(*p*-Amidinophenyl)methanesulfonyl Fluoride, an Irreversible Inhibitor of Serine Proteases†

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ABSTRACT: (*p*-Amidinophenyl)methanesulfonyl fluoride (*p*-APMSF) has been synthesized and shown to be a specific, irreversible inhibitor of the class of plasma serine proteases which demonstrate substrate specificity for the positively charged side chains of the amino acid lysine or arginine. In equimolar concentration, this compound causes immediate and complete irreversible inhibition of bovine trypsin and human thrombin. A 5–10-fold molar excess of reagent over enzyme is required to achieve complete irreversible inhibition of bovine Factor Xa, human plasmin, human C1$\bar{r}$, and human C1$\bar{s}$. The K_i of *p*-APMSF for all of the above-mentioned proteases is between 1 and 2 μM. In contrast, *p*-APMSF in large molar excess does not inactivate chymotrypsin or acetylcholinesterase. The unique reactivity of *p*-APMSF has been further shown in comparison with the related compound *p*-nitrophenyl (*p*-amidinophenyl)methanesulfonate which is an active-site titrant for thrombin but reacts poorly with Factor Xa, C1$\bar{r}$, and C1$\bar{s}$ and is not hydrolyzed by bovine trypsin or human plasmin. Similarly, (*p*-amidinophenyl)methanesulfonate has a K_i of 30 μM for thrombin but is a poor inhibitor of trypsin, Factor Xa, C1$\bar{r}$, C1$\bar{s}$, and plasmin. Studies with bovine trypsin have demonstrated that the inhibitory activity of *p*-APMSF is the result of its interaction with the diisopropyl fluorophosphate reactive site. The unique reactivity of this inhibitor classifies it as one of the most effective active site directed reagents for this class of serine proteases. Collectively, these results suggest that the primary substrate binding sites of these enzymes, which share a high degree of structural homology, do in fact significantly differ from each other in their ability to interact with low molecular weight inhibitors and synthetic substrates.

In 1963, Fahrney and Gold demonstrated that phenylmethanesulfonyl fluoride reacted covalently with chymotrypsin at the diisopropyl fluorophosphate reactive site, irreversibly inactivating the enzyme (Fahrney & Gold, 1963; Gold & Fahrney, 1964). Subsequently, benzenesulfonyl fluorides have been widely used as irreversible inhibitors of trypsin, thrombin, Factor Xa, kallikrein, plasmin, and the first component of complement (Baker & Erickson, 1967; Geratz, 1972; Walsmann et al., 1972; Cory et al., 1977). These compounds are crystalline solids and are active over a wide pH range. Although their use is limited by low solubility in aqueous media and low reactivity with serine proteases, these compounds are an attractive alternative to diisopropyl fluorophosphate (DIFP).[1] Baker and co-workers showed that the reactivity of aromatic sulfonyl fluorides could be increased by attaching a positively charged benzamidine or pyridinium moiety to the parent benzene ring (Baker & Erickson, 1969; Baker & Hurlbut, 1969; Baker & Cory, 1969, 1971; Cardinaud & Baker, 1970). Markwardt and co-workers have also shown that benzenesulfonyl fluorides can be made to react with a variety of serine proteases by the addition of amino alkyl or amidine side chains to the benzene ring (Markwardt et al., 1971, 1973; Markwardt, 1972; Kazmirowski, 1971).

It has been shown that sulfonyl fluorides can act by sulfonylation of the active-site serine with formation of an *O*-sulfonylserine (Gold, 1965). The serine in the active site (serine-195)[2] which reacts with sulfonyl fluorides is identical with the one which is derivatized by DIFP. This is also the same serine which forms the acyl intermediate during proteolysis and is derivatized by carboxylate and sulfonate esters that act as active-site titrants for the serine protease class of enzymes (Shaw, 1970; Glover et al., 1973). In contrast, the chloromethyl ketones described by Shaw and co-workers act by alkylation of the active-site histidine at N-3 (Kettner & Shaw, 1979; Shaw, 1975; Glover & Shaw, 1971).

Studies on the quantitative structure–activity relationships of a series of substituted benzamidines with serine proteases (Andrews et al., 1978) and particularly analysis of a computer-graphics representation of trypsin (Feldmann et al., 1978) led to the observation that an important parameter for acyl intermediate formation was the distance from the cationic nucleus, which confers specific reactivity, to the scissile bond locus in the substrate or inhibitor. This was calculated to be 0.65 nm by measurement on computer-generated models of synthetic substrates, such as *N*-carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester, N^{α}-carbobenzyloxy-L-lysine *p*-nitrophenyl ester, and the tripeptide *Bz*-Phe-Val-Arg-*p*-nitroanilide (R. Laura, M. Cory, and D. H. Bing, unpublished observations). We have used this information to design a benzamidine derivative containing a sulfonyl fluoride in which the distance between the reactive sulfonyl fluoride and the amidine is ~0.65 nm. Examination of a molecular surface graphics display with (*p*-amidinophenyl)methanesulfonyl fluoride occupying the binding site (Figure 1A) and Dreiding models of *p*-APMSF emphasizes the importance of this position and demonstrates that *p*-APMSF satisfies this criterion. This compound has been synthesized, and its interaction with chymotrypsin, acetylcholinesterase, and a variety of serine

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[1] Abbreviations used: DIFP, diisopropyl fluorophosphate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; CbzTyrONp, *N*-carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester; CbzLysONp, N^{α}-carbobenzyloxy-L-lysine *p*-nitrophenyl ester; CbzLysSBzl, N^{α}-carbobenzyloxy-L-lysine thiobenzyl ester; PMSF, phenylmethanesulfonyl fluoride; *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; *p*-APMS-OH, (*p*-amidinophenyl)-methanesulfonic acid; *p*-APMSNp, *p*-nitrophenyl (*p*-amidinophenyl)-methanesulfonate; *p*-APMS-trypsin, (*p*-amidinophenyl)methanesulfonyltrypsin; PMS-trypsin, phenylmethanesulfonyltrypsin.

[2] The chymotrypsin numbering system is used throughout this paper.

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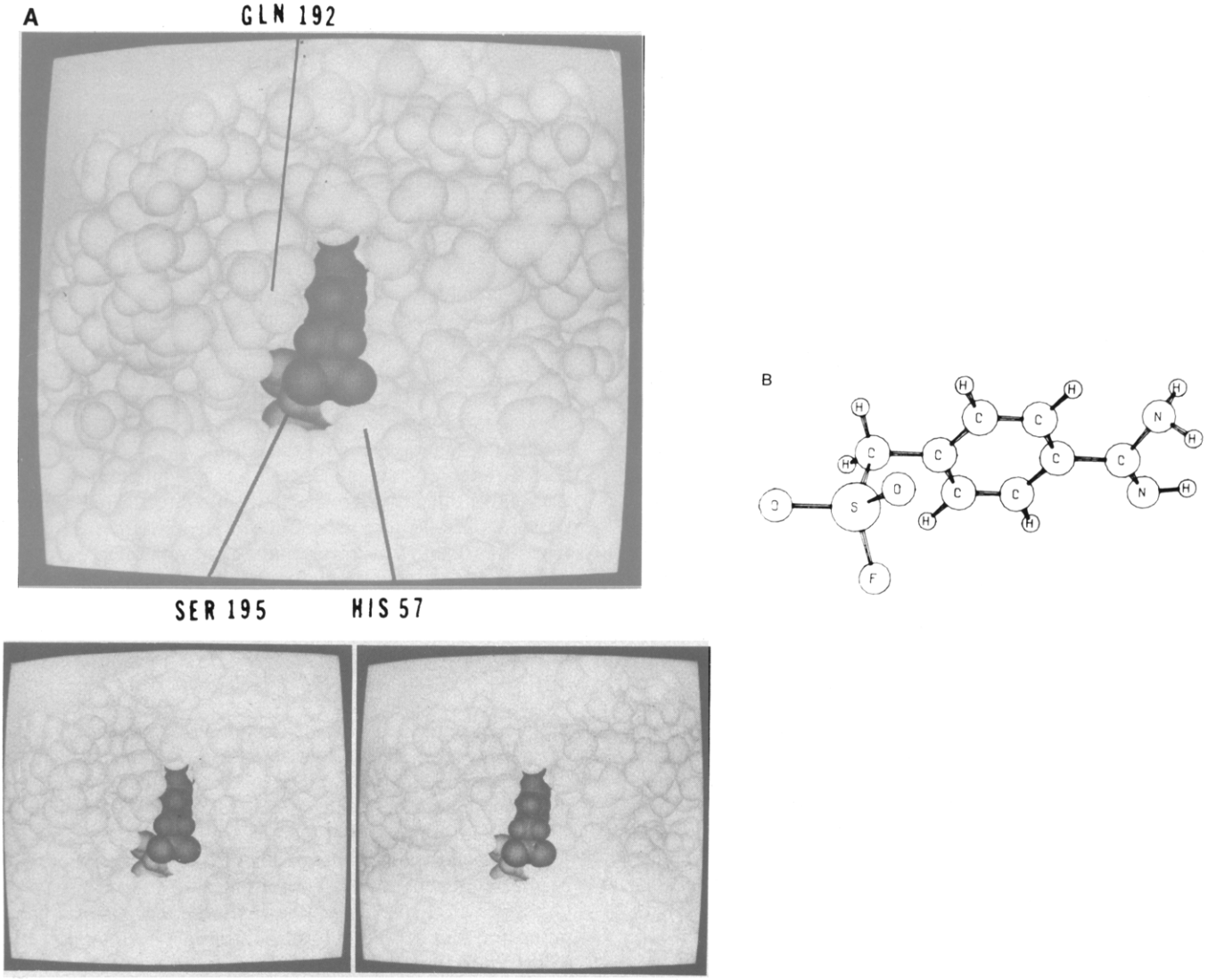


FIGURE 1: (A) A view of bovine trypsin in which *p*-APMSF has been inserted in the active site. Stereopair: *p*-APMSF, grey; serine-195, histidine-57, and Asp-102, black; the remainder of the protein is white. This picture was made by Richard Feldmann of the Division of Computer Research and Technology at NIH, Bethesda, MD, as described in Feldmann et al. (1978). (B) Model of (*p*-amidinophenyl)methanesulfonyl fluoride constructed with the aid of the Prophet computer.

proteases which are competitively inhibited by benzamidine has been examined. The unusual reactivity of this compound with trypsin-like serine proteases in general and with trypsin in particular is the subject of this report.

Materials and Methods

Proteins. Bovine trypsin 3× crystallized, acetylcholinesterase, and chymotrypsin 3× crystallized were obtained from Worthington Biochemicals (Freehold, NJ). Fully activated human complement proteases, $C1\bar{r}$ and $C1\bar{s}$, were purified as previously described (Bing et al., 1980). Human plasminogen was purified from outdated plasma as described by Robbins & Summaria (1976) except that divinyl sulfone was used to couple L-lysine to Sepharose as described by Porath & Sundberg (1972). Plasmin was activated from plasminogen by purified streptokinase (Castellino et al., 1976; Robbins & Summaria, 1976) and was used within 2–3 h of activation. Human α-thrombin (2.13 NIH clotting units/mg) was the generous gift of Dr. John W. Fenton, II, New York State Department of Health, Albany, NY.

Factor X was purified by DEAE Sephadex A-50 chromatography from fresh citrated bovine plasma as previously described (Furie & Furie, 1976; Esnouf et al., 1973; Bjaj et al., 1977; Robison et al., 1980). The initial collection and purification steps were carried out by the New England Enzyme Center, Tufts University School of Medicine, Boston, MA. Activated Factor X was prepared by reaction of Factor X in 200 mM Tris-HCl–100 mM NaCl buffer, pH 8.0, and 10 mM $CaCl_2$ with the venom coagulant protein from Russell's viper venom at a molar ratio of venom protein to Factor X of 0.01. The coagulant protein from Russell's viper venom was prepared by affinity chromatography with Nd(III) and bovine Factor X covalently bound to Sepharose (Furie & Furie, 1975). The activation reaction was stopped by the addition of Na_4EDTA to 20 mM. All proteins yielded a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in 12% gels under nonreducing conditions with 40-μg loads. The following E_{280} were used to estimate protein concentrations: acetylcholinesterase, 16.1; chymotrypsin, 20.4 (*Worthington Enzyme Manual*, 1972); $C1\bar{r}$, 11.5; $C1\bar{s}$, 9.3 (Bing et al., 1980); plasminogen, 17.1; streptokinase, 9.5 (Castellino et al., 1976); Factor Xa, 9.6 (Robison et al., 1980); α-thrombin, 17.3 (Thompson et al., 1977); trypsin, 15.4 (Keil, 1971). The percent activity relative to protein concentration of these enzymes estimated by NPGB titration (Chase & Shaw, 1969) was as follows: trypsin, 57.8%; thrombin, 85%; Factor Xa, 96%; plasmin, 48%; $C1\bar{r}$, 75%. $C1\bar{s}$ activity was estimated as greater than 85% with CbzTyrONp

(Bing, 1969). Chymotrypsin was 85% active by titration with CbzTyrONp (Kézdy & Kaiser, 1970); acetylcholinesterase had 1447 IU/mg.

All organic chemicals were obtained from Aldrich Chemical Co. (Metuchen, NJ). Buffers were made with ACS reagent-grade salts and pH was measured by reference to standard buffers. The substrates CbzTyrONp and NPGB were obtained from ICN Biochemicals (Cleveland, OH). CbzLysONp was obtained from Aldrich. [^{3}H]DIFP, 3 Ci/mmol, was obtained from New England Nuclear (Boston, MA). CbzLysSBzl and *p*-APMSNp were the kind gifts of Dr. Elliott Shaw, Brookhaven Laboratories, Upton, NY.

Enzyme Assays. NPGB titrations to determine the molarity of trypsin, plasmin, thrombin, $C1\bar{r}$, and Factor Xa and CbzTyrONp titration to determine molarity of chymotrypsin were performed with a Cary 118C UV–visible spectrophotometer. CbzTyrONp and CbzLysONp assays of $C1\bar{s}$ activity were done as described by Andrews et al. (1978) and employed a Cary 118C or a Gilford 240 spectrophotometer with a Heath SR-255B recorder. $C1\bar{r}$ and $C1\bar{s}$ were assayed in the presence of EDTA. Factor Xa was assayed as described by Robison et al. (1980). Assays with CbzLysSBzl were performed as described by Green & Shaw (1979). The inhibition of the enzymes by *p*-APMS-OH was analyzed by replotting the slopes derived from Lineweaver–Burk plots of the hydrolysis of CbzLysONp (Factor Xa; trypsin) or CbzLysSBzl ($C1\bar{s}$; thrombin; plasmin) at four or five *p*-APMS-OH concentrations. All lines were calculated by an unweighted linear regression and an r^2 value of 0.96 or greater. Acetylcholinesterase was assayed as described in *Worthington Enzyme Manual* (1972). Polyacrylamide gel electrophoresis was done in the presence of sodium dodecyl sulfate as described by Weber & Osborn (1975). Radiolabeled proteins were analyzed by sectioning the gel with a Gilson autogel divider and determining the radioactivity of the pulverized gel fractions suspended in 10 mL of Liquiscint (National Diagnostics) scintillation fluid with a Searle Mark III scintillation counter.

Synthesis of (p-Amidinophenyl)methanesulfonyl Fluoride (p-APMSF). α-Bromo-*p*-tolunitrile was converted to (*p*-amidinophenyl)methanesulfonyl chloride hydrochloride as described by Wong & Shaw (1974, 1976). A mixture of the sulfonyl chloride (1.0 g, 3.7 mmol) and sodium fluoride (0.5 g, 11.9 mmol) in acetonitrile (30 mL) was refluxed for 16 h with protection from moisture ($CaCl_2$ tube). After cooling, the mixture was filtered by gravity and the filtrate was concentrated to dryness under reduced pressure on a rotary evaporator. The resulting solid was dissolved in ~30 mL of acetone, 60 mL of 1% aqueous HCl was added, and the solution was concentrated to a small volume under reduced pressure on a rotary evaporator. After the solution was chilled, the product was obtained as fluffy white needles which were collected by suction filtration, washed with CH_2Cl_2, and dried in vacuo. The yield of (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, mp 190–191 °C, was 0.4 g (1.6 mmol; 43%): IR (Nujol mull) 3350 (amidine N–H), 1690 (amidine C=N), 1400 (SO_2), 1280, 1210 (SO_2), 1190, 1150 cm^{-1}. NMR (Me_2SO-d_6) δ 9.38 (br s, 4 H, amidine), 7.86 (q, 4 H, aromatic), 5.58 (d, J = 6 Hz, 2 H, methylene). Anal. Calcd for $C_8H_{10}ClFN_2O_2S$: C, 38.02; H, 4.00; F, 7.52; N, 11.09. Found: C, 38.11; H, 4.09; F, 7.58; N, 11.05. Infrared spectra were determined on a Perkin-Elmer 137B Infracord spectrometer, and proton magnetic resonance spectra were obtained on a Varian T-60 spectrometer.

The $t_{1/2}$ for spontaneous hydrolysis of *p*-APMSF at 23 °C was derived by monitoring the change of absorbancy at 260 nm in a Cary 118C spectrophotometer as a function of time in various buffers. The buffers used and values obtained were as follows: 0.1 M citrate, pH 6.0, 20 min; 0.1 M Tris-HCl, pH 7.0, 6.4 min; 0.1 M Tris–acetate, pH 8.0, 10^{-3} min. Stock solutions of *p*-APMSF (1–5 mM) were prepared by dissolving the inhibitor either in a small amount of dimethylformamide and diluting to volume with acetonitrile or in methanol for studies with Factor Xa. Solvent controls containing no *p*-APMSF were used in all studies but caused less than 5% nonspecific inactivation.

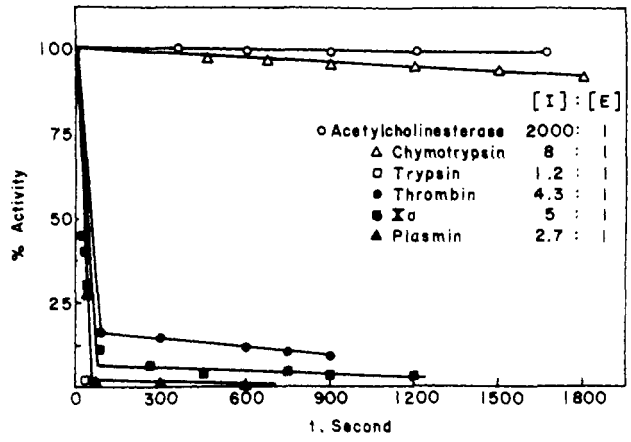


FIGURE 2: Inactivation of serine proteases by *p*-APMSF. (●) Bovine trypsin, 61.5 μM, pH 7.0, 100 mM Veronal buffer–20 mM NaCl; (▲) thrombin, 72.6 μM, 0.75 M NaCl; (■) plasmin, 6.9 μM, 100 mM lysine, 5 mM EDTA, and 100 mM Tris-HCl, pH 7.0; (▼) bovine Factor Xa, 2.7 μM, 100 mM Tris–acetate, pH 6.0; (○) bovine chymotrypsin, 78.5 μM, pH 7.0, 100 mM Veronal buffer; (□) bovine acetylcholinesterase, 1.56 μM, pH 7.0, 20 mM sodium phosphate buffer. Concentration is based on NPGB titration for bovine trypsin, thrombin, and plasmin.

Results and Discussion

Initial reactivity was examined at pH 7.0 for trypsin, chymotrypsin, acetylcholinesterase, thrombin, and plasmin and at pH 6.0 for Factor Xa over 10–15 min. At ~10-fold molar excess of reagent, all the enzymes were maximally inactivated within 2–5 min (Figure 2). Neither acetylcholinesterase nor chymotrypsin were significantly inactivated by large molar excess of *p*-APMSF, indicating that the reagent is specific for only those enzymes which are competitively inhibited by benzamidine.

It was determined that *p*-APMSF met the criteria established for active site directed irreversible inhibitors (Baker & Erickson, 1967; Shaw, 1970) in the following experiments. First, covalent derivatization of trypsin by [^{3}H]DIFP was completely blocked by preincubation with a slight molar excess of *p*-APMSF. In this experiment, trypsin was first completely inactivated with *p*-APMSF at pH 7.0. The concentration of trypsin was 61 μM as measured by active-site titration with NPGB. No NPGB reactivity was detectable following reaction with 67.6 μM *p*-APMSF. Following overnight dialysis against Veronal-buffered saline and 20 mM $CaCl_2$, both the *p*-APMS-trypsin and trypsin were incubated with 5.25 mM [^{3}H]DIFP at 23 °C. The excess [^{3}H]DIFP was removed by overnight dialysis against 1 mM HCl and 20 mM $CaCl_2$. Trypsin incorporated 0.79 mol of [^{3}H]DIP/mol, and *p*-APMS-trypsin incorporated 0.008 mol of [^{3}H]DIP/mol. Electrophoresis of the labeled proteins confirmed that [^{3}H]-DIFP was incorporated into trypsin but not into *p*-APMS-trypsin (Figure 3) and indicated that the DIFP reactive site (e.g., serine-195 at the active site) was being modified or blocked by *p*-APMSF.

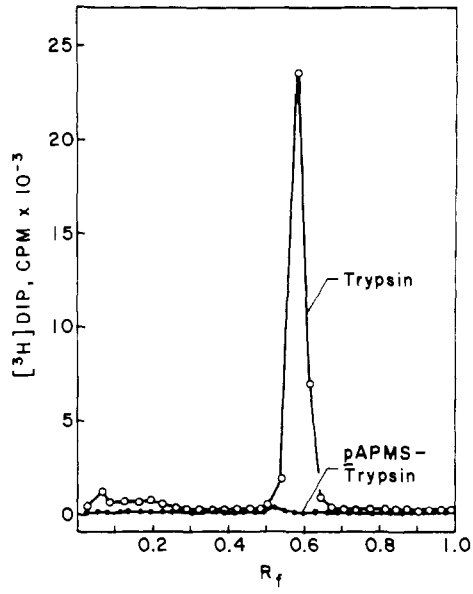


FIGURE 3: Incorporation of [^{3}H]DIFP into trypsin (O) and *p*-APMS-trypsin (■). The protein (20 μg) was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels as described under Materials and Methods.

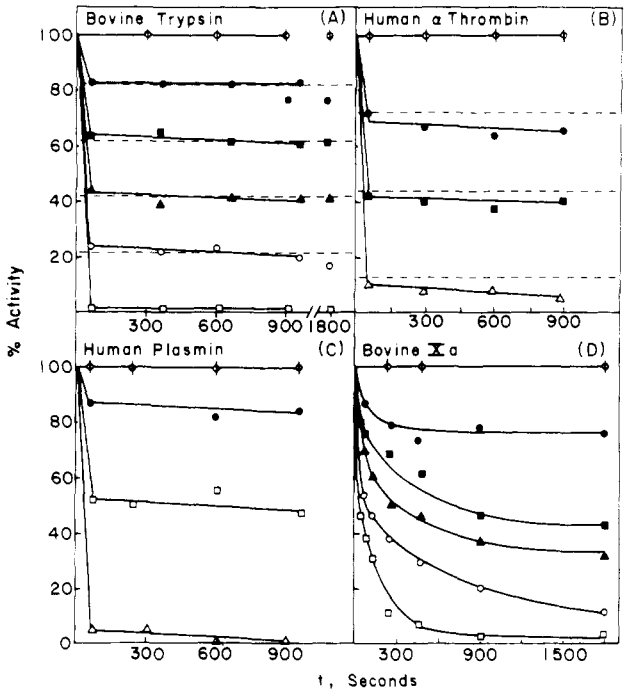


FIGURE 4: Inactivation of serine proteases by *p*-APMSF. (A) Trypsin, 1.23 μM, 100 mM Veronal buffer, pH 7.0; control (ϕ); *p*-APMSF, (●) 23, (■) 38, (▲) 69.1, (□) 92, and (○) 1.19 μM. (B) Thrombin, 69.2 μM, 750 mM NaCl, pH 7.0: control (ϕ); *p*-APMSF, (●) 19, (■) 38, and (△) 57 μM. (C) Plasmin, 6.9 μM, 100 mM lysine–5 mM EDTA and 100 mM Tris-HCl, pH 7.0: control (ϕ); *p*-APMSF, (●) 1.96, (□) 7.6, and (△) 19 μM. (D) Bovine Factor Xa, 6.5 μM, 100 mM Tris–acetate, pH 6.0: control (ϕ); *p*-APMSF, (●) 0.16, (■) 3.3 μM (▲) 6.5, (○) 13, and (□) 32.5 μM. The dashed line in panels A and B are calculated amounts of inhibition expected for the amount of inhibitor added.

Second, it could be shown that *p*-APMSF stoichiometrically inactivated the enzymes. These results are summarized in Figure 4. Maximal inactivation of trypsin and thrombin occurred within 60 s of the addition of the inhibitor and percent inactivation correlated with the concentration of *p*-APMSF added. Maximal inactivation of Factor Xa occurred within 300 s of the addition of inhibitor. At less than equivalent

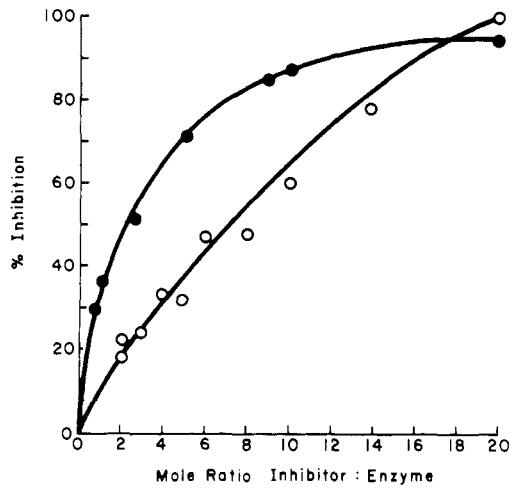


FIGURE 5: Inactivation of C1r̄ (●) and C1s̄ (○) by *p*-APMSF. C1r̄, 2 μM; C1s̄, 5 μM. 100 mM Tris–acetate and 1 mM EDTA, pH 7.0. Residual enzyme activity was measured with CbzTyrONp (C1s̄) or NPGB (C1r̄) after 30 min at 23 °C.

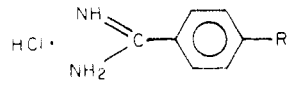
amounts of *p*-APMSF to Factor Xa, the degree of inhibition corresponded to the concentration of *p*-APMSF. It was necessary, however, to add a fivefold molar excess of *p*-APMSF over Factor Xa to achieve complete inhibition (Figure 4D). Plasmin behaved similarly; complete inhibition required a threefold molar excess of *p*-APMSF. The other two benzamidine inhibitable serine proteases, C1r̄ and C1s̄, also could be inhibited by *p*-APMSF (Figure 5). Although like the other enzymes maximal inhibition occurred within 60–120 s of addition of the inhibitor (data not shown), a 10-fold molar excess of *p*-APMSF over enzyme was required to achieve 100% inhibition.

Collectively, the ability of *p*-APMSF to irreversibly block incorporation of [^{3}H]DIFP into trypsin and the apparent specific and stoichiometric inactivation of these trypsin-like serine proteases establish *p*-APMSF as a potent active site directed irreversible inhibitor of the class of serine proteases which have substrate specificity for the positively charged side chains of arginine or lysine. Complete inactivation can be achieved under mild conditions with equi- to 10-fold molar excess of inhibitor over enzyme. The only other active site directed irreversible inhibitor with such potency is the peptide chloromethyl ketone recently described by Kettner & Shaw (1979). In contrast, we have noted in other studies that a 200–1000-fold molar excess of DIFP is required to achieve an equivalent degree of inactivation of C1r̄, C1s̄, and Factor Xa (R. Laura, D. H. Bing, and D. J. Robison, unpublished observations).[3]

Kinetic analysis of the reaction of *p*-APMSF was complicated by the fact that *p*-APMSF is unstable in the pH range in which the enzymes (with the exception of trypsin and Factor Xa) are most stable. While this property did not interfere with the enzyme inhibition reactions, it did preclude a kinetic analysis of the reaction either by the method described originally by Mares-Guia & Shaw (1967) or the method of Kitz & Wilson (1962). Both techniques rely on the accurate determination of a pseudo-first-order rate constant, k_L, for inactivation of a protease with the inhibitor present in large molar excess (Robison et al., 1980). It is possible, however,

[3] Conclusive proof would result from a demonstration of the stoichiometric uptake of radiolabeled *p*-APMSF and correlation of the degree of uptake with degree of inactivation. Efforts are now under way to prepare the suitably radiolabeled form of *p*-APMSF for use in such experiments.

Table I: Reactivity of Serine Proteases with (*p*-Amidinophenyl)methanesulfonyl Derivatives and Benzamidine



enzyme	K_i (μM)[c] (R = H)	K_i (μM)[d] (R = CH_2-SO_2F)	K_i (μM)[e] (R = CH_2-SO_3H)	act.[f] (%) (R = CH_2SO_2O-C_6H_4-*p*-NO_2)
trypsin[a]	16.6	1.02	>300	0
Factor Xa[a]	400	1.50	148	16.9
thrombin[b]	126	1.18	45	97.2
plasmin[b]	50.1	1.54	31	0[g]
C1$\bar{r}$[b]	>1000	1.58	ND[h]	0
C1$\bar{s}$[b]	63.1	2.35	468	0

[a] Bovine. [b] Human. [c] K_i values were obtained from the following references: trypsin, Mares-Guia (1968); thrombin, plasmin, and C1$\bar{s}$, Andrews et al. (1978); C1$\bar{r}$, Andrews & Baillie (1979); Factor Xa, Robison et al. (1980). [d] CbzLysONp at 50 μM was used to measure Factor Xa and trypsin activity. CbzTyrONp at 30 μM was used to measure residual C1$\bar{s}$ activity. NPGB was used to measure C1$\bar{r}$ and thrombin. Enzyme concentration, pH, and time of incubation were as follows: trypsin, 123 μM, pH 6.8, 30 min; thrombin, 69 μM, pH 7.0, 30 min; Factor Xa, 6.5 μM, pH 6.0, 120 min; plasmin, 6.6 μM, pH 7.0, 30 min; C1$\bar{s}$, 5 μM, pH 7.0, 30 min; C1$\bar{r}$, 2 μM, pH 7.0, 30 min. [e] CbzLysONp was used to assay Factor Xa and trypsin. CbzLysSBzl was used for all other enzymes. [f] Activity was expressed as percent NPGB titratable sites. Trypsin, 39 μM; C1$\bar{r}$, 19.6 μM; C1$\bar{s}$, 17.4 μM; Factor Xa, 6.9 μM; thrombin, 8.5 μM. [g] Wong & Shaw (1976). [h] ND, not determined.

with an unstable inhibitor such as *p*-APMSF to determine a dissociation constant K_i by measuring residual enzyme activity after allowing the reaction to proceed to a point at which the inhibitor has been completely hydrolyzed. As developed by Rakitzis (1974), for the labeling mechanism

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^* \qquad (1)$$

where EI* is the sulfonylated protease

$$\ln \frac{[E]_0}{[E]} = \frac{k}{c} \ln \frac{K_i + [I]_0}{K_i + [I]_0 e^{-ct}} \qquad (2)$$

where k is the first-order inactivation constant of the reversible enzyme inhibitor complex, c is the first-order constant of inhibitor disappearance, $[E]_0$ is the initial enzyme concentration, [E] is the enzyme concentration after reaction with inhibitor, $[I]_0$ is the initial inhibitor concentration, and K_i is the dissociation constant of the reversible inhibitor–enzyme complex. If the inhibitor is incubated with the enzyme long enough to allow for complete inhibitor disappearance, then t can be approximated as infinity and eq 2 reduces to

$$\log ([E]/[E]_0) = k/c \log [I]_0 - k/c \log K_i \qquad (3)$$

A plot of $\log ([E]_0/[E])$ vs. $\log [I]_0$ is then a straight line with an intercept of $\log K_i$ on the x axis and $k/c \log K_i$ on the y axis. The K_i of *p*-APMSF vs. trypsin, thrombin, Factor Xa, C1$\bar{s}$, C1$\bar{r}$, and plasmin was evaluated by this method, and these data are summarized in Table I.

It was of interest to compare these results with the inhibition of these enzymes by two other derivatives, namely, *p*-APMS-OH and *p*-APMSNp, and to compare all of these to the parent molecule, benzamidine. In *p*-APMS-OH fluoride has been removed by hydrolysis at pH 8, and in *p*-APMSNp there is a nitrophenol group in place of the fluoride. The latter compound is of particular interest as Wong & Shaw (1974) have reported it to be a specific titrant for thrombin. The results are also summarized in Table I. In every case the presence of the reactive fluoride converted the amidine to a potent inhibitor. The reactivity of the nitrophenyl ester parallelled the reactivity of the sulfonate. Wong & Shaw (1974, 1976) have suggested that the unique reactivity of thrombin with *p*-APMSNp is due to differences in structure at the active site of the enzyme. Examination of the computer-graphics representation of the active site of trypsin and the amino acid sequence of these enzymes in the immediate vicinity of the active-site serine shows that trypsin (Huber & Bode, 1977), Factor Xa (Titani et al., 1975), and plasmin (Robbins & Summaria, 1976) have a glutamine at position 192, whereas thrombin has a glutamate (Thompson et al., 1977) and C1$\bar{s}$ has a lysine or glycine (Cooper & Ziccardi, 1976; Reid & Porter, 1975). As this residue is located at the entrance to the binding pocket for the cationic arginine or lysine side chains (see Figure 1A), it can potentially influence the microenvironment at the catalytic center or the positioning of the susceptible bond in the substrate. In the case of thrombin, the presence of a negatively charged center at this position (e.g., glutamate-192) could significantly enhance the displacement of the nitrophenolate ion from *p*-APMSNp. As shown by Jencks & Carriuolo (1960), the rate of nucleophilic reaction of *p*-nitrophenyl acetate in aqueous solution is increased ~1000-fold in the presence of acetate ion. On the basis of available sequence data, only thrombin has a glutamic acid at this position, and it is possible the unique reactivity of thrombin with *p*-APMSNp may be influenced by the presence of a negatively charged center close to the active serine. On the other hand, the broad inhibitory activity of *p*-APMSF is similar to that exhibited by the ester methyl (*p*-amidinophenyl)methanesulfonate, a compound in which there is a small methoxy group in place of the fluoride. As reported by Wong & Shaw (1976), this methyl ester irreversibly inhibits equally well thrombin, trypsin, plasmin, and plasma kallikrein. In this case, however, the mechanism is different from the reaction with the nitrophenyl ester, as inactivation occurs via alkylation of a histidine rather than by sulfonylation of the active-site serine.

It is notable that the design and ultimate synthesis of this inhibitor derived from studies of computer-generated models of trypsin rather than evolving from quantitative structure–activity relationship studies of chemically related compounds, and to our knowledge this approach is unique. More traditional approaches to inhibitor design usually consider the relative effects of substituents on a parent inhibitor molecule and only potentially good inhibitors which arise from such an analysis are synthesized for further studies. Markwardt and co-workers (Markwardt et al., 1971, 1973; Markwardt, 1972) have synthesized amidino- and amino-substituted benzenesulfonyl fluorides which irreversibly inactivated some of the same enzymes used in our experiments, but one interpretation of the quantitative structure–activity relationships they derived for such compounds suggested that (*p*-amidinophenyl)-methanesulfonyl fluoride would have only marginal inhibitory activity with respect to these serine proteases. As seen in these studies, the opposite was found. The quantitative structure activity relationship approach as outlined by Hansch (1971) permits analysis of important parameters which may determine inhibition, but, as shown by Andrews et al. (1978), this analysis may also eliminate from consideration uniquely reactive inhibitors as well as poor inhibitors. The molecular modeling approach used in this study considers first the structure of the enzyme active site to predict whether an inhibitor may exhibit unique reactivity. We believe our results demonstrate the

advantage of using, where possible, the three-dimensional structure of an enzyme to design inhibitors and/or substrates. In addition, this approach may facilitate the design of inhibitors for other enzymes which share structural and functional similarities to the known enzyme but for which the structure of the natural substrate and/or crystal structure of the enzyme are unknown.

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