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Substrate Specificity of Two Chymotrypsin-like Proteases from Rat Mast Cells. Studies with Peptide 4-Nitroanilides and Comparison with Cathepsin G†

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ABSTRACT: The substrate specificity of chymotrypsin-like enzymes from typical mast cells, rat mast cell protease I (RMCP I), and from atypical mast cells, rat mast cell protease II (RMCP II), has been investigated with peptide 4-nitroanilide (NA) substrates and peptide chloromethyl ketone inhibitors. Suc-Phe-Pro-Phe-NA and Suc-Phe-Leu-Phe-NA are the best substrates for both RMCP I and RMCP II. In the case of RMCP II, both the P_3 (Phe) and the P_4 [succinyl (Suc)] groups are important. Succinyl tetrapeptides with nearly the same sequence were not as sensitive to either rat protease. The pH optimum of the hydrolysis of Suc-Phe-Leu-Phe-NA by RMCP II was pH 6.6 and by RMCP I was ca. pH 8.5. The S_2 subsite of RMCP II prefers Thr over either Leu or Pro. The subsite specificity of RMCP II may be explained on the basis of interaction with Asn-86 in S_2, a hydrophobic S_3 subsite due to the presence of Phe-178, and at pH 6.6, an interaction with a cationic His-200 in the S_4 subsite. Suc-Phe-Pro-Phe-NA is the best 4-nitroanilide substrate yet found for cathepsin G. The observed subsite preferences indicate that the S_3 and S_4 subsites of RMCP I, RMCP II, and cathepsin may be very similar. The k_{cat}/K_M values of RMCP II and cathepsin G are significantly lower than those observed with bovine chymotrypsin, RMCP I, and other serine proteases. It is not clear whether this difference is related to their physiological function or is due to a non-optimum substrate structure. Prolyl residues at the P_2 position enhance the effectiveness of substrates and inhibitors for RMCP I, RMCP II, and cathepsin G. Unlike other serine proteases, both rat enzymes can also accept Pro residues in position P_3 of substrates. A peptide chloromethyl ketone, Suc-Pro-Leu-PheCH_2Cl, was synthesized in order to take advantage of this phenomenon. This compound is an effective inhibitor of RMCP I. RMCP I is inhibited 10–93 times faster than RMCP II, cathepsin G, or bovine chymotrypsin. This inhibitor may be useful in elucidating the physiological function of RMCP I and RMCP II.

A number of intracellular chymotrypsin-like serine proteases have recently been isolated and characterized. These include a protease from rat small intestine (Katunuma et al., 1975), from rat skeletal muscle (Katunuma et al., 1975; Everitt & Neurath, 1979), and from human leukocyte cathepsin G. The protease from rat small intestine has been shown by immunofluorescent studies to be localized in atypical mast cells of the mucosa (Woodbury et al., 1978c) whereas the rat skeletal muscle enzyme is derived from typical mast cells (Woodbury et al., 1978b). The protease from typical mast cells is the same as the peritoneal mast cell protease, chymase, described by Benditt & Arase (1959). To distinguish the enzymes, the protease from typical mast cells is referred to as rat mast cell protease I (RMCP I),[1] and that from atypical cell is designated rat mast cell protease II (RMCP II). Cathepsin G is located in leukocyte granules.

The complete amino acid sequence of RMCP II has been determined and shows 33% identity with bovine chymotrypsin (Woodbury et al., 1978a). The catalytic triad of chymotrypsin is maintained in RMCP II, but there are a number of amino acid changes in the region of the primary and extended substrate binding sites. Although the primary structure of RMCP I is not complete, the two rat enzymes show 75% homology in the first 51 amino acid residues (Woodbury et al., 1978b). Comparison of the N-terminal 21 amino acid residues of cathepsin G with RMCP I and RMCP II reveals 4 and 6 changes, respectively.

The substrate specificity of both rat proteases has been studied by using natural peptide substrates such as glucagon and the oxidized insulin B chain (Kobayashi et al., 1978; Kobayashi & Katunuma, 1978). Both enzymes cleaved peptide bonds having Phe, Tyr, and Trp residues at position P_1,[2] but RMCP II showed greater selectivity. In addition, RMCP II is reported to have a lower catalytic rate toward substrates such as Ac-Tyr-OEt (Katunuma et al., 1975). The specificity of human leukocyte cathepsin G has been studied toward both the insulin B chain (Blow & Barrett, 1977) and peptide 4-nitroanilide substrates (Nakajima et al., 1979).

In this paper, we report a kinetic study of the hydrolysis of a series of peptide 4-nitroanilides by the rat mast cell proteases and human cathepsin G. Both rat enzymes are effective proteases toward such substrates. In addition, we have studied

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[1] Abbreviations used: NA, 4-nitroanilide; Ac, acetyl; Suc, succinyl; Glt, glutaryl; MeO-Suc, methoxysuccinyl; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate.

[2] 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).

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the reactivity of the three proteases, in particular RMCP II, toward a variety of peptide chloromethyl ketone inhibitors and have prepared an inhibitor which shows good selectivity for RMCP I.

Materials and Methods

Cathepsin G was a gift from Dr. James Travis and his research group of the University of Georgia. RMCP I was prepared by the method of Everitt & Neurath (1979). RMCP II was prepared by the method of Woodbury & Neurath (1978). Heparin from porcine intestinal mucosa, benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt), and pyridoxal 5'-phosphate were obtained from Sigma Chemical Co. *N,N'*-Dicyclohexylcarbodiimide and *p*-toluenesulfonyl fluoride were purchased from Aldrich Chemical Co. All other chemicals were reagent grade. The syntheses of many of the 4-nitroanilide substrates (Nakajima et al., 1979) and peptide chloromethyl ketone inhibitors (Powers et al., 1977) have been reported previously. The syntheses of all new compounds are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

4-Nitroanilide Kinetics. The rates of hydrolysis of the 4-nitroanilide substrates were measured by adding 10–50 μL of the appropriate enzyme solution to 2.0 mL of a substrate solution in 50 mM phosphate buffer, pH 8.0 or 6.6, containing 10% Me_2SO at 25 °C, unless otherwise specified. The increase in the absorbance at 410 nm was followed with a Beckman Model 25 spectrophotometer. An ϵ value of 8800 at 410 nm was used for the liberated 4-nitroaniline (Erlanger et al., 1961). Kinetic constants were determined from the initial rates of hydrolysis by using Lineweaver–Burk plots. Correlation constants were greater than 0.99 in all cases. The concentration of cathepsin G was determined with Suc-Ala-Ala-Pro-Phe-NA, applying kinetic constants based on active-site titration of the enzyme (Nakajima et al., 1979). The concentration of RMCP I was determined by using benzoyl-L-tyrosine ethyl ester as substrate and assuming 58.3 units/mg of protein for the enzymatic activity and 26 000 for the molecular weight (Everitt & Neurath, 1979). A molecular weight of 24 655 was assumed for RMCP II (Woodbury et al., 1978a). Protein concentrations were determined by using $E_{280}^{1\%} = 11.0$. Titration of our batch of enzyme with NPGB or human α_1-protease inhibitor yielded activity values of 21 and 21.4%, respectively. A value of 22% was obtained by using the Bz-Tyr-OEt assay and a specific activity of 8.5 units/mg of enzyme. The kinetic constants were calculated by using 21% for the activity of the enzyme.

Chloromethyl Ketone Inhibitions. All inhibition experiments were carried out under pseudo-first-order conditions at 30 °C in 50 mM phosphate buffer, pH 8.0 or 6.6, containing 10% Me_2SO. Inhibitor concentrations were at least 15-fold greater than the enzyme concentration. After the inhibitor and the enzyme solutions were mixed, aliquots were removed periodically from the reaction mixture. The remaining enzyme activity was measured with Suc-Ala-Ala-Phe-NA in 50 mM phosphate buffer, pH 6.6, containing 10% Me_2SO. Kinetic constants were determined by the equation $v_{inhibition} = k_{obsd}[E]$. The pseudo-first-order rate constants were obtained from plots of ln (initial velocities of substrate hydrolysis) vs. time by using a standard linear regression method. The second-order rate constant $k_{obsd}/[I]$ is suitable only for comparison of the relative reactivities of the different inhibitors since $k_{obsd}/[I]$ is suitable only for comparison of the relative reactivities of the different inhibitors since $k_{obsd}/[I]$ varies with [I] when [I] approaches K_I, the true dissociation constant of the enzyme–inhibitor complex (Kitz & Wilson, 1962).

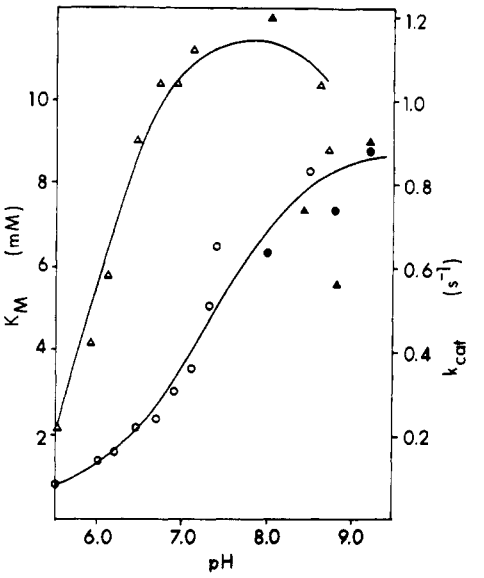

FIGURE 1: Plot of K_M (circles) and k_{cat} (triangles) as a function of pH for the hydrolysis of Suc-Ala-Ala-Pro-Phe-NA by RMCP II. The kinetics were measured in 50 mM phosphate buffer (○ and △) and 50 mM borate buffer (● and ▲). The standard deviation is ~1% for all the data reported.

Results

RMCP II. The susceptibilities of 13 peptide 4-nitroanilides toward RMCP II were investigated at pH 8.0 (Table A, supplementary material). Most of the good substrates were tri- and tetrapeptides containing Phe in position P_1. Bz-Tyr-NA, a typical chymotrypsin substrate, was slowly hydrolyzed, as was MeO-Suc-Ala-Ala-Pro-Met-NA, an excellent cathepsin G substrate (Nakajima et al., 1979). MeO-Suc-Ala-Ala-Pro-Val-NA, an excellent substrate for human leukocyte elastase, was not hydrolyzed. Suc-Ala-Pro-Leu-Phe-NA was hydrolyzed 150 times more effectively than Suc-Ala-Gly-Leu-Phe-NA, pointing to the possible importance of the Pro residue in position P_3.

The pH dependence of hydrolysis of Suc-Ala-Ala-Phe-NA by RMCP II showed a bell-shaped curve with a maximum at pH 6.6 (Figure A, supplementary material). Similar results were obtained with other succinyl peptide substrates. However, the pH optima for H-Ala-Ala-Phe-NA and MeO-Suc-Ala-Ala-Pro-Phe-NA were pH 8.0 and 7.0, respectively.

The pH dependences of k_{cat} and K_M for the hydrolysis of Suc-Ala-Ala-Pro-Phe-NA by RMCP II were determined and are shown in Figure 1. The maximum k_{cat} is reached at about pH 7.0, and the K_M increases as the pH is raised in the range studied. RMCP II begins to denature at ca. pH 9.0, and this may explain the considerable variation in the measured kinetic parameters at high pH values. Partial inactivation of the enzyme during the course of the high-pH kinetic experiments would introduce a significant error into the k_{cat} values, but would have less effect on K_M values.

Using data obtained in preliminary studies, we investigated the kinetics of hydrolysis of 18 tri- and tetrapeptide 4-nitroanilides by RMCP II as listed in Table I. The kinetic determination was carried out at both pH 6.6 and pH 8.0 since we were uncertain as to the optimum pH for each substrate.

The most effective substrates were Suc-Phe-Pro-Phe-NA and Suc-Phe-Leu-Phe-NA. Both have a succinyl group in position P_4 and a hydrophobic sequence. Both of these substrates were over 70-fold better than tetrapeptides such as Suc-Ala-Gly-Leu-Phe-NA which lack the hydrophobic residue in P_3 and the anionic succinyl group in P_4. Replacements of

Table I: Kinetic Constants for the Hydrolysis of 4-Nitroanilide Substrates by RMCP II[a]

substrate	pH 6.6			pH 8.0			ratio of
P_5 P_4 P_3 P_2 P_1	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M pH 6.6/pH 8.0
H-Ala-Ala-Phe-NA[b]	42	0.99	24	5.4	0.2	37	0.65
H-Ala-Pro-Phe-NA[c]	6.2	1.7	270				
Suc-Ala-Ala-Phe-NA[b]	1.2	1.1	920	110	62	560	1.6
Suc-Ala-Pro-Leu-NA[a]	1.2	0.34	280	6.6	0.86	130	2.2
Suc-Phe-Pro-Phe-NA[e]	0.23	8.2	36000	2.5	19	7600	4.7
Suc-Phe-Leu-Phe-NA[f]	0.21	6.4	30000	0.57	10	18000	1.7
Ac-Ala-Ala-Pro-Phe-NA[f]	5.2	1.6	310	3.2	0.76	240	1.3
H-Ala-Ala-Pro-Phe-NA[b]				1.4	0.59	420	
$NH_2(CH_2)_5CO$-Ala-Ala-Pro-Phe-NA[f]	9.8	1.8	180	4.6	0.91	200	0.9
MeO-Suc-Ala-Ala-Pro-Phe-NA[c]	1.4	0.53	380	2.3	0.77	340	1.1
Glt-Ala-Ala-Pro-Phe-NA[e]	1.6	0.94	590	7.9	2.5	320	1.8
Suc-Ala-Ala-Pro-Phe-NA[d]	2.4[g]	1.0[g]	420[g]	2.1[h]	0.58[h]	280[h]	1.5[i]
Suc-Ala-Gly-Leu-Phe-NA[e]	2.5	0.08	32	270	2.8	10	3.2
Suc-Ala-Phe-Leu-Phe-NA[f]	0.29	0.94	3200	0.47	0.85	1800	1.8
Suc-Ala-Pro-Leu-Phe-NA[e]	0.68	1.4	2100	2.7	2.0	760	2.8
Suc-Phe-Pro-Leu-Phe-NA[c]	7.1	1.1	150	3.1	0.27	85	1.8
Suc-Phe-Pro-Pro-Phe-NA[d]	2.2	0.36	160	8.6	0.75	88	1.8
Suc-Phe-Pro-Thr-Phe-NA[f]	13	5.4	420	29	5.6	190	2.2

[a] 50 mM phosphate, 10% dimethyl sulfoxide, enzyme concentration 1.56×10^{-7} M, 25 °C. Substrate concentration range: [b] 5.0–0.5 mM. [c] 0.60–0.03 mM. [d] 10 to ~1.0 mM. [e] 3.0–0.1 mM. [f] 1.5–0.05 mM. [g] pH 6.7. [h] pH 8.1. [i] Ratio of pH 6.7/pH 8.1.

Table II: Kinetic Constants for the Hydrolysis of 4-Nitroanilide Substrates by RMCP I[a]

substrate	substrate concn range (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
P_5 P_4 P_3 P_2 P_1				
Suc-Phe-Pro-Phe-NA	0.4–0.004	0.053	24	450 000
Suc-Phe-Leu-Phe-NA	0.05–0.007	0.023	19	830 000
Suc-Phe-Pro-Thr-Phe-NA	0.45–0.07	0.15	11	73 000
MeO-Suc-Ala-Ala-Pro-Phe-NA	0.65–0.13	0.42	10	24 000
$H_2N(CH_2)_5CO$-Ala-Ala-Pro-Phe-NA	1.0–0.20	1.2	13	11 000

[a] 50 mM phosphate (pH 8.0), 10% dimethyl sulfoxide, 25 °C.

the succinyl group by a hydrogen, a free amino acid residue, or an acylated amino acid residue resulted in significantly poorer substrates. Both the Phe residue in P_3 and the Leu or Pro residues in P_2 were favored over alanine residues. At P_2, a Thr residue was preferred over either Leu or Pro. Except for one substrate which has a free N terminus at P_3, all the substrates had larger k_{cat}/K_M values at pH 6.6 compared to pH 8.0.

At one time, RMCP II was postulated to be a "group specific" protease, thought to hydrolyze preferentially the apo form of pyridoxal phosphate proteins (Katunuma et al., 1975). We therefore investigated the effect of pyridoxal phosphate on the hydrolysis of Suc-Phe-Leu-Phe-NA by RMCP II. No effect was observed. RMCP II activity was also unaffected when 50 mM phosphate buffer was replaced by 50 mM citrate.

RMCP II was not acylated by Ac-NHN($CH_2C_6H_5$)CO-ONp, an excellent active-site titrant of chymotrypsin and cathepsin G.

RMCP I. The susceptibility of 23 peptide 4-nitroanilides toward RMCP I was investigated at pH 8.0 (Table B, supplementary material). RMCP I had a much higher catalytic activity than RMCP II, consistent with the observation by Katunuma et al. (1975). As in the case of RMCP II, all of the good substrates were tri- and tetrapeptides containing Phe residues at position P_1. The elastase substrate MeO-Suc-Ala-Ala-Pro-Val-NA was not hydrolyzed, and the cathepsin G substrate MeO-Suc-Ala-Ala-Pro-Met-NA was slowly hydrolyzed. A substrate with a proline residue in P_3 (Suc-Ala-Pro-Leu-Phe-NA) was just as effective as one without (Suc-Ala-Gly-Leu-Phe-NA).

The kinetic constants for five of the best substrates are listed in Table II. Once again, the best two substrates (Suc-Phe-Leu-Phe-NA and Suc-Phe-Pro-Phe-NA) contain Phe in P_3 and a succinyl group in P_4 and were 20-fold more sensitive than Suc-Ala-Ala-Phe-NA. The k_{cat}/K_M value ($\sim 1 \times 10^6$ M^{-1} s^{-1}) obtained with Suc-Phe-Leu-Phe-NA is certainly one of the highest recorded for any protease/synthetic amide substrate system tested. Replacement of Phe in P_3 by a Pro residue (Suc-Pro-Pro-Phe-NA) gave a 90-fold poorer substrate. A tetrapeptide with Phe in P_3 (Suc-Ala-Phe-Leu-Phe-NA) was almost as effective as the tripeptides (Table B, supplementary material). The kinetic constants for three tetrapeptides (Suc-Ala-Phe-Leu-Phe-NA, Glt-Ala-Ala-Pro-Phe-NA, and Suc-Ala-Ala-Pro-Phe-NA) could not be determined due to extensive curvature in the Michaelis–Menten plots. This may be an indication of multiple binding modes for these tetrapeptides.

The pH dependence of the hydrolysis of Suc-Phe-Leu-Phe-NA by RMCP I was investigated in a variety of buffers (Figure B, supplementary material). In phosphate buffers, the pH maximum was about 8.5. Similar results were obtained with MeO-Suc-Ala-Ala-Pro-Phe-NA. Hydrolysis rates were strongly influenced by the buffer used. Rates in phosphate, borate, diglycine, and glycine buffers (all 50 mM) were within a factor of two of each other. However, those in Tris and pyrophosphate were about 4- and 9-fold higher, respectively.

The effect of heparin on the hydrolysis of Suc-Phe-Leu-Phe-NA by RMCP I was studied in several buffers at pH 8.0 (Table C, supplementary material). Heparin had no effect in diglycine, increased the rate up to 2-fold in phosphate (50 mM), and inhibited the enzyme up to 25% in pyrophosphate (100 mM). In order to determine if the effect was purely due to ionic strength changes, the activity was measured in a 50 mM phosphate buffer, pH 8.0, containing 0.7 mM $NaSO_4$ or

Table III: Kinetic Constants for the Hydrolysis of 4-Nitroanilide Substrates by Human Cathepsin G[a]

substrate P_5 P_4 P_3 P_2 P_1	substrate concn range (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Suc-Phe-Pro-Phe-NA	2.0–0.40	1.5	5.3	3500
Suc-Phe-Leu-Phe-NA	0.20–0.05	0.62	0.95	1500
Suc-Ala-Phe-Leu-Phe-NA	0.15–0.03	4.8	2.6	540
Suc-Ala-Phe-Leu-Phe-NA[b]	0.20–0.04	0.64	0.31	480
Suc-Phe-Pro-Thr-Phe-Phe-NA	2.0–2.30	1.0	0.047	45
MeO-Suc-Ala-Ala-Pro-Met-NA[c]	2.1–0.10	0.31	0.52	1700

[a] 0.1 M Hepes (pH 7.5), 0.5 M NaCl, 10% dimethyl sulfoxide, 25 °C. [b] 50 mM phosphate (pH 8.0), 10% dimethyl sulfoxide, 25 °C. [c] Data of Nakajima et al. (1979).

0.1% Ampholine (pH 3.5–5.0). The enzyme activity was decreased, respectively, to 70% and 90% of the control value. The activity of RMCP I toward Suc-Phe-Leu-Phe-NA was not affected by the addition of pyridoxal phosphate.

Human Cathepsin G. Several of the better substrates for RMCP I and RMCP II were investigated with human cathepsin G, and the data are reported in Table III. The best 4-nitroanilide substrate previously reported was MeO-Suc-Ala-Ala-Pro-Met-NA (Nakajima et al., 1979). The tripeptide Suc-Phe-Leu-Phe-NA was almost as effective, while Suc-Phe-Pro-Phe-NA had a 2-fold higher k_{cat}/K_M value. Suc-Phe-Leu-Phe-NA was specific for cathepsin G and was not hydrolyzed by elastase, the other major leukocyte protease, in 0.1 M Hepes buffer, pH 7.5, containing 0.5 M NaCl.

The hydrolysis of several substrates containing Pro in P_3 by cathepsin G was investigated. Previously, Suc-Ala-Pro-Leu-Phe-NA has been shown to be cleaved at the Leu–Phe bond with k_{cat}/K_m = 8.9 M^{-1} s^{-1} (Nakajima et al., 1979). This implied that cathepsin G cannot accept a Pro in P_3. Suc-Pro-Pro-Phe-NA was not hydrolyzed by cathepsin G. During the hydrolysis of Suc-Phe-Pro-Pro-Phe-NA and Suc-Phe-Pro-Leu-Phe-NA, precipitates formed after 5 min at the higher substrate concentrations. With the former, nitroaniline was released after 2–3 days while none was observed with the latter. This indicates that enzymatic cleavage may be taking place at some other bond, possibly at the Phe–Pro or Leu–Phe bonds. The products may then have been further cleaved to release free nitroaniline. Suc-Phe-Pro-Thr-Phe-NA and Suc-Pro-Leu-Phe-NA were both hydrolyzed at approximately the same rate. The k_{cat}/K_M value for the hydrolysis of of Suc-Phe-Pro-Thr-Phe-NA (45 M^{-1} s^{-1}) was only 5-fold higher than the k_{cat}/K_M value for the cleavage of the Leu-Phe bond in Suc-Ala-Pro-Leu-Phe-NA, even though the former involves a much better leaving group (4-nitroaniline) than the latter.

Heparin had no effect on the hydrolysis of Suc-Phe-Leu-Phe-NA by cathepsin G in 0.1 M Hepes buffer, pH 7.5, containing 0.5 M NaCl.

Peptide Chloromethyl Ketone Inhibitors. The inhibition of RMCP II by a series of peptide chloromethyl ketones was studied at pH 8.0 (Table IV). Interestingly, *p*-toluenesulfonyl fluoride did not inhibit RMCP II. A number of peptide chloromethyl ketone inhibitors with Phe, Leu, or Trp residues in P_1 reacted with RMCP II. Elastase inhibitors which contained Ala or Val residues in P_1 were ineffective. All of the better inhibitors, such as Z-Gly-Leu-PheCH_2Cl, had both a Phe residue in P_1 and a Leu residue in P_2. However, the absolute rates of inhibition were quite small. Cathepsin G itself is slowly inhibited by peptide chloromethyl ketones, and reacts with Z-Gly-Leu-PheCH_2Cl with a $k_{obsd}/[I]$ value of 51 M^{-1} s^{-1} (Powers et al., 1977). This is a much lower value than

Table IV: Inhibition of RMCP II by Peptide Chloromethyl Ketones[a]

inhibitor P_4 P_3 P_2 P_1	[I] (mM)	$k_{obsd}/[I]$ (M^{-1} s^{-1})	$k_{obsd}/[I]$ (rel)
4-$CH_3C_6H_4SO_2F$		no inhibition	0
Ac-PheCH_2Cl	3.0	0.16	1
Z-TrpCH_2Cl	0.3	0.11	0.7
CHO-PheCH_2Cl	3.0	0.091	0.6
Z-Leu-PheCH_2Cl	0.05	1.60	10
$(CH_3)_2CHCH_2OCO$-Gly-PheCH_2Cl	0.05	0.19	1.2
Ac-Leu-PheCH_2Cl	0.3	0.060	0.4
Ac-Val-PheCH_2Cl	0.5	0.048	0.3
Ac-Ala-PheCH_2Cl	1.5	0.029	0.2
$(CH_3)_2CHCH_2CO$-Ala-PheCH_2Cl	0.05	0.025	0.2
Z-Gly-Leu-AlaCH_2Cl		0.002	0.01
Z-Gly-Leu-PheCH_2Cl	0.05	2.30	14
Boc-Gly-Leu-PheCH_2Cl	0.05	1.60	10
Boc-Ala-Gly-PheCH_2Cl	0.05	0.92	5.8
Z-Gly-Gly-PheCH_2Cl	0.05	0.37	2.3
Ac-Ala-Gly-PheCH_2Cl	1.0	0.52	3.2
Ac-Ala-Ala-Pro-ValCH_2Cl	0.50	0.001	0.01
Ac-Phe-Gly-Ala-LeuCH_2Cl	0.55	0.012	0.1
Ac-Phe-Gly-Ala-LeuCH_2Cl	0.55	0.014	0.1
Suc-Ala-Ala-Pro-ValCH_2Cl	2.0	no inhibition	0
Suc-Ala-Ala-Pro-ValCH_2Cl	2.0	no inhibition	0

[a] 50 mM phosphate (pH 8.0), 10% dimethyl sulfoxide, 3.34 μM enzyme, 30 °C. [b] Data at pH 6.6.

Table V: Inhibition of Chymotrypsin-like Enzymes by Suc-Pro-Leu-PheCH_2Cl

enzyme	k_{obsd} (s^{-1})	$k_{obsd}/[I]$ (M^{-1} s^{-1})	$k_{obsd}/[I]$ (rel)
RMCP I[a]	2.5×10^{-3}	37	93
RMCP II[a]	1.7×10^{-4}	0.4	1
cathepsin G[b]	4.8×10^{-4}	0.74	1.9
α-chymotrypsin[c]	2.4×10^{-3}	3.6	9

[a] pH 8.0, 0.05 M phosphate, 30 °C. [b] pH 7.5, 0.1M Hepes, 0.5 M NaCl, 30 °C. [c] pH 7.8, 0.08 M Tris, 0.1 M $CaCl_2$, 30 °C.

values (1600 M^{-1} s^{-1}) observed with elastase and its better inhibitors.

Since both RMCP I and RMCP II preferred substrates with Phe in P_1 and Leu in P_2 and could react with substrates with Pro in P_3, we decided to synthesize Suc-Pro-Leu-PheCH_2Cl. Previous studies have shown that many serine proteases will not react with substrates or peptide chloromethyl ketones containing a Pro in P_3. The inhibition rates with four chymotrypsin-like serine proteases are reported in Table V. A 93-fold difference in reactivity was observed in the rates when measured close to the optimum conditions for each enzyme. RMCP I was inhibited at least 10-fold faster than any of the other enzymes. Interestingly, both cathepsin G and α-chymotrypsin were inhibited by Suc-Pro-Leu-PheCH_2Cl, although the rates were at least 70-fold lower than those observed with good inhibitors.

Discussion

The presence of an extended substrate binding site in chymotrypsin is now well established by a combination of crystallographic (Segal et al., 1971) and kinetic studies with both substrates (Segal, 1972) and peptide chloromethyl ketone inhibitors (Kurachi et al., 1973). A section of the peptide chain (Ser-Trp-Gly-216) of the enzyme forms an antiparallel β structure with the P_3–P_1 residue of a substate or inhibitor. Hydrogen bonding occurs between the NH of the P_1 residue and between the NH and CO of the P_3 residue and this portion of the enzyme's backbone. A similar structural feature has

been found in trypsin (Sweet et al., 1974; Ruhlmann et al., 1973), subtilisin (Robertus et al., 1972), and elastase (Hassall et al., 1979). Additional interactions occur at the various subsites. In the case of chymotrysin, the side chain of Ile-99 can interact with bulky nonpolar residues at P_2.

RMCP II is homologous to bovine chymotrypsin with ca. 33% identity (Woodbury et al., 1978). A Ser-Tyr-Gly-199 sequence corresponds to the Ser-Trp-Gly-216 sequence of chymotrypsin, indicating the conservation of the extended substrate binding site in RMCP II. There are, however, a number of changes in the substrate binding site of RMCP II. Asn-86 is substituted for Ile-99 in chymotrypsin. In addition, a disulfide bond (Cys-191 to Cys-220) found in chymotrypsin and all other known serine proteases is replaced by Phe-178 and by a five-residue deletion immediately following Ser-Tyr-Gly-199 in RMCP II. This indicates the possibility of a significant change in the extended substrate binding site of RMCP II, particularly in the vicinity of S_3, and may explain the unusual ability of the protease to accept a P_3 prolyl residue in substrates. Yet another significant difference is the presence of a histidyl residue (His-200) immediately following the antiparallel β-structure-forming residues (Ser-Tyr-Gly-199). This could possibly affect the nature of the S_4 subsite in RMCP II.

Peptide 4-nitroanilide substrates are obviously interacting with an extended substrate binding site in RMCP II since alteration of the P_2, P_3, P_4, or P_5 residues resulted in significant changes in the kinetic parameters measured. The S_2 subsite preferred a Thr residue over either a Pro, which is a residue generally favorable for serine proteases, or a Leu in a series of three substrates (Suc-Phe-Pro-AA-Phe-NA) examined, with major differences being in k_{cat} values. This is consistent with the presence of Asn-86 in the S_2 subsite of RMCP II, which could interact favorably with a hydrophilic Thr residue in the substrates and less favorably with hydrophobic residues such as proline and leucine.

The two best RMCP II substrates (Suc-Phe-Pro-Phe-NA and Suc-Phe-Leu-Phe-NA) have a P_3 Phe residue. These have at least 5-fold lower K_M values than those with an Ala residue. This indicates the presence of a hydrophobic S_3 subsite and could be due to the presence of Phe-178. One might predict that Suc-Phe-Thr-Phe-NA would be an even better substrate since it contains a favorable P_2 residue, i.e., Thr.

The presence of a cationic group in the S_4 subsite of RMCP II is strongly supported by the present data. This group, probably His-200, interacts favorably with substrates containing the anionic succinyl group in P_4. This is not simply due to the presence of a charged residue since the anionic substrate Suc-Ala-Ala-Phe-NA binds 35-fold better than the cationic H-Ala-Ala-Phe-NA at pH 6.6. In fact, all of the substrates with a succinyl group in P_5 are significantly poorer than the analogous succinyl tripeptides even though the former could interact with more of the extended substrate binding site. This indicates that the interaction is a fairly specific S_4–P_4 interaction.

The K_M values for all the P_4 succinyl substrates are higher (3–93-fold) at pH 8.0 than at pH 6.6. Since the pK values for most histidyl residues in proteins are about 7, His-200 would be expected to be cationic at pH 6.6 and neutral at pH 8.0. This would result in a poorer interaction between the anionic succinyl group in P_4 and His-200 at the higher pH. H-Ala-Ala-Phe-NA, which has an N-terminal ammonium group in the vicinity of P_4–P_3, is the only substrate with a higher k_{cat}/K_M value at pH 8.0. Indeed, it has a 20-fold lower K_M value at pH 8.0 than Suc-Ala-Ala-Phe-NA. Possibly, interaction between the cationic substrate and the cationic His-200 hinders binding at the lower pH.

The natural substrate(s) of RMCP II is (are) not yet known. Kobayashi & Katunuma (1978) have shown that in small peptides the enzyme cleaves peptide bonds primarily following Phe, Tyr, and Trp residues, although one Arg–Arg bond reportedly was split. Our results would suggest that a substrate preferred by RMCP II would have an acidic residue (e.g., Asp, Glu, serine sulfate, or phosphate) at P_4 and an aromatic residue at P_3. The succinyl group in our substrates has the same structure as the side chain of an Asp residue in the P_4 site of a substrate.

RMCP I. The sequence of RMCP I is not yet complete. However, comparison of the first 51 amino-terminal residues shows a 75% identity with RMCP II. As in the case of RMCP II, the best substrates for RMCP I were the succinyl tripeptides Suc-Phe-Pro-Phe-NA and Suc-Phe-Leu-Phe-NA. This would indicate that the extended substrate binding sites of the two enzymes are quite similar.

A significant difference between RMCP I and RMCP II is the much higher enzymatic activity of RMCP I, having 12–173-fold higher k_{cat}/K_M values. In fact, the k_{cat}/K_M values observed with RMCP I are higher by a factor of 2–3 than those obtained with chymotrypsin and tetrapeptide 4-nitroanilides (Nakajima et al., 1979), while those observed with RMCP II are much lower. Another notable difference between the two proteases is that substrates containing a P_3 prolyl residue are especially favored by RMCP II when compared to analogous substrates without a Pro residue in P_3. Thus, whereas RMCP I hydrolyzed Suc-Ala-Pro-Leu-Phe-NA and Suc-Ala-Gly-Leu-Phe-NA equally well, RMCP II hydrolyzed the former substrate at a rate which was 65-fold higher than that for the latter one.

Cathepsin G and Chymotrypsin. Human leukocyte cathepsin G is also a poor peptidase as evidenced by low k_{cat}/K_M values exhibited with a wide variety of substrates examined (Nakajima et al., 1979). Suc-Phe-Pro-Phe-NA has a twofold higher k_{cat}/K_M value than the previous best 4-nitroanilide cathepsin G substrate (MeO-Suc-Ala-Ala-Pro-Met-NA). This may indicate the S_4 and S_3 subsite of cathepsin G is quite similar to that of RMCP II and RMCP I. Interestingly, it has been suggested that cathepsin G and mast cell protease (RMCP I) are in fact one and the same enzyme (Starkey, 1978).

Since Suc-Phe-Leu-Phe-NA was one of the best substrates for RMCP II, RMCP I, and cathepsin G, we decided to test it toward bovine chymotrypsin A_α. The kinetic constants under conditions identical with those of Nakajima et al. (1979) were $K_M = 0.31$ mM, $k_{cat} = 0.65\ s^{-1}$, and $k_{cat}/K_M = 2100\ M^{-1}\ s^{-1}$, over 150-fold lower than values obtained with tetrapeptides such as MeO-Suc-Ala-Ala-Pro-Phe-NA ($k_{cat}/K_M = 380\,000\ M^{-1}\ s^{-1}$). There appears little doubt that the S_4 and S_3 subsites of chymotrypsin are significantly different than those of RMCP II and RMCP I.

Of the four chymotrypsin-like enzymes examined, cathepsin G is the poorest peptidase with low k_{cat}/K_M values, i.e., 3500 $M^{-1}\ s^{-1}$, for its best 4-nitroanilide substrate. The corresponding values are 36 000 $M^{-1}\ s^{-1}$ for RMCP II, 380 000 $M^{-1}\ s^{-1}$ for bovine chymotrypsin, and 830 000 $M^{-1}\ s^{-1}$ for RMCP I and their best 4-nitroanilide substrates. These rates would suggest that cathepsin G and RMCP II are probably much more discriminating in their choice of substrates than chymotrypsin and RMCP I. It may be that when the correct substrate sequence is discovered these two enzymes will exhibit rates of the same order as other serine proteases. Alternately, their

low catalytic efficiency may be related to their respective physiological role.

Proline Residues. A proline residue at P_3 in a chymotrypsin substrate disrupts the interaction at the S_3 subsite due to loss of a hydrogen bond with the NH group and unfavorable stereochemical interactions with the pyrrolidine ring. A tripeptide ester with a Pro residue in P_3 binds 30-fold less tightly to chymotrypsin than the one containing Gly, but is still hydrolyzed (Segal, 1972). A substrate containing Pro in P_2 on the other hand interacts favorably with chymotrypsin.

Cathepsin G also prefers Pro at P_2 and will cleave Suc-Ala-Pro-Leu-Phe-NA at the Leu–Phe bond rather than the Phe–NA bond (Nakajima et al., 1979). A number of substrates with a Pro residue in P_3 were tested with cathepsin G and found not to release 4-nitroaniline. Of the two substrates which were cleaved, the rates were extremely slow. In fact, the rates with these two 4-nitroanilides were much smaller than those observed when simple peptide bonds are cleaved.

With both RMCP I and RMCP II, substrates containing Pro at P_2 produced favorable interactions with the enzymes. Interestingly, both enzymes would accept substrates with a P_3 residue. With RMCP I, Suc-Phe-Pro-Thr-Phe-NA was a better substrate than the other tetrapeptides examined. With RMCP II, all substrates containing Pro residues in P_3 were cleaved at reasonable rates, as was one containing Pro residues in both P_3 and P_2. In fact, the fourth best substrate for RMCP II has a Pro residue in P_3. It is possible that changes in the S_3 subsite of RMCP II due to the loss of the Cys-191 to Cys-220 disulfide bond result in a less unfavorable interaction with the pyrrolidine ring of a Pro in P_3 than is the case with other serine proteases such as chymotrypsin and elastase (Thompson & Blout, 1973).

Inhibitors. In order to develop selective inhibitors for RMCP II, we studied a variety of peptide chloromethyl ketone inhibitors and found the best inhibitor to be Z-Gly-Leu-PheCH_2Cl. This is also the best chloromethyl ketone inhibitor of cathepsin G found thus far (Powers et al., 1977); with cathepsin G, it has about a 4-fold higher inhibition rate. We then synthesized Suc-Pro-Leu-PheCH_2Cl as a possible inhibitor. The proper placement of a prolyl residue can be used to introduce selectivity into inhibitors. For example, peptide chloromethyl ketones with a Pro residue in P_3 will not inhibit porcine pancreatic elastase (Powers & Tuhy, 1973), but will inhibit human leukocyte elastase (Tuhy & Powers, 1975). As can be seen from Table V, Suc-Pro-Leu-PheCH_2Cl is very selective for RMCP I. In view of the fact that substrates containing a Pro residue in P_3 are readily hydrolyzed by RMCP II, it is surprising that this inhibitor is not more efficient toward RMCP II. Because the inhibition reaction was carried out at pH 8, however, it is likely that the presence of the succinyl group in P_4 resulted in a relatively high K_i for this inhibitor.

Inhibitors of this type (P_3 Pro) may be useful for elucidating the function(s) of RMCP I and RMCP II, particularly of RMCP I, since rat peritioneal mast cells can be isolated and may be studied in vitro.

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Supplementary Material Available

Experimental details for the synthesis of the new compounds reported, Tables A–C, and Figures A and B (11 pages). Ordering information is given on any current masthead page.

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