TETRA-*p*-AMIDINOPHENOXY-PROPANE AS A PROBE OF THE SPECIFICITY SITE OF SERINE PROTEASES

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1. Introduction

The increasing knowledge of the physio-pathological importance of serine proteases has stimulated interest in the search for effective and selective inhibitors of these enzymes. The interest is also justified by the potential therapeutic value as drugs, of the inhibitors [1].

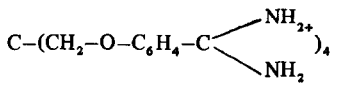
The development of synthetic competitive inhibitors of trypsin-like proteases has been greatly influenced by Mares-Guia and Shaw's discovery of the effectiveness of benzamidine as an anti-trypsin agent [2].

A significant advance is represented by the finding that the link-up of 2 or 3 benzamidine moieties by aliphatic or arylaliphatic chains markedly enhances the inhibitory effect, both in vitro and in vivo (beyond that of mono-aminidines) on serine protease catalysis [3–6].

The in vivo effect of bis- and tris-amidines, as inhibitors of serine proteases, directed our attention to an even higher protonated compound: tetra-*p*-amidinophenoxy-propane (TAPP). This compound inhibits, in vitro, the 'cancer coagulation factor' produced by the Walker carcinoma in Wistar rats and, in vivo, the growth of sarcoma 180 tumors implanted in hybrid mice [7].

This paper reports the inhibition by TAPP of the catalysed hydrolysis of *p*-nitrophenyl esters by bovine β-trypsin, bovine thrombin, human urinary kallikrein, human urokinase, bovine α-chymotrypsin, and bovine pancreatic elastase, in comparison with benzamidine. The results indicate that the interaction with TAPP reflects structural differences in the specificity site of the various enzymes.

Abbreviations: ZLysONp, α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester; ZAlaONp, α-carbobenzoxy-L-alanine-*p*-nitrophenyl ester; ZTyrONp, α-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester; TAPP, tetra-*p*-amidinophenoxy-propane:



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2. Materials and methods

Human urinary kallikrein, kindly provided by 'Lepetit S.p.A.', was prepared according to [8]. Bovine β-trypsin (treated with diphenylcarbamyl chloride to abolish chymotryptic activity), bovine thrombin and porcine pancreatic elastase were obtained from Sigma Chemical Co. Both forms of human urokinase (33 000 and 54 000 M_r) were provided by 'Serono S.p.A.' (details in [9]). Bovine α-chymotrypsin was obtained from Worthington Chemical Co. The characterization of the enzyme preparations used has been reported in [10–12].

The substrates ZLysONp, ZTyrONp and ZAlaONp were obtained from Sigma Chemical Co. Benzamidine was obtained from Merck. TAPP was synthetised according to [7].

Values of the dissociation constants for TAPP and benzamidine binding to the enzymes examined have been determined:

(i) By the inhibitory effect (K_i) on the bovine β-trypsin, human urinary kallikrein, human urokinase

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and bovine thrombin-catalysed hydrolysis of ZLysONp; on bovine α-chymotrypsin-catalysed hydrolysis of ZLysONp and ZTyrONp and on porcine pancreatic elastase-catalysed hydrolysis of ZAlaONp;

(ii) By spectrophotometric titrations over 220–270 nm, (K_s), as reported in [11,12].

Under all the experimental conditions, the inhibition of the enzyme activity and the spectrophotometric titrations conform to a simple equilibrium ($E + I \rightleftharpoons EI$).

Calcium was never present in the reaction mixtures.

The pH profile was explored using the following buffers: phosphate (pH 2–3.5), acetate (pH 3.5–6), phosphate (pH 6–8.5), all at 0.1 M (sodium salts).

The spectrophotometric measurements were carried out with a double-beam spectrophotometer (Cary 118 or 219).

All the measurements were performed at 21 ± 0.5°C.

The biological assay of bovine thrombin was done as in [13].

3. Results

The pH dependence of the dissociation constants (expressed as K_i and K_s) for TAPP and benzamidine binding to bovine β-trypsin, human urinary kallikrein and human urokinase (fig.1) may be fitted with a simple pH transition with av. pK_a 4.1. No pH dependence of the values of the dissociation constants for bovine α-chymotrypsin was observed. TAPP shows a lower affinity than benzamidine for all these enzymes.

The pH dependence of the dissociation constant for the benzamidine binding to bovine thrombin (fig.2) also corresponds to a simple pH transition with pK_a 4.1. However, the pH dependence of the dissociation constant for TAPP to bovine thrombin (fig.2) has a mid-point at pH 4.1, but is much steeper than that corresponding to pK changes of a single ionizing group.

Contrary to the other enzymes, at pH > 3.9, TAPP has an higher affinity than benzamidine for bovine thrombin. At pH 7, TAPP has an inhibitory effect higher than benzamidine (~1 order of magnitude) on the bovine thrombin-catalysed interconversion of fibrinogen to fibrin.

The stoichiometry of the reaction of TAPP with bovine thrombin (1:1) was verified, by spectrophotometric titrations, at pH 3.3 and 6.5 at 600 and 50 μM bovine thrombin, respectively (i.e., at concentrations much higher than K_i and K_s).

In agreement with the absence of the inhibitory effect of TAPP and benzamidine on porcine pancreatic elastase catalysis, no spectral changes reflecting the binding of these 2 cations have been observed.

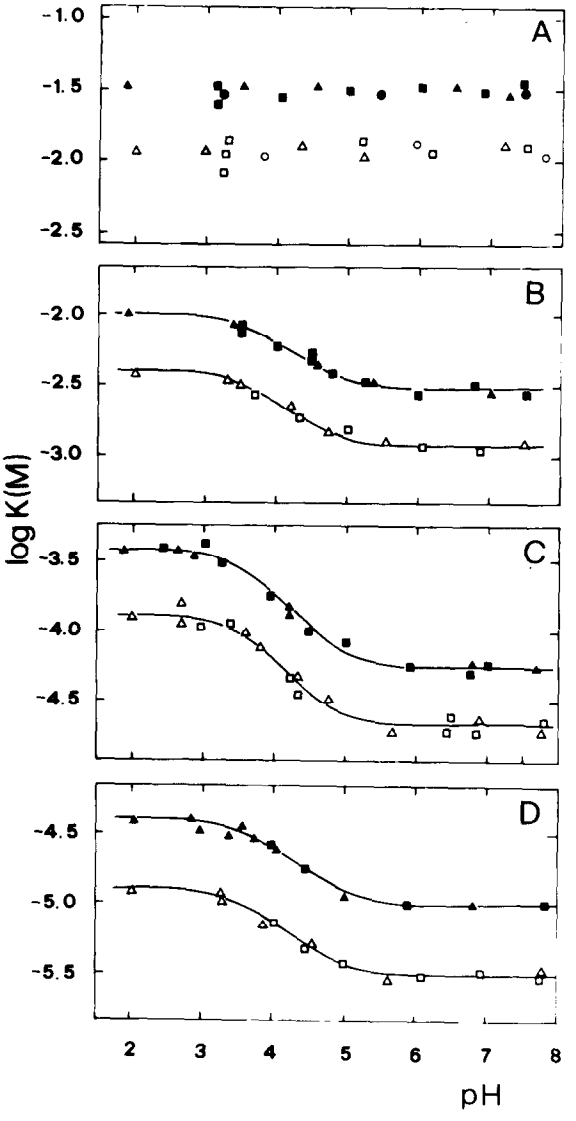

Fig.1. pH-Dependence of the dissociation constants for the reaction of TAPP (filled symbols) and benzamidine (open symbols) with bovine α-chymotrypsin (A), human urokinase (B), bovine β-trypsin (C) and human urinary kallikrein (D) at 21°C. Both forms of human urokinase (33 000 and 54 000 M_r) show indistinguishable ligand binding constants. The data have been obtained: (i) by the inhibition effect on the enzymatic hydrolysis of ZLysONp (squares) and ZTyrONp (circles); (ii) by spectrophotometric titrations (triangles). Continuous lines were calculated from eq. (1) with values of K_{UNL} and K_{LIG} shown in table 1.

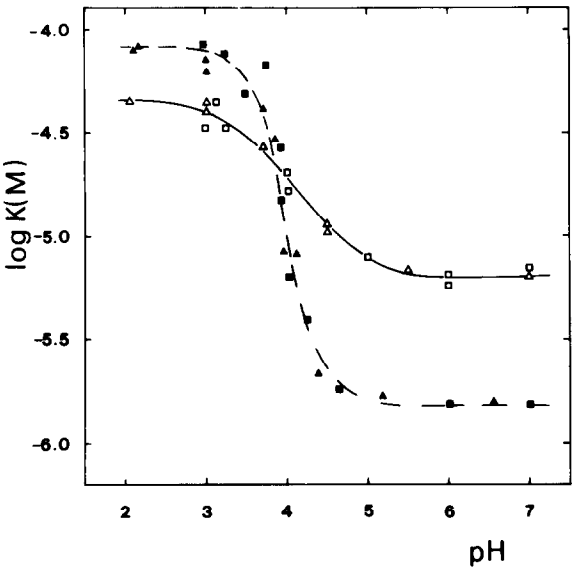

Fig.2. pH Dependence of the dissociation constants for the reaction of TAPP and benzamidine with bovine thrombin at 21°C. Dotted line was calculated from eq. (2) with values of K_{UNL} and K_{LIG} shown in table 1. For the other symbols and conventions see fig.1.

4. Discussion

This paper provides a comparison of the inhibitory effects of TAPP and benzamidine on the catalytic properties of bovine thrombin, bovine β-trypsin, human urokinase, human urinary kallikrein, all acting on cationic substrates, and bovine α-chymotrypsin and porcine pancreatic elastase, both acting on neutral substrates.

The higher affinity of bovine thrombin and human urinary kallikrein for TAPP and benzamidine than bovine β-trypsin and human urokinase, is in agreement with the greater ability of the former to catalyse the hydrolysis of substrates containing arginine rather than lysine residues [11,12].

The increase in the value of the dissociation constants for the binding of benzamidine to bovine thrombin, human urinary kallikrein, bovine β-trypsin and human urokinase, over pH 3–6, reflects according to linkage relations [14], an acid-shift in the pK of a group on binding the cation. Also TAPP binding to human urinary kallikrein, human urokinase and bovine β-trypsin is similarly affected by pH.

The pH dependence of the equilibrium constant for benzamidine binding to bovine β-trypsin agrees with the results of proton release experiments [15].

The simple model, involving a pK-shift of a single ionizable group on ligand binding leads to the following expression:

$$\log K = C + \log \frac{(K_{UNL} + [H^+])}{(K_{LIG} + [H^+])} \quad (1)$$

where C is a constant that corresponds to the acid asymptote of log K, and K_{UNL} and K_{LIG} are the values of the proton dissociation constant for the ligand-free and ligand-bound enzymes, respectively (fig.1,2).

In the TAPP binding to bovine thrombin the situation is different. The curve for the pH dependence is so steep in relation to its amplitude that it cannot be fitted with eq. (1) by any choice of constants. In this case the involvement of at least two TAPP-linked proton binding groups must be postulated. If the groups are inherently the same, the pH dependence may be expressed by the relation [14]:

$$\log K = C + \log \frac{(K_{UNL} + [H^+])^2}{(K_{LIG} + [H^+])^2} \quad (2)$$

The good agreement between the calculated curve and the observations (fig.2) supports the idea that TAPP binding to bovine thrombin involves 2 equivalent proton-binding groups.

The pK-values of the groups involved in TAPP and benzamidine binding (table 1) are closely similar for the various enzymes and agree very well with the pK-

Table 1
pK-values of ligand-free (pK_{UNL}) and ligand-bound (pK_{LIG}) enzymes at 21°C

	TAPP	Benzamidine
Human urokinase	pK_{UNL} = 4.4 pK_{LIG} = 3.9	pK_{UNL} = 4.4 pK_{LIG} = 3.9
Bovine β-trypsin	pK_{UNL} = 4.6 pK_{LIG} = 3.8	pK_{UNL} = 4.5 pK_{LIG} = 3.7
Human urinary kallikrein	pK_{UNL} = 4.6 pK_{LIG} = 4.0	pK_{UNL} = 4.5 pK_{LIG} = 3.9
Bovine thrombin	pK_{UNL} = 4.5 pK_{LIG} = 3.7	pK_{UNL} = 4.6 pK_{LIG} = 3.7

pK-Values were determined by curve fitting from eq. (1) and (2); data in fig.1 and 2

values of the group involved, between pH 3–6, in the binding of cationic substrates [11,12].

Inspection of the amino acid sequences [10,16–19] and of the three-dimensional structures [10,20,21] of the various enzymes studied here suggest that the ionizable group regulating TAPP binding to bovine β-trypsin, human urinary kallikrein and human urokinase can be identified with Asp-189. Moreover, also one of the 2 ionizations affecting TAPP binding to bovine thrombin can be assigned to Asp-189. This residue is known to interact with positively charged benzamidine in the specificity pocket of bovine β-trypsin [21]; it is common to the primary structures of the 4 above-mentioned enzymes, and it has no counterpart in bovine α-chymotrypsin and in porcine pancreatic elastase, acting on neutral substrates. This fact is in agreement with the pH independence of the equilibrium constant for TAPP and benzamidine binding to bovine α-chymotrypsin. In addition, the presence, at the recognition site, of 2 residues showing an high steric hindrance, notably Val-216 and Thr-226, explains the absence of any inhibitory effect of TAPP and benzamidine on porcine pancreatic elastase catalysis.

A tentative assignment can be given also for the second ionizing group found to affect TAPP binding to bovine thrombin. On the basis of simple stereochemical considerations, drawn on the analogous three-dimensional structures of bovine β-trypsin and of sequence comparisons, only Glu-149 and Asp-192 residues of bovine thrombin seem to meet the requirements for productive binding of a second TAPP benzamidine substituent. These 2 residues are unique to bovine thrombin, while no other negatively charged side chains appear to be accessible to TAPP once one of its benzamidine moiety is bound into the enzyme specificity pocket.

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