Biochimica et Biophysica Acta, 525 (1978) 171—179

BBA 68478

SPECIFICITY AND REACTIVITY OF HUMAN GRANULOCYTE ELASTASE AND CATHEPSIN G, PORCINE PANCREATIC ELASTASE, BOVINE CHYMOTRYPSIN AND TRYPSIN TOWARD INHIBITION WITH SULFONYL FLUORIDES

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(Received November 28th, 1977)
(Revised manuscript received March 6th, 1978)

Summary

Human granulocyte elastase (EC 3.4.21.11) and cathepsin G (EC 3.4.21.20), porcine pancreatic elastase (EC 3.4.4.7), and bovine chymotrypsin-A_α (EC 3.4.4.3) and trypsin (EC 3.4.4.4) are inhibited by aryl and alkyl sulfonyl fluorides at different rates. The 26 000-fold variance in inhibition rates reflects the intrinsic reactivity and specificity differences of these closely related serine proteases. Human granulocyte elastase is inhibited more rapidly than porcine pancreatic elastase by all of the sulfonyl fluorides studied. In contrast, human cathepsin G reacts more slowly with the series of sulfonyl fluorides than does bovine chymotrypsin-A_α. Trypsin is the least reactive of the five enzymes, reflecting its relatively more stringent specificity requirements. Reagents such as CH_3SO_2F, 4-$CH_3C_6H_4SO_2F$, 3-$CH_3COC_6H_4SO_2F$ and 2-$NH_2C_6H_4SO_2F$ exhibit little or no selectivity toward the five enzymes studied. On the other hand, phenylmethane sulfonyl fluoride shows a range of reactivity of 137-fold toward the various proteases and is most reactive with bovine chymotrypsin-A_α. The peptide sulfonyl fluoride 2-(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$ had a reactivity range of 41, with granulocyte elastase being inhibited most rapidly. Although none of the sulfonyl fluorides are completely specific, the examples reported in this paper show that structural changes have considerable potential for increasing both the selectivity of sulfonylating agents toward various serine proteases and their reactivities.

Abbreviations: Tris · HCl, tris-(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Introduction

The neutral proteases isolated from the granule fraction of human polymorphonuclear leukocytes have been the objects of much research because of their acknowledged involvement in diseases such as emphysema and bronchitis [1—3]. Elastase [4,5] and a chymotrypsin-like enzyme, cathepsin G [6—9], are found in the granules of human polymorphonuclear leukocytes. The selective and specific inhibition of these enzymes has been studied because of the potential such inhibitors would have as therapeutic agents in the control of these diseases [10].

Granulocyte elastase and cathepsin G are serine proteases. Much information has been gathered over many years of study of this family of enzymes and their reaction mechanism is now well understood [11]. Serine proteases are inhibited by peptide chloromethyl ketones [12,13] and by various alkyl and aryl sulfonyl fluorides. The latter reaction occurs with the active site serine to yield a sulfonylated enzyme which is no longer catalytically active [14—17]. A study of the rates of inhibition of the human granulocyte enzymes by a series of related reagents should yield further insight into the relative reactivities and specificities of these important enzymes. Information obtained from such studies should be useful in the design of more specific and selective agents of potential therapeutic and investigative value. In an effort to further the understanding of the reactivity differences of these important granulocyte enzymes, a systematic study of the reactions of a series of sulfonyl fluorides with human granulocyte elastase and cathepsin G, bovine chymotrypsin-A_α and trypsin and porcine pancreatic elastase was undertaken.

Materials and Methods

Reagents used in this study were obtained from the following sources: *p*-toluenesulfonyl fluoride, 3-acetylbenzenesulfonyl fluoride, and 2-aminobenzenesulfonyl fluoride from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin; methanesulfonyl fluoride and phenylmethanesulfonyl fluoride from Eastman Kodak Co., Rochester, N.Y.; Boc-L-Ala-ONp and Boc-L-Tyr-ONp from BACHEM, Inc., Torrance, Calif.; Bz-L-Arg-OEt · HCl and twice crystallized, lyophilized bovine trypsin (lot No. 102C-1920) from Sigma Chemical Co., St. Louis, Mo.; porcine pancreatic elastase (Lot No. 56H577) and 6 times crystallized, salt-free bovine chymotrypsin-A_α (Lot No. 111C-8170) from Worthington Biochemical Corporation, Freehold, N.J. All common chemicals and solvents used were reagent grade. Human granulocyte elastase and cathepsin G preparations were generously supplied by Drs. J. Travis and R. Baugh of the University of Georgia, Athens, Ga. Elemental microanalyses were performed by Atlantic Microlabs of Atlanta, Ga.

Preparation of 2-(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$

2-Fluorosulfonylphenyl isocyanate was prepared by refluxing 2-aminobenzenesulfonyl fluoride (4.4 g, 25 mmol) in 12.5% phosgene in benzene (125 ml) for 2 h. A crude solid product was obtained upon evaporation of the benzene at 45°C. Low pressure distillation of the crude product yielded 2.6

gm (51%) of a white solid, m.p. 59.5—62°C. The product showed a strong infrared isocyanate band at 2250 cm^{-1} and was used without subsequent purification. 2-Fluorosulfonylphenyl isocyanate (0.52 g, 2.6 mmol) and Ac-Ala-Ala-NH-$NHCH_3$ [18] (0.59 g, 2.6 mmol) in 50 ml tetrahydrofuran (freshly distilled over sodium) were stirred for 12 h at 25°C, refluxed for 2.5 h, filtered and the solvent was removed by rotary evaporation yielding a crude white solid. The product was twice recrystallized from hot methanol yielding 1 g (88%) of colorless needle crystals, m.p. 234—236°C, decompn. The product migrated as a single spot on thin layer silica gel chromatography developed in *n*-butanol : acetic acid : water (4 : 1 : 2, v/v) and was visualized by spraying with 10% $FeCl_3$ followed by 5% potassium ferricyanide [19]. Hydrazides and their derivatives yield a deep blue color when treated with these reagents. The NMR spectrum was consistent with the assigned structure. Elemental analysis: calculated for $C_{16}H_{22}FN_5O_6S$: C, 44.54; H, 5.14; N, 16.23; S, 7.43; found: C, 44.46; H, 5.14; N, 16.04; S, 7.40.

Enzyme assays

All enzyme assays were performed spectrophotometrically using a Beckman model 25 spectrophotometer with a thermostated cell compartment maintained at 25°C. Human granulocyte elastase and porcine pancreatic elastase activities were monitored using the chromogenic substrate Boc-L-Ala-ONp [20]. Human granulocyte cathepsin G and bovine chymotrypsin were assayed using Boc-L-Tyr-ONp [21]. Trypsin activity was determined spectrophotometrically using Bz-L-Arg-OEt · HCl [22]. Because the 1.0 mM Bz-Arg-OEt used in the reported method had an absorbance at 253 nm very near the upper detection limit of the spectrophotometer and because hydrolysis of the substrate leads to an increase in this already too high absorbance, the substrate concentration was reduced to 0.50 mM.

Protein concentrations were determined by ultraviolet absorbance using the following values for $E^{1\%}_{1cm,280}$: porcine elastase, 22.2 [23]; human granulocyte elastase, 9.85 [4]; human granulocyte cathepsin G, 6.64 [21]; chymotrypsin-A_α, 20.5 [24]; and trypsin, 15.4 [25]. The cathepsin G concentration was also calculated using the reported specific activity of 450 nkat/mg [21] and indicated that the enzyme preparation was 53% active.

Stock solutions of porcine elastase, trypsin, and chymotrypsin were prepared in 1 mM HCl. Leukocyte elastase was stored in 0.05 M citrate (pH 5.0) containing 0.4 M NaCl. Cathepsin G was stored in 0.05 M Tris · HCl (pH 7.0) containing 0.8 M NaCl. All enzyme solutions were kept at 4°C.

Inhibition reactions

All reactions were performed at 30°C and pH 7.50 in a constant temperature circulator. The reaction buffer (0.80 ml) and 200 μl stock enzyme solution were incubated for at least 10 min prior to the addition of 100 μl stock inhibitor solution in methanol. The final concentration of methanol was 9.1% (v/v). The concentration of inhibitor was varied from 0.025 to 18 mM, depending on the rate of the particular reaction. The final compositions of the reaction mixtures for the enzymes were: (1) 3 μM porcine elastase, 0.10 M HEPES; (2) 1 μM human granulocyte elastase, 0.10 M HEPES, 0.3 M NaCl; (3) 1 μM human

cathepsin G, 0.10 M HEPES, 1.0 M NaCl; (4) 1 μM chymotrypsin, 0.10 M HEPES, 1.0 M NaCl; (5) 1 μM trypsin, 0.05 M Tris · HCl, 0.01 M $CaCl_2$.

At appropriate time intervals, aliquots were removed from the reaction mixture and rapidly diluted into the assay solution so that the residual enzyme activity could be determined. The initial enzyme rates thus determined were evaluated as pseudo-first order rates according to the equation

$$v_{initial} = k_{obsd}\,[E].$$

The pseudo-first order rate constants were calculated from plots of ln $v_{initial}$ vs. time using a standard linear regression analysis method. Correlation coefficients were all greater than 0.995 in the data sets reported. The pseudo-second order rate constant, $k_{obsd}/[I]$, is appropriate only for comparison of relative reactivities of the different inhibitors since $k_{obsd}/[I]$ varies with [I] when the inhibitor concentration chosen is in the region of K_I, the true dissociation constant of the enzyme · inhibitor complex [26].

Results and Discussion

Sulfonyl fluorides are active-site-directed irreversible inhibitors for the serine proteases [14—17,27—30]. These reagents have been shown to be very specific and not to react to any significant degree with sites other than the active site serine even when high concentrations are used for long periods of time [29]. A number of the sulfonyl derivatives formed have been investigated by X-ray crystallography. These include tosyl bovine chymotrypsin-A_α [31] and tosyl porcine elastase [32]. In this paper we report a study of the reactions of a variety of sulfonyl fluorides with the granulocyte serine proteases elastase and cathepsin G and a systematic study of the specificity of these reagents.

All of the serine proteases examined in this study were irreversibly inhibited by all of the sulfonyl fluorides examined. However, significant differences in the rates of reaction were observed. The slowest reaction was the inhibition of cathepsin G by methanesulfonyl fluoride, while the fastest reaction was that of phenylmethanesulfonyl fluoride with bovine chymotrypsin-A_α. The inhibition rates differed by a factor of over 26 000.

The reactions of many active site directed inhibitors, including sulfonyl fluorides, have been shown to involve the initial formation of an enzyme · inhibitor complex followed by bond formation and irreversible inhibition [14,29,33]. Fahrney and Gold [14] reached the conclusion that the reaction of chymotrypsin-A_α with sulfonyl fluorides is strongly influenced by binding and proper orientation.

No attempt was made in the present study to determine the concentration dependence of the inhibition reactions. The second order rate constant ($k_{obsd}/[I]$) for the various inhibitors could thus be affected by changes in the binding of the inhibitor to the enzyme (K_I), by changes in the rate of bond formation within the complex, or by both.

Human granulocyte and porcine pancreatic elastase

The rates of inhibition of human granulocyte and porcine pancreatic elastase are listed in Table I. The best inhibitor of the porcine enzyme was 3-$CH_3COC_6H_4SO_2F$ while $C_6H_5CH_2SO_2F$ was the best inhibitor for the granulocyte elas-

TABLE I

INHIBITION OF PROTEASES BY SULFONYL FLUORIDES

Sulfonyl fluoride	$k_{obsd}/[I]$ ($M^{-1} \cdot s^{-1}$)				
	Porcine [a] pancreatic elastase	Human [b] granulocyte elastase	Human [c] granulocyte cathepsin G	Bovine [c] chymotrypsin-A_α	Bovine [d] trypsin
CH_3SO_2F [e]	0.020	0.030	0.014	0.036 (0.022) [i]	0.017 (0.012) [i]
$C_6H_5CH_2SO_2F$ [f]	2.7	21 [g]	14 [h]	370 [j] (250) [i]	2.9 (4.5) [i]
4-$CH_3C_6H_4SO_2F$ [f]	2.7	3.2	1.5	8.6	0.59
3-$CH_3COC_6H_4SO_2F$ [f]	3.2	11	2.5	19 [h]	0.96
2-$NH_2C_6H_4SO_2F$ [f]	0.45	1.1	0.16	0.35	0.093
2-(Ac-Ala-Ala-NHN-(CH_3)CONH)-$C_6H_4SO_2F$ [f]	1.1	4.1 [h]	0.49	3.9	0.10

[a] 3 μM elastase, 0.10 M HEPES (pH 7.50), 30°C, 9% (v/v) methanol.
[b] 1 μM elastase, 0.10 M HEPES (pH 7.50), 0.3 M NaCl, 30°C, 9% (v/v) methanol.
[c] 1 μM enzyme, 0.10 M HEPES (pH 7.50), 1.0 M NaCl, 30°C, 9% (v/v) methanol.
[d] 1 μM trypsin, 0.05 M Tris · HCl (pH 7.50), 10 mM $CaCl_2$, 30°C, 9% (v/v) methanol.
[e] [I] = 18 mM.
[f] [I] = 1.0 mM, unless otherwise noted.
[g] [I] = 0.27 mM.
[h] [I] = 0.50 mM.
[i] Calculated from data of Fahrney and Gold [14], pH 7.0, 25°C, 5% 2-propanol.
[j] [I] = 25 μM.

tase. In the latter case a 700-fold difference in rates was observed between the fastest and slowest (CH_3SO_2F) inhibitor.

Examination of the data reveals that the granulocyte elastase is more reactive with these sulfonyl fluorides than is the porcine enzyme. This is the opposite of the situation observed with most peptide chloromethyl ketones where the porcine elastase is the more reactive enzyme [34,35]. This may indicate significant differences in the active site geometries of the two enzymes favoring the transition state for sulfonylation in one case and that for alkylation in the other. Alternatively, the sequences of the chloromethyl ketone inhibitors investigated thus far may not be optimum for the granulocyte enzyme and as yet undicovered chloromethyl ketone inhibitors may in fact more effectively inhibit the granulocyte enzyme.

Recent chloromethyl ketone studies [21] have shown that inhibitors containing somewhat larger side chains at the P_1 (notation of Schechter and Berger [36]) position, such as Val or Ile, react faster with human granulocyte elastase than with porcine elastase. It therefore seems likely that the granulocyte elastase has a larger S_1 binding site than the porcine enzyme. This observation is strengthened by the data presented here. The granulocyte elastase is inhibited 3.4—7.8 times more rapidly than porcine elastase by all of the sulfonyl fluorides containing a phenyl ring with the exception of the 4-toluenesulfonyl fluoride. The rates with 4-toluenesulfonyl fluoride and methane sulfonyl fluoride are almost equal for the two enzymes. This seems to indicate that the active site serine of the granulocyte enzyme is more accessible to these larger, more

hydrophobic reagents than is the porcine active center.

It is interesting to compare the reaction of 2-aminobenzenesulfonyl fluoride with 2-(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$. The latter compound was prepared in an attempt to increase the reactivity of the sulfonyl fluoride by adding an extended side chain that could provide some secondary binding interaction with the enzyme and a subsequent increase in reaction rate. This compound inhibited the granulocyte enzyme almost 4 times faster than the unsubstituted 2-aminobenzenesulfonyl fluoride. An increase of only a factor of 2 was observed with the porcine enzyme. It is difficult to ascribe such small increases to increased binding alone in the absence of more detailed kinetics data. The increase could simply be due to the acylation of the amino group resulting in a structure that is more reactive. Of the series of sulfonyl fluorides used in this study, 2-aminobenzenesulfonyl fluoride is the most stable in aqueous solution and the second least reactive inhibitor of the enzymes; only CH_3SO_2F was less reactive. In a solution of dioxane : water (40 : 60, w/w) at 45°C, no hydrolysis of 2-$NH_2C_6H_4SO_2F$ could be detected after 2 months [37]. However, 2-acetamidobenzenesulfonyl fluoride, a structure analogous to the peptide amino benzene sulfonyl fluoride derivative, was found to be more readily hydrolyzed in aqueous solution than either 2-aminobenzenesulfonyl fluoride or 3- or 4-acetamidobenzenesulfonyl fluoride. This observation was explained by the suggestion that the amide proton provides intramolecular catalysis by acting as as general base via hydrogen bonding with the adjacent fluorine atom [37]. A similar observation was made by Baker and Hurlbut [38] in a study of the inhibition of chymotrypsin-A_α by 2- and 3-(3-chlorophenoxyacetamido)benzenesulfonyl fluoride. The ortho isomer, in contrast to the meta isomer, was found to be unstable in water and even more unstable to Tris buffer at pH 7.4. For these reasons, it is most reasonable to ascribe the rate differences to an increase in the inherent greater reactivity of the acylated aminobenzenesulfonyl fluoride rather than an interaction with the extended substrate binding site of elastase.

An attempt was made to correlate the $k_{obsd}/[I]$ values by means of the Taft-relationship [39]. No correlation was possible. This indicates that the various rate differences were not simply caused by electronic effects of the various substituents on the reactivity of the sulfonyl fluoride functional group.

Human granulocyte cathepsin G, bovine chymotrypsin-A_α, and bovine trypsin

The order of reaction of the six sulfonyl fluorides was identical with these three enzymes: $C_6H_5CH_2SO_2F$ > 3-$CH_3COC_6H_4SO_2F$ > 4-$CH_3C_6H_4SO_2F$ > 2-(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$ > 2-$NH_2C_6H_4SO_2F$ > CH_3SO_2F (see the table). In terms of relative enzyme reactivity, chymotrypsin was by far the most reactive, followed by cathepsin G and finally trypsin. Cathepsin G was less reactive than the bovine chymotrypsin, in contrast to the observation that the human enzyme was the more reactive of the elastases. This finding is consistent with the hydrolysis rates observed by Zimmerman and Ashe [40] for the hydrolysis of Ac-Ala-Ala-Pro-Phe-*p*-nitroanilide by human granulocyte and bovine chymotrypsins. The ratio of the two k_{cat}/K_m values obtained for these enzymes was 280 : 1, bovine : human. Phenylmethanesulfonyl fluoride is 26 times more reactive with bovine chymotrypsin than with cathepsin G.

The extended peptide chain of 2(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$ increased the rate of reaction with chymotrypsin-A_α by a factor of 11. This may reflect secondary binding interactions with the extended substrate binding site of the enzyme resulting in better binding and a higher rate of inhibition. With cathepsin G the rate increase for the change from 2-$NH_2C_6H_4SO_2F$ to the same compound was only a factor of three. It is more difficult here to invoke greater binding as the cause of this modest increase in rate.

Trypsin was the least reactive enzyme with all of the inhibitors examined. This may well be because of the stringent specificity requirements of this enzyme. None of the compounds examined contain a positively charged grouping that could interact with the S_1 specificity pocket of trypsin. For this reason, it is not surprising that the rates of inhibition were slow. Phenylmethanesulfonyl fluoride was the fastest inhibitor of trypsin and this was at a rate 130 times slower than the reaction of this compound with chymotrypsin. It is probable that the binding of these compounds to trypsin is very weak and the reaction rates observed reflect the intrinsic reactivities of this group of sulfonyl fluorides with the active site serine hydroxyl group.

Conclusions

Because of the involvement of these granulocyte proteases in certain diseases such as emphysema [1—3], an understanding of their relative reactivities and specificity toward various reagents is necessary for the design of effective therapeutic agents aimed at the control of these enzymes in situations where their proteolysis has become uncontrolled. In order to be useful, an inhibitor should be fairly reactive and specific for one protease or a small group of proteases. Competing side reactions with other nucleophiles in physiological systems should be minimal. In this regard sulfonyl fluorides offer a significant advantage since they are known to react almost solely with the active site serine residue in serine proteases and mechanistically related enzymes such as acetylcholine esterase. In addition, thiol proteases will react as evidenced by the inhibition of papain by phenylmethanesulfonyl fluoride [41].

Non-specific reactions with other nucleophilic groups present under physiological conditions have been shown to be minimal. Exposure of chymotrypsinogen to a 20 fold excess of [^{14}C]-phenylmethanesulfonyl fluoride over a 24 h period resulted in no incorporation of the inhibitor, while the active enzyme chymotrypsin incorporated only one equivalent [29]. The ability of free thiols to react with sulfonyl fluorides under physiological circumstances is uncertain. Peptide chloromethyl ketones on the other hand will react with thiols in addition to serine and thiol proteases [12].

None of the sulfonyl fluorides was completely specific since they all reacted with all the serine proteases investigated. The specificity toward the various proteases was however quite variable. Methanesulfonyl fluoride was almost equally reactive toward all of the enzymes. The spread in rates of reaction for 4-$CH_3C_6H_4SO_2F$, 3-$CH_3COC_6H_4SO_2F$ and 2-$NH_2C_6H_4SO_2F$ was slightly larger, but still varied by only 12—20 fold with the five enzymes studied. Phenylmethanesulfonyl fluoride and 2(Ac-Ala-Ala-NHN(CH_3)CONH)$C_6H_4SO_2F$ had the greatest range in reactivities, being 137- and 41-fold, respectively. Some of the peptide chloromethyl ketones such as MeO-Suc-Ala-Ala-Pro-ValCH_2Cl and

Z-Gly-Leu-PheCH_2Cl recently studied with granulocyte elastase and cathepsin G, and porcine elastase are much more specific and in addition are more reactive than sulfonyl fluorides [21]. MeO-Suc-Ala-Ala-Pro-ValCH_2Cl will inhibit granulocyte elastase 74 times faster than phenylmethane sulfonyl fluoride, the best sulfonyl fluoride inhibitor. And Z-Gly-Leu-PheCH_2Cl will inhibit cathepsin G 4 times faster than phenylmethanesulfonyl fluoride. However it is likely that the specificity of sulfonylating agent can be increased by changes in the nature of the leaving group as has been demonstrated with trypsin-like enzymes [42]. The peptide aminobenzene sulfonyl fluoride offers the possibility of changes in the size and nature of the peptide chain and even in the case of phenylmethanesulfonyl fluoride, substituent changes can be envisioned to increase the specificity of the inhibitor. At present studies are underway in our laboratory aimed at increasing both the specificity and reactivity of sulfonylating agents related to those reported in this paper.

Acknowledgement

This research was supported by U.S. Public Health Services grant HL18679. We would like to express our sincere appreciation to Drs. Robert Baugh and James Travis (University of Georgia, Athens, Ga.) for gifts of the human granulocyte enzymes used in this research.

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