J. Enzyme Inhibition, 1987, Vol. 2, pp. 23–30
Photocopying permitted by license only

INHIBITION OF SERINE PROTEINASES BY TETRA-*p*-AMIDINOPHENOXY-*neo*-PENTANE: THERMODYNAMIC AND MOLECULAR MODELING STUDY

ENEA MENEGATTI, ROBERTO FERRONI, SANTO SCALIA and MARIO GUARNERI

Department of Pharmaceutical Sciences, University of Ferrara, Via Scandiana 21, 44100 Ferrara, Italy

MARTINO BOLOGNESI

Department of Genetics and Microbiology, Section of Crystallography, University of Pavia, Via Taramelli 16, 27100 Pavia, Italy

PAOLO ASCENZI and GINO AMICONI*

C.N.R., Center for Molecular Biology, Department of Biochemical Sciences, University of Rome "La Sapienza", Piazzale Aldo Moro 5, 00185 Roma, Italy

(Received 21 October 1986)

The inhibitory effect of the aromatic tetra-benzamidine derivative tetra-*p*-amidinophenoxy-*neo*-pentane (TAPP) on the catalytic properties of β-trypsin (EC 3.4.21.4), α-thrombin (EC 3.4.21.5), factor Xa (EC 3.4.21.6), Lys^{77}-plasmin (EC 3.4.21.7) and β-kallikrein-B (EC 3.4.21.35) was investigated (between pH 2 and 8, I = 0.1 M; T = 37 $\pm$ 0.5°C), and analyzed in parallel with that of benzamidine, commonly taken as a molecular inhibitor model of serine proteinases. Over the whole pH range explored, TAPP and benzamidine show the same values of the dissociation inhibition constant (K_i) for β-trypsin; at variance with the affinity of TAPP for α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B which is higher than that found for benzamidine association around neutrality, but tends to converge in the acidic pH limb. On lowering the pH from 5.5 to 3.0, values of K_i for TAPP binding to β-trypsin as well as for benzamidine association to all the enzymes investigated decreased thus reflecting the pK-shift, upon inhibitor binding, of a single ionizing group. Over the same pH range, values of K_i for TAPP binding to α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B may be described as depending on the pK-shift, upon inhibitor association, of two equivalent proton-binding amino acid residues. Considering the X-ray three-dimensional structures and the computer-generated molecular models of serine proteinases:TAPP and :benzamidine adducts, the observed binding behaviour of TAPP and benzamidine to the enzymes considered has been related to the inferred stereochemistry of proteinase: inhibitor contact region(s).

KEY WORDS: Tetra-*p*-amidinophenoxy-*neo*-pentane, benzamidine, serine proteinase inhibitors, serine proteinase: inhibitor adduct formation (thermodynamics of), serine proteinase: inhibitor adducts (molecular modeling of).

ABBREVIATIONS: BzArgNHNp, *N*-α-benzoyl-L-arginine *p*-nitroanilide; BzIleGluGlyArgNHNp, *N*-α-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide; TAPP, tetra-*p*-amidinophenoxy-*neo*-pentane.

*Correspondence.

INTRODUCTION

Serine proteinases play a central role in several biological processes, and therefore the possibility of selectively influencing their activities by specific inhibitors appears of considerable interest in view of their potential therapeutic value as drugs.[1]

The development of most synthetic competitive inhibitors of serine proteinases so far has been based on the discovery of the effectiveness of benzamidine as an anti-trypsin agent by Mares-Guia and Shaw in 1965.[2] Significant progress in the development of this class of compounds has been made by the finding that bis-, tris- and tetra-benzamidine derivatives are generally stronger inhibitors of serine proteinases than mono-benzamidine compounds.[3–7] Among aromatic tetra-benzamidine derivatives, tetra-*p*-amidinophenoxy-*neo*-pentane (TAPP) inhibits, (i) *in vivo*, the growth of sarcoma 180 implanted in hybrid mice[3] and, (ii) *in vitro*, the blood coagulation induced by the "cancer coagulation factor" produced by the Walker carcinoma in Wistar rats,[3] the fibrinogen-to-fibrin conversion,[6] and the hydrolysis of esters and anilides of amino acids catalyzed by serine proteinases acting on cationic and non-cationic substrates.[6,7]

In order to shed more light on the inhibitory mechanism of benzamidine derivatives, the effect of pH (between pH 2 and 8; I = 0.1 M) on the values of the dissociation inhibition constant (K_i) for the binding of TAPP and benzamidine to β-trypsin, α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B has been investigated at 37 $\pm$ 0.5°C. Analysis of three-dimensional structures[8,9] as well as of computer-generated molecular models (present study) of serine proteinase: inhibitor adducts has allowed the binding behaviour of TAPP and benzamidine to be related to the stereochemistry of enzyme: inhibitor contact region(s).

MATERIAL AND METHODS

Materials

Bovine β-trypsin (treated with diphenylcarbamyl chloride in order to abolish chymotryptic activity) was purified from commercial preparations (from Sigma Chemical Co., St. Louis, U.S.A.) according to Luthy *et al.*[10] Bovine α-thrombin was isolated from commercial samples (from Sigma Chemical Co., St. Louis, U.S.A.) according to Lundblad *et al.*[11] Bovine factor Xa was obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Human Lys^{77}-plasmin was prepared from the Lys^{77}-zymogen by activation with urokinase-substituted-Sepharose-4B, as previously detailed.[12] Porcine pancreatic neuraminidase-treated β-kallikrein-B was isolated from commercial preparations (a kind gift of Bayer AG, Wuppertal, F.R.G.) according to Fiedler *et al.*[13]

BzArgNHNp and benzamidine were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). BzIleGluGlyArgNHNp was obtained from Ortho Diagnostic Inc. (Stockholm, Sweden). TAPP was synthesized according to the published method.[7] All the other reagents were obtained from Merck AG (Darmstadt, F.R.G.). All chemicals were of analytical grade and used without further purification.

The characterization of enzymes, substrates and inhibitors has been reported elsewhere.[2,5,7,10,12–15]

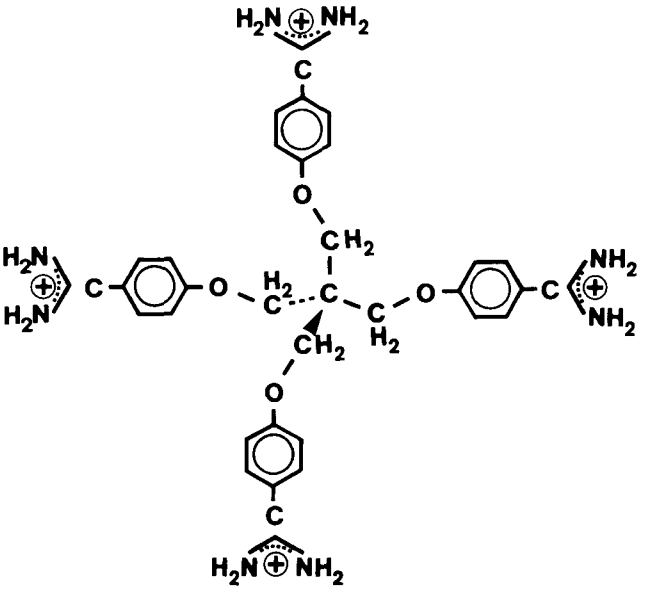

FIGURE 1 Chemical structure of TAPP (modified from Ferroni *et al.*[7]).

Determination of the Inhibition Constant

Values of the dissociation inhibition constant (K_i) for TAPP and benzamidine binding to the serine proteinases examined were determined, between pH 2 and 8 ($I = 0.1$ M) and 37 $\pm$ 0.5°C, by assay systems using, (i) BzArgNHNp as substrate for β-trypsin, α-thrombin, Lys[77]-plasmin and β-kallikrein-B and, (ii) BzIleGluGlyArgNHNp as substrate for factor Xa. The detailed biochemical procedures have been published previously.[5]

Values of K_i were determined using the Dixon graphical method.[16] An average error value of $\pm$ 8% was evaluated for K_i values according to Sagnella.[17]

Preparation of Computer-generated Molecular Models

For the preparation of the molecular models mentioned the program FRODO was used throughout,[18] in conjunction with an Evans and Sutherland PS300 graphics station. Atomic coordinates for β-trypsin:benzamidine[8] and β-kallikrein-B:benzamidine[9] adducts were recovered from the Protein Data Bank distribution tape.[19] Central site models of α-thrombin, factor Xa and Lys[77]-plasmin were obtained by substitution of the relevant aminoacid side chains of β-trypsin[8,20] according to the sequence informations available.[20–22] The atomic model of TAPP was prepared on the basis of its chemical structure (see Figure 1), allowing for proper torsional angles, and using tabulated bond lengths and angles.[23] TAPP was assumed to bind to the primary specificity subsite (i.e., S_1) of the serine proteinases considered with one of its four benzamidine moieties orientated as observed in the β-trypsin:benzamidine[8] and β-kallikrein-B:benzamidine[9] complexes. Given this location of one of the four arms of TAPP, additional possible enzyme:inhibitor interactions were explored directly on

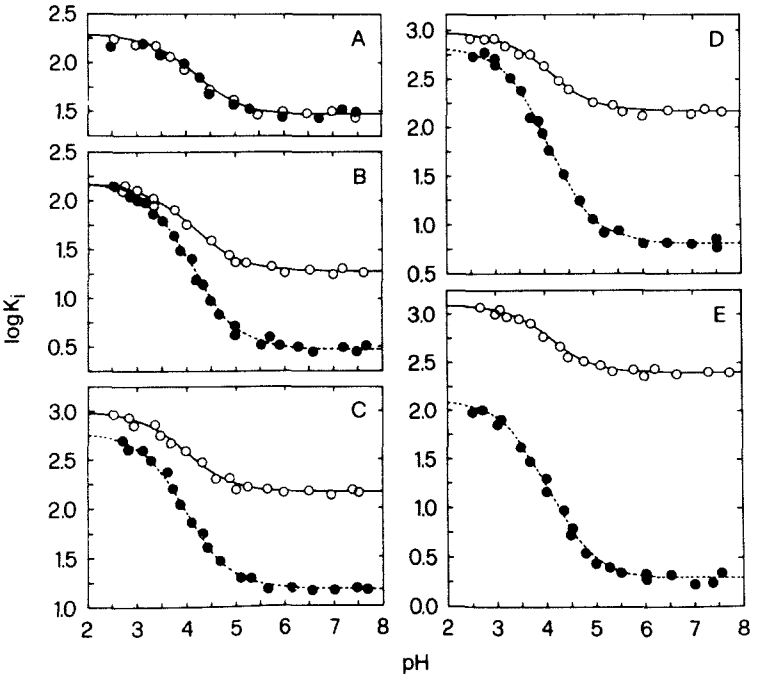

FIGURE 2 pH dependence of the dissociation inhibition constant (K_i, μM) for benzamidine (◇) and TAPP (●) binding to β-trypsin (A), α-thrombin (B), factor Xa (C), Lys^{77}-plasmin (D) and β-kallikrein-B (E), at 37 ± 0.5°C. The continuous and dashed lines, calculated from eqns. (1) and (2), respectively, with values of pK_{UNL} and pK_{LIG} shown in Table I, were obtained with an iterative non-linear least-squares curve-fitting procedure. The pH profile was explored using the following buffers: phosphate (pH 2 to 3.5); acetate (pH 3.5 to 6.0); and phosphate (pH 6.0 to 8); all at I = 0.1 M (sodium salts). No specific ion effects were found using different buffers with overlapping pH values.

the graphics screen considering the torsional degrees of freedom of the inhibitor molecule.

RESULTS AND DISCUSSION

For all the enzyme:inhibitor systems examined, the inhibition patterns were strictly competitive, and titrations conformed to simple equilibria. The stoichiometry of the reaction of TAPP with β-trypsin, α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B (1:1 in molar terms) was also verified by titrations performed at 100 μM proteinase concentration (i.e., at a concentration higher than K_i).

For each proteinase, values of K_i for TAPP and benzamidine binding were independent of the enzyme concentration, and compared well with those reported in the literature,[5–7] if differences in enzyme preparations, inhibitor assay procedures, temperature, as well as pH and buffer composition are taken into account.

Data shown in Figure 2A indicate that TAPP and benzamidine show the same affinity (i.e., K_i values) for β-trypsin, over the whole pH range explored. At variance, values of K_i for TAPP binding to α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallik-

TABLE I

pK values of the inhibitor-free (pK_{UNL}) and inhibitor-bound (pK_{LIG}) β-trypsin, α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B at 37°C (I = 0.1 M)

Proteinase	Benzamidine	TAPP
β-trypsin	$pK_{UNL} = 4.60$ $pK_{LIG} = 3.80$	$pK_{UNL} = 4.60$ $pK_{LIG} = 3.80$
α-thrombin	$pK_{UNL} = 4.55$ $pK_{LIG} = 3.70$	$pK_{UNL} = 4.55$ $pK_{LIG} = 3.70$
Factor Xa	$pK_{UNL} = 4.40$ $pK_{LIG} = 3.60$	$pK_{UNL} = 4.40$ $pK_{LIG} = 3.60$
Lys^{77}-plasmin	$pK_{UNL} = 4.50$ $pK_{LIG} = 3.70$	$pK_{UNL} = 4.60$ $pK_{LIG} = 3.60$
β-kallikrein-B	$pK_{UNL} = 4.45$ $pK_{LIG} = 3.75$	$pK_{UNL} = 4.50$ $pK_{LIG} = 3.60$

pK values were determined by curve fitting from eqns. (1) and (2). Data in Figure 2A–E. An average error value of ± 15% was evaluated for K_{UNL} and K_{LIG} values according to the iterative non-linear least-squares curve-fitting procedure (see Figure 2).

rein-B are lower than those for benzamidine association, around neutrality, but tend to converge in the acidic pH limb (see Figure 2B–E).

Lowering the pH from 5.5 to 3.0, the increase in the values of K_i for benzamidine binding to β-trypsin, α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B as well as for TAPP association to β-trypsin reflects, according to linkage relations,[24] the acid pK-shift of a single ionizing group on inhibitor binding. This simple model leads to the following equation (1):

$$\log K_i = C + \log\{([H^+] + 10^{-pK_{UNL}})/([H^+] + 10^{-pK_{LIG}})\} + |\Delta pK| \quad (1)$$

where C is a constant that corresponds to the alkaline asymptote of $\log K_i$, and pK_{UNL} and pK_{LIG} are the pK values of the proton dissociation constants for the inhibitor-free (K_{UNL}) and inhibitor-bound (K_{LIG}) enzymes, respectively. Eqn (1) has been used to generate the continuous lines shown in Figure 2A–E with the choice of parameters given in Table I. In spite of the uncertainty in the evaluation of the acid asymptotes, the agreement with the experimental data is satisfactory (see Figure 2A–E).

At variance, TAPP association to α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B appears to be modulated by two equivalent TAPP-linked proton-binding groups. On the basis of such a model, which appears to be the simplest one describing the data, the pH dependence of K_i may be expressed by eqn. (2):[24]

$$\log K_i = C + \log\{([H^+] + 10^{-pK_{UNL}})^2/([H^+] + 10^{-pK_{LIG}})^2\} + |2\Delta pK| \quad (2)$$

As shown in Figure 2B–E, dashed lines, calculated according to eqn. (2) with the choice of parameters given in Table I, fit the experimental data well.

Moreover, data shown in Figure 2A–E indicate that TAPP and benzamidine preferably bind the unprotonated species of the enzymes considered.

The pK values of group(s) involved in TAPP and benzamidine binding (see Table I) are closely similar for the enzymes investigated, and agree very well with the pK

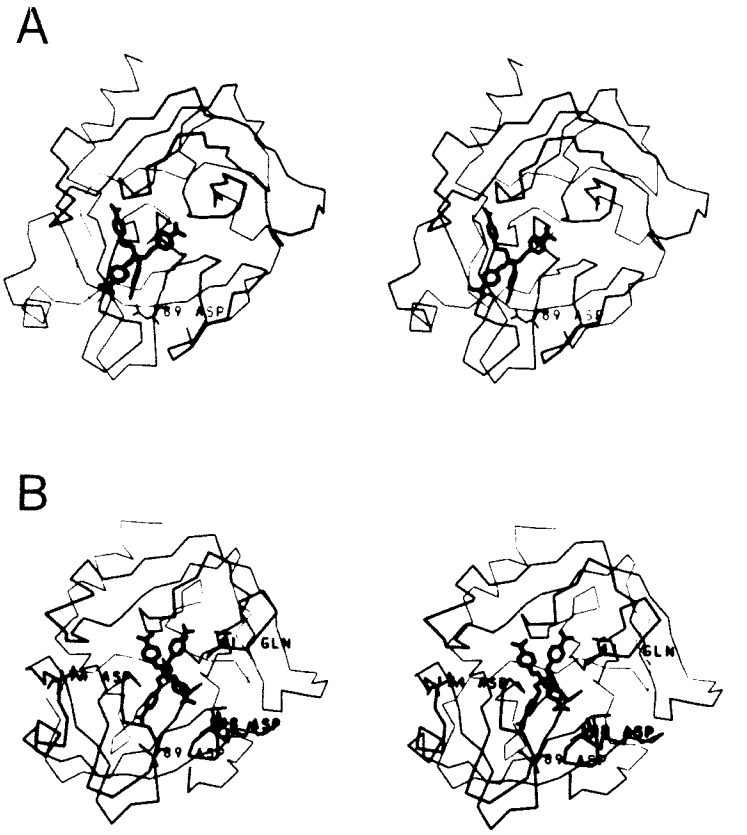

FIGURE 3 Stereo view of the three-dimensional models of β-trypsin: TAPP (A) and β-kallikrein-B:TAPP (B) adducts; both views have been drawn approximately in the same orientation. The polypeptide chain of the enzymes has been drawn as C-alpha skeleton. Amino acid residue(s) modulating TAPP binding to β-trypsin and β-kallikrein-B is (are) labeled.

values of amino acid residue(s) modulating, between pH 3.0 and 5.5, spectral, inhibitor binding and catalytic properties of serine proteinases acting on cationic substrates.[25,26]

Inspection of the amino acid sequences,[20–22,27] X-ray three-dimensional structures[8,9] and computer-generated molecular models (present study) of serine proteinase:TAPP and :benzamidine adducts suggests that the ionizable group affecting benzamidine binding can be identified with the invariant aspartyl residue present at the S_1 subsite of β-trypsin (see Figure 3A), α-thrombin, and β-kallikrein-B (see Figure 3B) (at position 189), factor Xa (at position 227), and Lys^{77}-plasmin (at position 735) (see note 28). Also TAPP binding to β-trypsin is probably modulated by the acid-base equilibrium of Asp189 (see Figure 3A). Similarly, one of the two ionizations affecting TAPP association to α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B can be assigned to the invariant aspartyl residue involved in the S_1 subsite of all serine proteinases investigated. In fact, it is known that this residue interacts with the

positively charged amidino group of benzamidine (see note 29) in the S_1 subsite of β-trypsin[8] and β-kallikrein-B[9] (see Figures 3A, B).

The analysis of computer-generated models of proteinase:TAPP adducts allowed the tentative assignment of the second ionizing group affecting TAPP binding to α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B. Thus, only Glu-39, Glu-149 or Glu-217 in α-thrombin, Glu-75 or Glu-255 in factor Xa, Glu-605 or Glu-640 in Lys^{77}-plasmin, and Asp-148 or Asp-174 in β-kallikrein-B (see Figure 3B) meet the charge and steric requirements for the binding of a second TAPP substituent, once the first benzamidine group is bound into the S_1 subsite of these enzymes. Moreover, TAPP binding to β-kallikrein-B can take advantages of polar pH-independent interactions between one of the exposed benzamidine substituents and residue Gln-41 (see Figure 3B), which is Phe in β-trypsin, factor Xa and Lys^{77}-plasmin, and Leu in α-thrombin. Accordingly, in β-trypsin, no aminoacid residues meet the stereochemical requirements for the productive ionic binding of additional TAPP moieties (see Figure 3A). This interpretation agrees with the pH independence of K_i values for TAPP and benzamidine binding to bovine α-chymotrypsin,[6] where aspartyl and/or glutamyl residue(s), whose ionization(s) affects inhibitor association, have no counterpart(s).[31]

Finally, although enzyme:inhibitor affinity cannot be directly derived from stereochemical considerations, possible multiple electrostatic interactions occurring only in the adducts of α-thrombin, factor Xa, Lys^{77}-plasmin and β-kallikrein-B with TAPP may be taken as partially responsible for the higher effect shown around neutrality by this tetra-benzamidine derivative as compared to that reported for monobenzamidine, the last compound exerting its functional property only through interaction(s) within the S_1 subsite. In addition to these ionic forces (clearly evidenced by the pH dependence of benzamidine and TAPP inhibitory effect; see Figure 2A–E), hydrophobic interaction(s) as well as van der Waals' contacts (the latter related to the different steric adaptability of the molecular surfaces of the various proteinases and inhibitors)[4,5,32–39] can play a significant role in the binding of TAPP to serine enzymes. There is in fact clearcut evidence[4,5,32–39] in support of the notion that, (i) significant differences exist in the topography of the binding sites of the various serine proteinases for synthetic amidino inhibitors and, (ii) the inhibitory potency and specificity of bis- and tris-benzamidine derivatives are affected by a number of modifications such as variation in the length of the alkane chain, the substitution of halogen on the benzene moieties, alteration of the position of amidino groups, and inclusion of aromatic rings. Thus, the present results emphasize the relevance of electrostatic interactions between TAPP and some serine proteinases.

On the whole, if there is to be binding discrimination among close molecular species (such as the proteinases studied), one single contribution, however strong, cannot confer the desired specificity. The present results suggest that a fine degree of recognition in the binding of synthetic inhibitors to serine proteinases may also be obtained through appropriate interactions with residues different from those in the so-called active site. From this point of view, TAPP association to serine proteinases appears to be a powerful model in the study of multiple (pro)-enzyme:inhibitor or :substrate interactions.

References

1. Barrett, A.J. (1980) In Sandler, M. (ed.), *Enzyme Inhibitors as Drugs*, p. 219. London: McMillan.
2. Mares-Guia, M. and Shaw, E. (1965) *J. Biol. Chem.*, **240**, 1579.
3. Parsons, D.G. (1972) Brit. Pat. 1, 288 376; C.A., **78**, 3980r.
4. Geratz, J.D. and Tidwell, R.R. (1978) *Haemostasis* **7**, 170.
5. Geratz, J.D., Stevens, F.M., Polakoski, K.L., Parrish, R.F. and Tidwell, R.R. (1979) *Archs. Biochem. Biophys.*, **197**, 551.
6. Menegatti, E., Guarneri, M., Ferroni, R., Bolognesi, M., Ascenzi, P. and Antonini, E. (1982) *FEBS Letters*, **141**, 33.
7. Ferroni, R., Menegatti, E., Guarneri, M., Taddeo, U., Bolognesi, M., Ascenzi, P. and Amiconi, G. (1984) *Il Farmaco – Ed. Sc.*, **39**, 901.
8. Bode, W. and Schwager, P. (1975) *J. Mol. Biol.*, **98**, 693.
9. Bode, W., Chen, Z., Bartels, K., Kutzback, C., Schmidt-Kastner, G. and Bartunik, H. (1983) *J. Mol. Biol.*, **164**, 237.
10. Luthy, J.A., Praissman, M., Finkenstadt, W.R. and Laskowski Jr, M. (1973) *J. Biol. Chem.*, **248**, 1760.
11. Lundblad, R.L., Uhteg, L.C., Vogel, C.N., Kingdon, H.S. and Mann, K.G. (1975) *Biochem. Biophys. Res. Commun.*, **66**, 482.
12. Ascenzi, P., Torroni, A., Menegatti, E., Guarneri, M. and Amiconi, G. (1985) *Biochim. Biophys. Acta*, **832**, 215.
13. Fiedler, F., Fink, E., Tschesche, H. and Fritz, H. (1981) *Meth. Enzymol.*, **80**, 493.
14. Jesty, J. and Nemerson, Y. (1976) *Meth. Enzymol.*, **45**, 95.
15. Ascenzi, P., Menegatti, E., Bolognesi, M., Guarneri, M. and Amiconi, G. (1986) *Biochim. Biophys. Acta*, **871**, 319.
16. Dixon, M. (1953) *Biochem. J.*, **55**, 170.
17. Sagnella, G.A. (1985) *Trends Biochem. Sci.*, **10**, 100.
18. Jones, T.A. (1978) *J. Appl. Crystallogr.*, **11**, 268.
19. Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer Jr, E.F., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) *J. Mol. Biol.*, **112**, 535.
20. Hartley, B.S. and Shotton, D.M. (1971) In Boyer, P.D. (ed.), *The Enzymes*, 3rd edn, Vol. 3, p. 323. New York and London: Academic Press.
21. Titani, K., Fujikawa, K., Enfield, D.L., Ericsson, L.H., Walsh, A. and Neurath, H. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3082.
22. Castellino, F.J. and Powell, J.R. (1981) *Meth. Enzymol.*, **80**, 365.
23. McGillavry, C.H. and Rieck, G.D. (1962) In Lansdale, K. (ed.), *International Tables for X-ray Crystallography*, Vol. 3. Birmingham: Kynoch Press.
24. Wyman, J. (1964) *Adv. Prot. Chem.*, **19**, 223.
25. Antonini, E., Ascenzi, P., Bolognesi, M., Guarneri, M. and Menegatti, E. (1984) In Elödi, P. (ed.), *Proteinase Action*, p. 325. Budapest: Akadémiai Kiadó.
26. Ascenzi, P., Bertollini, A., Bolognesi, M., Guarneri, M., Menegatti, E. and Antonini, E. (1984) In Elödi, P., (ed.), *Proteinase Action*, p. 339. Budapest: Akadémiai Kiadó.
27. Tschesche, H., Mair, G., Godec, G., Fielder, F., Ehret, W., Hirschauer, C., Lemon, M., Fritz, H., Schmidt-Kastner, G. and Kutzback, C. (1979) *Adv. Exp. Med. Biol.*, **120** A, 245.
28. Amino acid residues have been identified by their three-letter code and by their sequence number. The numbering of β-trypsin, α-thrombin and β-kallikrein-B refers to that of bovine chymotrypsinogens A and B[20]. The numbering of factor Xa refers to that of its heavy chain[21]. The numbering of Lys^{77}-plasmin refers to that of the complete form (Glu^{1}) of human plasminogen[22].
29. The pK values of amidino group(s) of TAPP and benzamidine are higher[7,30] than 10; therefore, over the whole pH range explored (2 to 8), the inhibitors examined are always in the cationic form.
30. East, E.J. and Trowbridge, C.G. (1968) *Archs. Biochem. Biophys.*, **125**, 334.
31. Tsukada, H. and Blow, D.M. (1985) *J. Mol. Biol.*, **184**, 703.
32. Geratz, J.D., Whitmore, A.C., Cheng, M.C.-F. and Piantadosi, C. (1973) *J. Med. Chem.*, **16**, 970.
33. Loeffler, L.J., Mar, E.-C., Geratz, J.D. and Fox, L.L. (1975) *J. Med. Chem.*, **18**, 287.
34. Geratz, J.D., Cheng, M.C.-F. and Tidwell, R.R. (1975) *J. Med. Chem.*, **18**, 477.
35. Geratz, J.D., Cheng, M.C.-F. and Tidwell, R.R. (1976) J. Med. Chem., **19**, 634.
36. Tidwell, R.R., Fox, L.L. and Geratz, J.D. (1976) *Biochim. Biophys. Acta*, **445**, 729.
37. Geratz, J.D. and Tidwell, R.R. (1977) In Lundblad, R., Fenton II, J.W. and Mann, K.G. (eds.), *Chemistry and Biology of Thrombin*, p. 179 Ann Arbor-Michigan: Ann Arbor Science Publ. Inc.
38. Walsmann, P., Markwardt, F., Strurzebecher, J., Vieweg, H. and Wagner, G. (1979) *Pharmazie*, **34**, 837.
39. Robison, D.J., Furie, B., Furie, B.C. and Bing, D.H. (1980) *J. Biol. Chem.*, **255**, 2014.