzyl ester derivative of the most reactive tripeptide 4-nitroanilide investigated here is an excellent substrate for all of the enzymes tested and is 6–171 times more reactive than the nitroanilide derivative. The human skin enzyme is most easily inhibited by chymostatin and N-substituted saccharin derivatives while RMCP I is the most susceptible to inactivation by substituted benzenesulfonyl fluoride derivatives. The search for a highly reactive inhibitor for RMCP II and cathepsin G was unsuccessful. The low reactivity of RMCP II and cathepsin G toward substrates and inhibitors, compared to other serine proteases, indicates that the catalytic apparatus of these enzymes may not be optimally functional. Alternatively, these proteases may require extremely specific substrate sequences or conformations for proper alignment of the catalytic residues and maximal catalytic activity.

Although the proteases studied here have the same general substrate specificity, their varied reactivities toward the various substrates and inhibitors investigated here demonstrate that the extended binding sites and active sites of all are unique. The distinctiveness in reactive site structure may result from differences in biological function, as would be expected for the case of proteases isolated from mast cells, the pancreas, and leukocytes, respectively. Aside from chymotrypsin, the biological roles and physiological substrates of these proteases are not known. The substrates and inhibitors described here not only provide a useful tool kit to compare the active sites of chymotrypsin-like enzymes but also provide a means to elucidate potential biological substrates and inhibitors.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Travis and his research group at the University of Georgia for supplying the human leukocyte cathepsin G used in this study and to Dr. M. Zimmerman of Merck Sharp & Dohme Research Laboratories, Rahway, NJ, for the saccharin derivatives used in this study.

Registry No. Suc-Val-Pro-Phe-NA, 95192-11-3; Suc-Val-Pro-Tyr-NA, 95192-12-4; Suc-Val-Pro-Trp-NA, 95192-13-5; Suc-Val-Pro-Leu-NA, 95192-14-6; Suc-Val-Pro-Met-NA, 95192-15-7; Suc-Phe-Leu-Phe-NA, 75651-69-3; Suc-Phe-Pro-Phe-NA, 75651-68-2; Suc-Phe-Val-Phe-NA, 95192-18-0; Suc-Phe-Lys-Phe-NA, 95192-19-1; Suc-Phe-Thr-Phe-NA, 95192-20-4; Suc-Phe-Ala-Phe-NA, 95192-21-5; Suc-Phe-Met-Phe-NA, 95192-22-6; Suc-Phe-Gln-Phe-NA, 95192-23-7; Suc-Phe-Ser-Phe-NA, 95192-24-8; Suc-Phe-Glu-Phe-NA, 95192-25-9; Suc-Met-Pro-Phe-NA, 95192-26-0; Suc-Thr-Pro-Phe-NA, 95192-27-1; Suc-Leu-Pro-Phe-NA, 95192-28-2; Suc-Gln-Pro-Phe-NA, 95192-29-3; Suc-Ser-Pro-Phe-NA, 95192-30-6; Suc-Glu-Pro-Phe-NA, 95192-31-7; Suc-Ala-Pro-Phe-NA, 83329-41-3; Suc-Lys-Pro-Phe-NA, 95192-33-9; Suc-D-Val-Pro-Phe-NA, 95192-34-0; Suc-MeVal-Pro-Phe-NA, 95192-35-1; Suc-D-MeVal-Pro-Phe-NA, 95192-36-2; Suc-Met-Val-Pro-Phe-NA, 95192-37-3; Suc-Ala-Val-Pro-Phe-NA, 95192-38-4; Suc-Phe-Val-Pro-Phe-NA, 95192-39-5; Suc-Leu-Val-Pro-Phe-NA, 95192-40-8; Suc-Glu-Val-Pro-Phe-NA, 95192-41-9; Suc-Lys-Val-Pro-Phe-NA, 95192-42-0; Suc-Phe-Lys(Pic)-Phe-NA, 95192-43-1; Suc-Lys(Pic)-Pro-Phe-NA, 95192-44-2; Suc-Lys-(Pic)-Val-Pro-Phe-NA, 95192-45-3; Suc-His-Pro-Phe-NA, 95192-46-4; Suc-Ile-His-Pro-Phe-NA, 95192-47-5; Suc-Ile-Thr-Leu-Leu-Ser-NA, 95192-48-6; Suc-Ile-Thr-Leu-Leu-Phe-NA, 95192-49-7; Suc-Ile-Thr-Leu-Leu-NA, 95192-50-0; Suc-Val-Pro-Phe-SBzl, 95192-51-1; Z-Leu-Phe-CH_2Cl, 52386-46-6; Ac-Ala-Gly-Phe-CH_2Cl, 35172-52-2; BOC-Gly-Leu-Phe-CH_2Cl, 41658-43-9; Suc-Pro-Leu-Phe-CH_2Cl, 75651-76-2; MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, 65144-34-5; $C_6H_5CH_2SO_2F$, 329-98-6; 4-$CH_3C_6H_4SO_2F$, 455-16-3; 3-$CF_3C_6H_4SO_2F$, 32466-24-3; 3-$C_6H_5CONHC_6H_4SO_2F$, 82422-63-7; 2-$(C_6H_5CONHCH_2CONH)C_6H_4SO_2F$, 95192-52-2; 2-$(C_6H_5CH_2NHCONH)C_6H_4SO_2F$, 95192-53-3; 2-(Z-$NHCH_2CONH)C_6H_4SO_2F$, 82422-64-8; 2-[Z-NH-$(CH_2)_2CONH]C_6H_4SO_2F$, 82422-66-0; 2-[Z-NH-$(CH_2)_5CONH]C_6H_4SO_2F$, 82422-70-6; chymostatin, 9076-44-2; *N*-furoylsaccharin, 63633-79-4; *N*-(2,4-dicyanophenyl)saccharin, 78492-52-1; benzoylglycine, 495-69-2; pentachlorophosphorane, 10026-13-8; 2-aminobenzenesulfonyl fluoride, 392-86-9; 2-(fluorosulfonyl)phenyl isocyanate, 59651-55-7; benzylamine, 100-46-9; proteinase, 9001-92-7; rat mast cell proteinase, 75496-62-7; chymotrypsin, 9004-07-3; cathepsin G, 56645-49-9.

REFERENCES

Antonaccio, M., & Cushman, D. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 2275–2284.

Ashe, B. M., Clark, R. L., Jones, H., & Zimmerman, M. (1981) *J. Biol. Chem. 256*, 11603–11606.

Barrett, A. J., & McDonald, J. K. (1980) in *Mammalian Proteases*, pp 197–204, Academic Press, New York.

Boudier, C., Holle, C., & Bieth, J. G. (1981) *J. Biol. Chem. 256*, 10256–10258.

Briggaman, R. A., Schechter, N. M., Fraki, J. E., & Lazarus, G. S. (1984) *J. Exp. Med. 160*, 1027–1042.

Bunning, P., Holmquist, B., & Riordan, J. F. (1983) *Biochemistry 22*, 103–110.

Castillo, M. J., Nakajima, K., Zimmerman, M., & Powers, J. C. (1979) *Anal. Biochem. 99*, 53–64.

Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961) *Arch. Biochem. Biophys. 95*, 271–278.

Everitt, M. T., & Neurath, H. (1979) *Biochimie 61*, 653–662.

Fraki, J. E., Briggman, R. A., & Lazarus, G. S. (1982) *Science (Washington, D.C.) 215*, 685–686.

Fraki, J. E., Schechter, N. M., & Lazarus, G. S. (1983) *Br. J. Dermatol. 109 (Suppl. No. 25)*, 72–76.

Gupton, B. F., Carroll, D. L., Tuhy, P. M., Kam, C. M., & Powers, J. C. (1984) *J. Biol. Chem. 259*, 4279–4287.

Harper, J. W., Ramirez, G., & Powers, J. C. (1981) *Anal. Biochem. 118*, 382–387.

Harper, J. W., Cook, R. R., Roberts, C. J., McLaughlin, B. H., & Powers, J. C. (1984) *Biochemistry 23*, 2995–3002.

Johnson, D. A., & Travis, J. (1978) *J. Biol. Chem. 253*, 7142–7144.

Kettner, C., & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.

Klickstein, L. B., Kaempfer, C. E., & Wintroub, B. U. (1982) *J. Biol. Chem. 257*, 15042–15046.

Kobayashi, K., & Katunuma, N. (1978) *J. Biochem. (Tokyo) 84*, 65–74.

Kurachi, K., Powers, J. C., & Wilcox, P. E. (1973) *Biochemistry 12*, 771–777.

Lively, M. O., & Powers, J. C. (1978) *Biochim. Biophys. Acta 525*, 171–179.

Lottenberg, R., Christensen, U., Jackson, C. M., & Coleman, P. L. (1981) *Methods Enzymol. 80*, 341–361.

Marossy, K., Szabo, G. C., Pozsgay, M., & Elodi, P. (1980) *Biochem. Biophys. Res. Commun. 96*, 762–769.

McRae, B., Nakajima, K., Travis, J., & Powers, J. C. (1980) *Biochemistry 19*, 3973–3978.

Miller, H. R. P., Woodbury, R. G., Huntley, J. F., & Newlands, G. (1983) *Immunology 49*, 471–480.

Morii, H., & Travis, J. (1983) *J. Biol. Chem. 258*, 12749–12752.

Nakajima, K., Powers, J. C., Ashe, B. M., & Zimmerman, M. (1979) *J. Biol. Chem. 254*, 4027–4032.

Powers, J. C., Gupton, B. F., Harley, A. D., Nishino, N., & Whitley, R. F. (1977) *Biochim. Biophys. Acta 485*, 156-166.
Reilly, C. F., Tewksbury, D. A., Schechter, N. M., & Travis, J. (1982) *J. Biol. Chem. 257*, 8619-8622.
Schechter, I., & Berger, A. C. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Schechter, N. M., Fraki, J. E., Geesin, J. C., & Lazarus, G. S. (1983) *J. Biol. Chem. 258*, 2973-2978.
Stein, R. L., Viscarello, B. R., & Wildonger, R. A. (1984) *J. Am. Chem. Soc. 106*, 796-798.
Szabo, G. C., Pozsgay, M., Gaspar, R., & Elodi, P. (1980) *Acta Biochim. Biophys. Acad. Sci. Hung. 15*, 263-273.
Tanaka, T., Minematsu, Y., Reilly, C. F., Travis, J., & Powers, J. C. (1985) *Biochemistry* (preceding paper in this issue).
Travis, J., Bowen, J., & Baugh, R. (1978) *Biochemistry 17*, 5651-5656.
Umezawa, H., & Aoyagi, T. (1977) in *Proteases in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 637-662, North-Holland Publishing Co., New York.
Wintroub, B. U., Klickstein, L. B., Dzau, V. J., & Watt, K. W. K. (1984) *Biochemistry 23*, 227-232.
Woodbury, R. G., & Neurath, H. (1978) *Biochemistry 17*, 4298-4304.
Woodbury, R. G., & Neurath, H. (1980) *FEBS Lett. 114*, 189-196.
Woodbury, R. G., Katunuma, N., Kobayashi, K., Tatani, K., & Neurath, H. (1978a) *Biochemistry 17*, 811-819.
Woodbury, R. G., Everitt, M. T., Sanada, Y., Katunuma, N., Lagunoff, D., & Neurath, H. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5311-5313.
Woodbury, R. G., Everitt, M. T., & Neurath, H. (1981) *Methods Enzymol. 80*, 588-609.
Yasutake, A., & Powers, J. C. (1981) *Biochemistry 20*, 3675-3679.
Yoshida, N., Everitt, M. T., Neurath, H., Woodbury, R. G., & Powers, J. C. (1980) *Biochemistry 19*, 5799-5804.
Yoshimura, T., Barker, L. N., & Powers, J. C. (1982) *J. Biol. Chem. 257*, 5077-5084.
Zimmerman, M., Morman, H., Mulvey, D., Jones, H., Frankshun, R., & Ashe, B. M. (1980) *J. Biol. Chem. 255*, 9848-9851.

Nuclear Magnetic Resonance and Neutron Diffraction Studies of the Complex of Ribonuclease A with Uridine Vanadate, a Transition-State Analogue

Babul Borah,[‡] Chi-wan Chen,[‡] William Egan,[§] Maria Miller,[∥] Alexander Wlodawer,[∥] and Jack S. Cohen*[,‡]

Biophysical Pharmacology Section, Clinical Pharmacology Branch, National Cancer Institute, Office of Biologics Research and Review, Food and Drug Administration, and Laboratory of Molecular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205, and Center for Chemical Physics, National Bureau of Standards, Gaithersburg, Maryland 20899

Received July 10, 1984; Revised Manuscript Received November 5, 1984

ABSTRACT: The complex of ribonuclease A (RNase A) with uridine vanadate (U-V), a transition-state analogue, has been studied with ^{51}V and proton NMR spectroscopy in solution and by neutron diffraction in the crystalline state. Upon the addition of aliquots of U-V at pH 6.6, the C^{ϵ}-H resonances of the two active-site histidine residues 119 and 12 decrease in intensity while four new resonances appear. Above pH 8 and below pH 5, these four resonances decrease in intensity as the complex dissociates. These four resonances are assigned to His-119 and His-12 in protonated and unprotonated forms in the RNase-U-V complex. These resonances do not titrate or change in relative area in the pH range 5-8, indicating a slow protonation process, and the extent of protonation remains constant with ca. 58% of His-12 and ca. 26% of His-119 being protonated. The results of diffraction studies show that both His-12 and His-119 occupy well-defined positions in the RNase-U-V complex and that both are protonated. However, while the classic interpretation of the mechanism of action of RNase based on the proposal of Findlay et al. [Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R., & Ross, C. A. (1962) *Biochem. J. 85*, 152-153] requires both His-12 and His-119 to be in axial positions relative to the pentacoordinate transition state, in the diffraction structure His-12 is found to be in an equatorial position, while Lys-41 is close to an axial position. Hydrogen exchange data show that the mobility and accessibility of amides in the RNase-U-V complex do not significantly differ from what was observed in the native enzyme. The results of both proton NMR in solution and neutron diffraction in the crystal are compared and interpreted in terms of the mechanism of action of RNase.

The structure and mechanism of action of ribonuclease (RNase)[1] continue to be subjects of active interest. The recent reinvestigation of crystals of RNase A by X-ray diffraction (Wlodawer et al., 1982) and by neutron diffraction (Wlodawer & Sjolin, 1983) clarified certain features of the structure of the active site. In general, this provided a coherent picture of the structure as interpreted by both diffraction and proton NMR solution studies (Cohen & Wlodawer, 1982). A somewhat unusual finding of the NMR studies was the ab-

[‡] National Cancer Institute.
[§] Food and Drug Administration.
[∥] National Bureau of Standards.

[1] Abbreviations: RNase, ribonuclease; U-V, uridine vanadate; TSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$; NOE, nuclear Overhauser enhancement; F, fermi unit of scattering length (10^{-13} cm).

This article not subject to U.S. Copyright. Published 1985 by the American Chemical Society