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SPECIFICITY OF PORCINE PANCREATIC ELASTASE, HUMAN LEUKOCYTE ELASTASE AND CATHEPSIN G

INHIBITION WITH PEPTIDE CHLOROMETHYL KETONES

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Summary

In order to design more effective elastase (EC 3.4.21.11) inhibitors, the specificity of porcine pancreatic elastase toward peptide substrates was analyzed by examining cleavages of peptides of known sequence which were reported in the literature. Elastase cleaved 99 peptide bonds in the sample of 419 bonds. The cleavage probability (number of peptide bonds cleaved/total peptide bonds in the sample) was highest for peptides with P_1 residue Ile, Val and Ala, the probability being Ile (0.42), Val (0.39) and Ala (0.35). No bonds with a P_3 Pro were cleaved. Based on the observed cleavage probabilities, several new chloromethyl ketones Ac-Ala-Ala-Pro-AACH_2Cl (AA = Ile, Val, Thr) were synthesized. Porcine pancreatic elastase was inhibited most rapidly by the Ile chloromethyl ketone, but there was little difference in rates of inhibition ($k_{obs}/[I]$) between the Ala, Val and Ile inhibitors. The Thr compound was poor. Human leukocyte elastase was inhibited more rapidly by the Ile and Val chloromethyl ketones compared to Ala inhibitor by factors of 49 and 40 respectively at pH 6.5. Excellent correlation was observed between rates of chloromethyl ketone inhibition, peptide nitroanilide hydrolysis and peptide bond cleavage for leukocyte elastase. The water soluble inhibitor MeO-Suc-Ala-Ala-Pro-ValCH_2Cl was found to be the most effective chloromethyl ketone inhibitor of leukocyte elastase yet reported. A number of chloromethyl ketones were surveyed as inhibitors for cathepsin G (a leukocyte chymotrypsin-like enzyme). The most effective was Z-Gly-Leu-PheCH_2Cl. Rates of reaction of several of chloromethyl ketones with bovine trypsin and chymotrypsin were determined to elucidate the specificity of the inhibitors. In addition, the rate of the reaction of MeO-Suc-Ala-Ala-Pro-ValCH_2Cl with glutathione was measured as a model for non-

Abbreviations: Anle = -NHN(n-C_3H_7)CO-; NA, p-nitroanilide; HONbz, p-nitrobenzyl alcohol; XCH_2Cl, The chloromethyl ketone derivative of peptide X.

specific alkylation reactions which might occur when chloromethyl ketones are used in physiological systems. MeO-Suc-Ala-Ala-Pro-ValCH_2Cl reacted over 1770 times faster with leukocyte elastase than with glutathione and would not inhibit cathepsin G. Z-Gly-Leu-PheCH_2Cl inhibited cathepsin G, but not leukocyte elastase. The results demonstrate that it possible to construct highly reactive and selective inhibitors for use in physiological situations.

Introduction

The proteases of human leukocytes have attracted considerable interest recently since it is generally acknowledged that these enzymes are responsible for the tissue destruction that occurs in diseases such as emphysema and bronchitis [1—3]. Several proteolytic enzymes have been isolated from the granule fraction of human leukocytes. These include elastase (EC 3.4.21.11) [4—7], collagenase [8,9] and capthepsin G, a chymotrypsin like enzyme [7,10—14]. Although it is extremely difficult to demonstrate experimentally, elastase and cathepsin G either individually or in concert, are probably responsible for tissue destruction in pulmonary emphysema. Elastase and cathepsin G inhibitors obviously have considerable potential in therapy [15].

Peptide chloromethyl ketones have proved to be very effective inhibitors of porcine pancreatic elastase [16—18], human leukocyte elastase [19] and horse leukocyte elastase [20]. These inhibitors have been used by several investigators to study the physiological and pathological role of elastase [5,21] and to identify elastases or elastase like enzymes obtained from new sources [22,23]. Cathepsin G on the other hand has been reported to be unreactive toward Tos-PheCH_2Cl but could be inhibited slowly by one of our chymotrypsin inhibitors [10,7,24].

This study reports an analysis of the specificity of porcine pancreatic elastase which led to the belief that this enzyme might prefer residues besides alanine as the P_1 residue (nomenclature of Schechter and Burger [25]) of a substrate. This data was utilized in designing and synthesizing several new chloromethyl ketone elastase inhibitors which were tested with both porcine pancreatic and human leukocyte elastase. Since little specificity data is available for cathepsin G, a variety of peptide chloromethyl ketones have been surveyed for the most effective inhibitor. The specificity of some of the inhibitors for elastase, cathepsin G, chymotrypsin and trypsin are reported. The reaction of glutathione with a chloromethyl ketone was also measured as a model for possible side reactions which might occur when these compounds are used under physiological situations.

Materials and Methods

Porcine pancreatic elastase (lot 55B405) was obtained from Whatman Biochemicals Ltd. and used without further purification; its substrate, Boc-Ala-ONp from Sigma Chemical Co. Human neutrophil leukocyte elastase was gratuitously provided by Dr. A. Janoff at S.U.N.Y. at Stony Brook and by Drs. J. Travis and R. Baugh of the U. of Georgia. Cathepsin G was also kindly

provided by Drs. Travis and Baugh and its substrate, Boc-Tyr-ONp, was obtained from Bachem. Chymotrypsin A_α (lot CDIOBK) was obtained from Worthington Biochemical Co., and its substrate, Ac-Tyr-OEt, was synthesized in our laboratory and had m.p. 80—81°C. Trypsin (lot 102C-1920) and its substrate, Bz-Arg-OEt, were obtained from Sigma Chemical Co. All other reagents and solvents were analytical grade.

Chloromethyl ketone inhibitors. The synthesis of many of the peptide chloromethyl ketone inhibitors utilized in the present study are reported in earlier papers [16,24,26]. The procedure for the synthesis of Ac-Ala-Ala-Pro-ValCH_2Cl is given below. Ac-Ala-Ala-Pro-IleCH_2Cl (m.p. 207—208°C (d), recrystallized from methanol) and Ac-Ala-Ala-Pro-ThrCH_2Cl (m.p. 200—201°C (d), recrystallized from ethyl acetate/methanol (100:1)) were prepared by a similar procedure. All three compounds were pure by thin layer chromatography on Merck Silica Gel G plates and had combustion analyses, mass spectra and proton magnetic resonance (PMR) spectra which were consistent with the assigned structres. Suc-Ala-Ala-Pro-ValCH_2Cl (m.p. 99—102°C, recrystallized from ethanol) was prepared from Boc-Ala-Ala-Pro-ValCH_2Cl (m.p. 172—173°C (d), recrystallized from methanol/ether) by removal of the Boc group with HCl in dioxane followed by succinylation with succinic anhydride. The methyl ester MeO-Suc-Ala-Pro-ValCH_2Cl (m.p. 133—134°C) was prepared from Suc-Ala-Ala-Pro-ValCH_2Cl by treatment with methanol and HCl in dioxane for 1 h. The analysis and PMR spectra were consistent with the assinged structures. Both the succinyl and methyl succinyl inhibitors were water-soluble with the methyl succinyl being completely soluble to about 1 mM concentrations.

N-acetyl-L-alanyl-L-alanyl-L-prolyl-L-valine chloromethyl ketone (Ac-Ala-Ala-Pro-ValCH_2Cl. The diazo ketone Boc-ValCHN_2 was prepared from Boc-Val-OH (10.0 g, 45 mmol) and diazomethane using a mixed anhydride method [27]. Anhydrous HCl was bubbled through the solution of Boc-ValCHN_2 in ether/tetrahydrofuran at 5°C for 15 min as the solution turned from yellow to colorless. L-Valine chloromethyl ketone hydrochloride was isolated, recrystallized from THF/ether and dried in vacuo to give 4.5 g (55%) of an extremely hygroscopic white solid.

Ac-Ala-Ala-Pro-OH (1.5 g, 5 mmol) was dissolved in 50 ml anhydrous tetrahydrofuran and stirred at 15°C while *N*-methylmorpholine (0.55 ml, 5 mmol) and isobutyl chloroformate (0.65 ml, 5 mmol) were added [28]. After 10 min, the *N*-methylmorpholine salts were filtered and H-Val$CH_2Cl \cdot HCl$ (0.93 g, 5 mmol in 5 ml DMF) and *N*-methylmorpholine (0.55 ml, 5 mmol) were added. Stirring was continued for 4 h as the mixture warmed to 25°C. Then the solvent was removed in vacuo and the resulting yellow oil was dissolved in methanol and chromatographed on Merck silica gel 60 (0.063—0.2 mm). The product was isolated after elution with 8% methanol in chloroform. Recrystallization from methanol gave 1.1 g (49%) of white solid, m.p. 195—196°C (d), R_F 0.41 ($CHCl_3/CH_3OH$, 9:1).

Analysis Calculated for $C_{19}H_{31}N_4O_5Cl$: C, 52.96; H, 7.20; N, 13.00. Found: C, 52.67; H, 7.30; N, 12.96.

Enzyme assays. The activity of porcine pancreatic and human leukocyte elastase was measured using the Boc-Ala-ONp spectrophometric assay [29]. Chymotrypsin was assayed using a Radiometer automated pH-stat to measure

the initial hydrolysis of 0.01 M Ac-Tyr-OEt at pH 7.8 using 0.5420 M NaOH as the titrant [30]. Trypsin was assayed similarly using Bz-Arg-OEt as the substrate [31]. Initial velocities were obtained from the pH-stat recorder tracing of base consumption with respect to time [32].

Cathepsin G assay. The activity of cathepsin G was measured with the substrate Boc-Tyr-ONp. The substrate stock solution consisted of 0.056 mM Boc-Tyr-ONp in 1.0 M NaCl/0.05 M sodium citrate buffer (pH 6.5) containing 17% ethanol and was good for up to 2 h. If the buffer was prepared with 10% dioxane instead of ethanol, the rate was decreased by 30% and the substrate solution was less stable. To each of the two standard ultraviolet cells was added 2.0 ml of the substrate stock solution. A 50-μl portion of 0.1 M NaCl/0.04 M KH_2PO_4 buffer (pH 7.5) was added to the reference cell followed by the addition of a 50-μl aliquot of the enzyme solution to the sample cell and the production of *p*-nitrophenol measured at 347.5 nm. The final concentrations were as follows: 0.05 mM substrate, $5.1 \cdot 10^{-3}$ μM enzyme, 0.05 M sodium citrate buffer (pH 6.5), 1.0 M NaCl, 17% ethanol (v/v). The activity towards Boc-Tyr-ONp was found to be directly proportional to enzyme concentration over a range of $1.6 \cdot 10^{-3}$ to $1.8 \cdot 10^{-2}$ μM enzyme. The specific activity for this method was found to be $4.1 \cdot 10^2$ nkat/mg of enzyme. The enzyme concentraton was determined from the absorbance at 280 nm based on $E_{1\%} = 6.64$. The activity of the enzyme was 92% by active site titration with Ac-Ala-Ala-Anle-ONp (Powers, J.C. and Gupton, B.F., Unpublished) which gives a corrected specific activity of $4.5 \cdot 10^2$ nkat/mg of enzyme.

Reaction of proteases with inhibitors. All inhibition reactions were carried out under psuedo first order conditions with the inhibitor concentration at least 5-fold (and usually more) greater than the enzyme concentration. The inhibitor was dissolved in methanol and then diluted with the appropriate buffer. Aqueous solutions of the inhibitors were used the same day to avoid possible erros due to hydrolysis of the chloromethyl ketones. The inhibition reaction was started by mixing the inhibitor solution and the enzyme solution, and was followed for at least two half-lives. The final concentrations of enzyme, inhibitor, organic solvent and buffers are given in the tables. Enzyme concentrations were determined by ultraviolet absorbance at 280 nm. At periodic time intervals, aliquots were removed from the inhibition solution and rapidly mixed with the assay solution to determine the residual enzyme activity. Kinetic results for each inhibitor were calculated from the equation

$$v_{\text{inhibition}} = k_{\text{obsd}} [\text{E}]$$

with the aid of a line-fitting computer program as described earlier [16]. Correlation coefficients of greater than 0.995, 0.998, 0.995, and 0.991 were obtained respectively for porcine pancreatic elastase, human leukocyte elastase, cathepsin G, and chymotrypsin inhibition reactions. The second order rate constant $k_{\text{obsd}}/[\text{I}]$ is suitable only for the purpose of calculating approximate reactivity among inhibitors, since $k_{\text{obsd}}/[\text{I}]$ becomes non-linear with [I] when [I] becomes close to K_{I}, the dissociation constant of the enzyme-inhibitor complex [16].

Reaction of glutathione with MeO-Suc-Ala-Ala-Pro-ValCHCl. Glutathione (0.1 mM) and MeO-Suc-Ala-Ala-Pro-ValCH_2Cl (1 mM) in 0.05 M phosphate

buffer pH 7.5 was incubated at 30°C while bubbling N_2 through the solution. After appropriate intervals, 2-ml aliquots were withdrawn and the remaining thiol groups were measured employing 5,5-dithiobis-(2-nitrobenzoic acid) at 412 nm [33]. No autoxidation of glutathione was observed during the incubation. The reaction proceeded with psuedo first order kinetics and the results were treated in the same manner as the protease chloromethyl ketone reactions.

Results and Discussion

The reactivity of peptide chloromethyl ketones with serine proteases has been shown to closely parallel the reactivity of the corresponding substrates [24,26,16,18]. In the case of porcine pancreatic elastase, peptide chloromethyl ketones with Ala as the P_1 residue have been synthesized and shown to be irreversible inhibitors of this enzyme [16—18]. The choice of Ala as the P_1 residue was based on reports in the literature that small synthetic substrates with a P_1 Ala were hydrolyzed most rapidly by porcine elastase [34]. Since it was possible that the reactivity of elastase toward larger peptide substrates and small synthetic substrates might be different, we decided to analyze the specificity of elastase toward peptides with the expectation that the data would be useful in the design of new inhibitors.

Primary specificity of porcine elastase. Papers published in the major biochemical journals during 1967—72 and a few of more recent origin were searched for peptides of defined structure which had been digested with elastase in the course sequence studies. The sample, which was collected in this manner, contained 419 peptide bonds of which 99 (24%) were cleaved by elastase. Since so few elastase cleavages of peptides have been reported in the literature, we utilized the sequences of peptides obtained from an elastase digestion of dihydrofolate reductase [35] and thus the sequence of some of the 99 bonds cleaved, is known on only one side of the split peptide bond.

Table I lists the probability of cleavage of any particular AA-X peptide bond, the base probability where AA equals any amino acid residue is 0.24. Cleavage probabilities are potentially related to rates of peptide bond hydrolysis. Bonds with high hydrolysis rates are more likely to be hydrolyzed to a great enough extent to be recognized as being cleaved during the course of an elastase digestion than those with low hydrolysis rates. If enough bonds are considered, any affects due to unusual sequences in the sample or sampling problems would be eliminated and the correlation between cleavage probabilities and hydrolysis rates should be quite good. However 99 bonds cleaved is a fairly small sample and one additional bond cleaved or not cleaved would change the relative cleavage probabilities of some amino acids. For example the presence of Trp high on the list is surprising since elastase's S_1 binding pocket is not large enough to accommodate such a bulky residue [36]. Since all three Trp cleavages appeared in one paper [35], we are inclined to discount this result even though a highly purified elastase sample was utilized. Although other such pertibations may exist, the data is quite useful for suggesting future experiments.

The preference at P_1 in a polypeptide substrate for elastase is Ile > Val > Ala > Trp > Thr > Leu. This is in contrast to the order observed in the hydrolysis rates of Z-AA-ONp where Ala > Leu > Gly > Val > Ile [34]. A similar result was observed in elastase hydrolysis of Bz-AA-OMe where AA = Ala > Leu ≅ Val ≅ Ile [37]. With peptide p-nitrobenzyl esters H-Ala_n-AA-ONbz, the order was AA = Ala > Leu > Val > Gly > Phe for n = 1 or 2. When n = 3, Ala ≅ Leu [38]. With the peptide *p*-nitroanilides Ac-Ala-Ala-Pro-AA-NA, the order AA = Ala > Leu > Val was observed [39]. In almost of all of the above examples, there were substantial rate differences between the peptide with Ala at P_1 and its nearest competitor. Evidently there is either little correlation between cleavage probabilties and elastase hydrolysis rates or the relative rates of bond cleavage by elastase of peptide substrates differ signficantly from those observed in the small substrates investigated to this point. Further experiments are necessary to clarify the rationale for this difference.

The P_1 preference of porcine pancreatic elastase is Glu (0.43) > Met (0.40) > Arg (0.33) > Phe (0.29) > Lys (0.24) > Asp (0.23) > Ala (0.21) = Asn (0.21) > Trp (0.20) = Gln (0.20). With regard to the specificity at other subsites, the sample in most cases is insufficiently large to permit valid correlations. However one observation should be permitted us. Out of 19 -Pro-AA-AA*AA- bonds in the sample (AA = any amino acid, asterisk indicates cleavage site), not one was cleaved compared to an expected 5 computed using 0.24 as the cleavage probability of any bond in the sample. This observation was signficant by a chi squared test at the 95% confidence level. Of course a Pro at P_3 in an synthetic elastase substrate or inhibitor is known to prevent hydrolysis or inhibition [40,16].

Porcine pancreatic elastase inhibitors. The inhibitors Ac-Ala-Ala-Pro-AACH_2Cl (AA = Ile, Val, and Thr) were synthesized with the expectation that their order of reactivity would closely follow the probabilities listed Table I. In fact the most reactive inhibitor is the Ile compounds Ac-Ala-Ala-Pro-IleCH_2Cl which is slightly faster than our previous best inhibitor Ac-Ala-Ala-Pro-AlaCH_2Cl (Table II). The variation between the Ala, Val and Ile chloromethyl ketones is very slight. On the other hand, the Thr inhibitor which is isosteric with the Val inhibitor, is 450 times less realtive. Either the Thr OH is hydrogen-bonded with the carbonyl group of the chloromethyl ketone moiety resulting in a less reactive conformation for the alkylation reaction or the Thr side chain OH hydrogen bonds with some group on the enzyme producing a less reactive enzyme-inhibitor complex than in the case of the Val compound. No significant effect is observed in the inhibition rate when the P_5 residue is changed from an acetyl to a succinyl or methyl succinyl moiety.

Human leukocyte elastase inhibitors. The rates of inhibition of human leukocyte elastase with a series of peptide chloromethyl ketones at pH 6.5 and 7.5 are listed in Table III. The relative rates (at pH 6.5) are also listed using the $k_{obsd}/[I]$ for Ac-Ala-Ala-Pro-AlaCH_2Cl, the previous best inhibitor for this enzyme [19], as the standard. Once again the Thr compound is unreactive toward the human elastase, but the Val and Ile chloromethyl ketones are respectively 49 and 40 times more reactive at pH 6.5 than the Ala chloromethyl ketone. The difference between the Val and Ala inhibitors is slightly less marked (23 fold) at pH 7.5. These results correspond nicely with rates of

TABLE I

PROBABILITY OF PORCINE ELASTASE CLEAVAGE OF ANY AA-X BOND

AA residue	Probability *	Number of bonds in sample
Ile	0.42	12
Val	0.39	39
Ala	0.35	34
Trp	0.30	10
Thr	0.29	24
Leu	0.27	41
Gln	0.27	15
Ser	0.18	17
Tyr	0.17	12
Phe	0.12	17
Lys	0.12	17
Asn	0.07	14
Arg	0.06	15
Pro	0.05	20
Gly	0.04	24
His	0	11
Met	0	5
Asp	0	13
Glu	0	14

* The probability of a particular bond being cleaved is the number of AA-X bonds split/total of AA-X bonds observed in sample.

Ac-Ala-Ala-Pro-AA-NA hydrolysis where the substrates with AA=Val or Ile are respectively 8 and 1.5 times more rapidly hydrolyzed than the Ala p-nitroanilide [39]. In addition, a preference for Val over Ala at P_1 has been observed in the digestion of the oxidized insulin B chain with human lysosomal elastase [41]. Thus a direct correlation is observed between chloromethyl ketone inhibition, *p*-nitroanilide hydrolysis and peptide bond cleavage.

Comparison of the inhibition rates of porcine pancreatic and human leukocyte elastase points to significant differences in the S_1-binding subsite of the two enzymes. The human enzyme prefers much large side chains in the S_1 site than the porcine enzyme. Distinguishing differences in inhibition behavior between the human and porcine enzymes have also been observed at subsites other than S_1[19] and with inhibitors other than chloromethyl ketones

TABLE II

INHIBITION OF PORCINE PANCREATIC ELASTASE WITH PEPTIDE CHLOROMETHYL KETONES

Elastase concentration 5 μM, 0.05 M acetate buffer at pH 5.0, 30°C, 5% (v/v) methanol.

Inhibitor	[I] (mM)	$k_{obsd}/[I]$ ($M^{-1} \cdot s^{-1}$)	$k_{obsd}/[I]$ (rel.)
Ac-Ala-Ala-Pro-AlaCH_2Cl	0.05	40 *	1.0
Ac-Ala-Ala-Pro-ValCH_2Cl	0.05	35 *	0.9
Suc-Ala-Ala-Pro-ValCH_2Cl	0.05	73 *, **	1.8
MeO-Suc-Ala-Ala-Pro-ValCH_2Cl	0.05	55 *, **	1.4
Ac-Ala-Ala-Pro- IleCH_2Cl	0.05	48 *	1.2
Ac-Ala-Ala-Pro-ThrCH_2Cl	5.0	0.08 *	0.002

* Average of three runs.

** No methanol was used in the buffer.

TABLE III

INHIBITION OF HUMAN LEUKOCYTE ELASTASE WITH PEPTIDE CHLOROMETHYL KETONES

pH 6.5 0.1 M phosphate, 0.06 M NaCl, elastase 10 μM, 30°C, 5% (v/v) methanol; pH 7.5 0.05 M phosphate 0.035 M NaCl elastase 2.1 μ M, 30°C, 4.3% (v/v) methanol.

Inhibitor P_4 P_3 P_2 P_1	pH	[I] (mM)	$k_{obsd}/[I]$ ($M^{-1} \cdot s^{-1}$)	$k_{obsd}/[I]$ (rel.)
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	6.5	0.20	3.4	
	6.5	0.40	3.3	1.0
	7.5	0.11	9.7	
Ac-Ala-Ala-Pro-$ValCH_2Cl$	6.5	0.05	160 *	49
	7.5	0.10	219	
Suc-Ala-Ala-Pro-$ValCH_2Cl$ **	6.5	0.025	320	97
	7.5	0.025	1400	
MeO-Suc-Ala-Ala-Pro-$ValCH_2Cl$ **	6.5	0.025	922	280
	7.5	0.025	1560	
Ac-Ala-Ala-Pro-$IleCH_2Cl$	6.5	0.05	133 ***	40
Ac-Ala-Ala-Pro-$ThrCH_2Cl$	6.5	10.0	0.15	0.05
Z-Gly-Leu-$PheCH_2Cl$	7.5	0.10	0 †	

* Average of three runs.

** 0.1 M phosphate, 0.035 M NaCl, elastase 0.94 μM, 30°C.

*** Average of two runs.

† Enzyme showed no measurable decrease in activity over a 4-day period.

[42,39]. It is now clear that the human enzyme prefers a P_2 Leu or Pro in a substrate. The best inhibitors contain one of these two residues and two of the major cleavages in the oxidized insulin B chain had a P_2 Leu (and a P_1 Val) and the anomalous minor cleavage also occurred at a site with a P_2 Leu (P_1 was cysteic acid) [41].

The observation that Suc-Ala-Ala-Pro-$ValCH_2Cl$ and MeO-Suc-Ala-Ala-Pro-$ValCH_2Cl$ rapidly inhibited elastase was quite astonishing to us. These inhibitors, which were synthesized to increase water solubility, are 7—8 times more reactive than the acetyl compound at pH 7.5. MeO-Suc-Ala-Ala-Pro-$ValCH_2Cl$ is the best leukocyte elastase chloromethyl ketone inhibitor thus far reported. It would appear that the S_5 subsite of leukocyte elastase contains some structural feature which interacts favorably with a succinyl or methyl succinyl grouping. This points out another significant difference between the two enzymes, since the change from acetyl to succinyl or methyl succinyl has a relatively minor affect on the rate of inhibition of the porcine enzyme.

Human cathepsin G. The effectiveness of over a dozen chloromethyl ketone inhibitors of human cathepsin G (leukocyte chymotrypsin-like enzyme) are listed in Table IV. The $k_{obsd}/[I]$ values are reported using the elastase inhibitor Ac-Ala-Ala-Pro-$AlaCH_2Cl$ as a standard. The most effective inhibitor is Z-Gly-Leu-$PheCH_2Cl$ which is also an effective inhibitor of bovine chymotrypsin [24]. Both the P_1 Phe (ratio of $k_{obsd}/[I]$ for Z-Gly-Leu-$PheCH_2Cl$/Z-Gly-Leu-$AlaCH_2Cl$ = 20) and the P_2 Leu (ratio for Z-Gly-Leu-$PheCH_2Cl$/Z-Gly-Gly-$PheCH_2Cl$ = 12.5) are important features of this inhibitor. The P_4 benzyloxycarbonyl (Z) group has an insignificant effect (Z-Gly-Gly-$PheCH_2Cl$/Ac-Ala-Gly-$PheCH_2Cl$ = 1). Except for this one inhibitor (Z-Gly-Leu-$PheCH_2Cl$) there is very little spread in the reactivities of the other inhibitors tested.

TABLE IV

REACTION OF HUMAN LEUKOCYTE CATHEPSIN G WITH PEPTIDE CHLOROMETHYL KETONES

Cathepsin G concentration 0.21 μM, 0.02 M phosphate buffer at pH 7.5, 1.0 M NaCl, 30°C, 4.3% (v/v) methanol.

Inhibitor	[I] (μM)	$k_{obsd}/[I]$ ($M^{-1} \cdot s^{-1}$)	$k_{obsd}/[I]$ (rel.)
P_4 P_3 P_2 P_1			
Z-Gly-Leu-Ala CH_2Cl	9.7	2.6	1.3
Ac-Ala-Ala-Pro-Ala CH_2Cl	9.8	2.0	1.0
Ac-Ala-Ala-Pro-Val CH_2Cl	10.1	3.7	1.8
MeO-Suc-Ala-Ala-Pro-Val CH_2Cl	10.0	0 *	0
Ac-Ala-Ala-Pro-Ile CH_2Cl	9.2	4.2 **	2.1
Ac-Phe-Gly-Ala Leu CH_2Cl	9.5	3.2 **	1.6
Ac-Ala-Phe CH_2Cl	13.2	3.5 **	1.8
$(CH_3)_2$)$CHCH_2CO$-Ala-Phe CH_2Cl	11.2	6.2	3.1
Z-Leu-Phe CH_2Cl	10.8	4.7	2.3
Z-Gly-Gly-Phe CH_2Cl	9.2	4.1 **	2.1
Ac-Ala-Gly-Phe CH_2Cl	11.8	4.0	2.0
Z-Gly-Leu-Phe CH_2Cl	8.2	51.2	26.0
Ac-Gly-Gly-Ala-Phe CH_2Cl	10.0	6.8 **	3.4
Z-Trp CH_2Cl	11.1	5.7 **	2.9

* Enzyme showed no measurable decrease in activity over a 2 day period.

** Approximate value based on three data points.

The inhibition of cathepsin G by Z-PheCH_2Cl and Z-Gly-Gly-PheCH_2Cl has been reported [10]. The observed rate for Z-Gly-Gly-PheCH_2Cl was approximately 1/4 of that reported in this study, probably due to high DMSO concentration utilized. The more reactive Z-PheCH_2Cl had a $k_{obs}/[I]$ of approx. 1.8. Tos-PheCH_2Cl has been reported by two groups to have no affect on cathepsin G [10,7].

Cathepsin G cleavages of the oxidized insulin B chain occur at sites with a P_1 Leu, Tyr or Phe [43]. In the case of *p*-nitroanilides Ac-Ala-Ala-Pro-AA-NA, hydrolysis occurred only with the AA=Phe and Leu, *p*-nitroanilides with the Ala, Val and Ile being untouched by the enzyme [39]. Chloromethyl ketones and the *p*-nitroanilide with a P_1 Phe were the most reactive. With substrates a change in the P_1 residue can determine whether hydrolysis occurs or not. For example the Ile *p*-nitroanlide is not cleaved while the Leu analog is a good substrate. In the case of chloromethyl ketones, changes at P_1 have little effect while subsite interactions are more significant. For example a P_5 change of an acetyl to methyl succinyl group prevents inhibition and the various features of Z-Gly-Leu-PheCH_2Cl (discussed above) accelerate the inhibition rate. The data suggest that differences are not simply due to changes in the binding of the inhibitors (K_I) or substrates (K_S), but are due to significant differences in k_{cat} (substrates) or k_2 (the limiting rate of inactivation for chloromethyl ketones). Or in other words, subsite interactions are affecting the catalytic site and binding alone cannot explain the observed differences. Future experiments may clarify this point.

Specificity of the inhibitors. In order to determine how specific the various inhibitors were for a particular enzyme, we measured the rates of inhibition of

TABLE V

INHIBITION OF TRYPSIN AND CHYMOTRYPSIN WITH PEPTIDE CHLOROMETHYL KETONES

Enzyme concentration 10 μM, 0.1 M citrate buffer pH 6.5, 30°C, 5% (v/v) methanol.

Inhibitor	[I](mM)	Trypsin activity (%) *	Chymotrypsin	
P_4 P_3 P_2 P_1			$k_{obsd}/[I]$ ($M^{-1} \cdot s^{-1}$)	rel. value
Ac-Ala-Ala-Pro-AlaCH_2Cl	0.20	87	1.1	1.0
Ac-Ala-Ala-Pro-ValCH_2Cl	0.05	90	2.0	1.8
Ac-Ala-Ala-Pro-Ile CH_2Cl	0.05	88	6.4	5.8
Ac-Ala-Ala-Pro-ThrCH_2Cl	10	89	0.05	0.05
Z-Gly-Leu-PheCH_2Cl	—	—	2.99 **	
none		89		

* Enzyme activity after 4 h measured as percent of initial activity at $t = 0$.

** pH 5.8, 30% (v/v) 1,2 dimethoxyethane [24]. This would be expected to be 2 or 3 times higher at pH 6.5.

trypsin and chymotrypsin with the new inhibitors synthesized (Tabel V). As expected none of chloromethyl ketones inhibited trypsin even after long incuation periods. Some of the inhibitors reacted with bovine chymotrypsin.

It is now possible to inhibit selectively the two enzymes of human leukocytes. Z-Gly-Leu-PheCH_2Cl rapidly inhibits cathepsin G but showed no reactivity toward elastase under identical conditions. This inhibitor will also react with pancreatic chymotrypsin, but not trypsin. The most effective inhibitor of the leukocyte elastase is MeO-Suc-Ala-Ala-Pro-ValCH_2Cl. This inhibitor does not inhibit cathepsin G. Less discrimination is observed in the case of Ac-Ala-Ala-Pro-ValCH_2Cl (elastase/cathepsin G = 219/3.7) and Ac-Ala-Ala-Pro-AlaCH_2Cl (9.7/2.0). On the other hand if the goal is inhibition of both enzymes, Ac-Ala-Ala-Pro-ValCH_2Cl would probably be the most suitable since it is relatively effective at inhibiting both enzymes.

Under physiological conditions, chloromethyl ketones can react not only with serine proteases but with other nucleophilic groups. In particular, alkylation of sulfhydryl groups could be a significant side reaction. In order to evaluate the significance of such side reactions, the rate of reaction of glutathione with MeO-Suc-Ala-Ala-Pro-ValCH_2Cl was measured at pH 7.5 (The reaction of Tos-LysCH_2Cl with glutathione has also been studied but no rates have been reported [43]). The $k_{obs}[I]$ was 0.88. This means that the inhibitor would descriminate in favor of leukocyte elastase over glutathione by a factor of 1770 if the concentrations were equivalent. The results obtained with MeO-Suc-Ala-Ala-Pro-ValCH_2Cl demonstrate that it is quite feasible to construct a highly reactive and selective inhibitor for use in physiological situations. However, it should be noted that cellular thiol proteases such as cathepsin B1 also can react with chloromethyl ketones [45] and it remains to be seen whether an inhibitor can be constructed which would be able to discriminate between elastase and cellular thiol proteases.

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