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pH dependence of bovine mast cell tryptase catalytic activity and of its inhibition by 4′,6-diamidino-2-phenylindole

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Abstract Tryptases are oligomeric enzymes localized in the secretory granules of mast cells. Their role(s) in vivo has yet to be clarified and the lack of powerful and specific inhibitors has hampered the comprehension of the biological functions of these enzymes. In this paper, we identify 4′,6-diamidino-2-phenylindole as a potent inhibitor for bovine tryptase. This inhibitory effect and the enzyme catalyzed hydrolysis of the synthetic substrate Boc-Phe-Ser-Arg-methyl-coumarin were investigated in the pH range of 6.0–9.0. On the basis of the pK shifts occurring upon formation of the inhibitor(substrate)/enzyme complexes, some aminoacidic groups are proposed to play a role in such interactions.

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Key words: Bovine tryptase; 4′,6-Diamidino-2-phenylindole; Catalytic activity; Inhibitor

1. Introduction

Tryptases are oligomeric trypsin-like enzymes present as major constituents of dog [1] and human [2–4] mast cells and, to a lesser extent, in rat [5,6] and bovine [7,8] mast cells. These enzymes are localized in the secretory granules of mast cells and they appear to be potentially involved in a number of biochemical reactions in vitro. They may cleave fibrinogen [9] and high-molecular mass kininogen [10] and induce activation of prostromelysin [11]. In addition, these enzymes inactivate vasoactive intestinal peptide [12] and other neuropeptides, such as vasopressin and neurotensin [6]. Although these and other in vitro studies suggest that tryptase may have potentially important biological functions, the true role(s) of tryptase has not yet been demonstrated due to the lack of powerful and specific inhibitors. To date, the inhibition of tryptase by low-molecular mass synthetic inhibitors has not been studied in detail and only the inhibition of human tryptase by some benzamidine-related compounds, has been reported by other authors [13,14]. In an effort to clarify the recognition mechanism operative in bovine tryptase and to identify new inhibitors of mast cell tryptases, we tested a derivative of benzamidine, DAPI, a fluorescent dye capable of binding double-stranded DNA [15], which is known as an inhibitor of β-trypsin, α-thrombin and β-kallikrein-B [16]. Indeed, this compund behaves as a potent inhibitor of bovine tryptase catalytic activity as well. Therefore, in order to shed more light on the catalytic mechanism of bovine tryptase and on the inhibitory mechanism of benzamidine derivatives, the effect of pH (range 6.0–9.0) on the interactions of the synthetic substrate Boc-Phe-Ser-Arg-MCA and DAPI was investigated at 25°C.

2. Materials and methods

2.1. Materials

4′,6-Diamidino-2-phenylindole, Boc-Phe-Ser-Arg-MCA and 7-amino-4-methyl-coumarin were purchased from Sigma-Aldrich. [^{3}H]DFP was purchased from NEN Life Science Products, Du Pont De Nemours. All the other chemical products were of analytical grade.

2.2. Bovine tryptase preparation

Bovine tryptase was purified from liver capsule and the concentration was determined by both titration with [^{3}H]DFP of the active enzyme as previously described [7] and the optical absorption at 280 nm (tryptase $E_{1cm}^{1\%} = 2.5$). The values obtained with both methods were routinely within 10% of each other, suggesting that purified tryptase was at least 90% active.

2.3. pH dependence of tryptase activity on substrate hydrolysis and of its inhbition by DAPI

The steady-state parameters k_{cat} and K_m for the tryptase-catalyzed hydrolysis of Boc-Phe-Ser-Arg-MCA were calculated by non-linear regression analysis of the data obtained by measuring the initial rates at different $[S]$ with $[S_0] \gg [E_0]$. The substrate was added to the buffered reaction mixtures containing 5 nM active sites of tryptase. Substrate hydrolysis was measured by monitoring the fluorescence of MCA released from the substrate as described previously [17]. The pH profile was investigated using the following buffers: phosphate, pH 6.0–8.0; borate, pH 8.5–9.0; all at I = 0.1 M, as sodium salts. No specific ion effects were found using different buffers overlapping in pH and no appreciable alkaline hydrolysis of the substrate was observed over the explored pH range. All experiments were performed at 25°C.

For the inhibition experiments in the same pH range, purified tryptase (final concentration, 5 nM active sites) and increasing concentrations of DAPI were mixed in 2 ml of the same buffered reaction mixtures and maintained at 25°C for 10 min. After the addition of the substrate, residual activity was measured monitoring the fluorescence of MCA released from the substrate, as above, by comparison with an identical enzyme incubation mixture containing no inhibitor. Comparison of DAPI and benzamidine inhibitory activity was performed under the same conditions in 0.1 M Tris-HCl pH 8.0.

2.4. Data analysis

Data analysis of the pH dependence was carried out acccording a linkage scheme [18]. In the case of k_{cat}, the following equation was used:

$$k_{cat}^{obs} = k_{cat}^{\varnothing}/p + k_{cat}^{1}K_1[H^+]/p + k_{cat}^{2}K_1K_2[H^+]^2/p \quad (1)$$

where k_{cat}^{obs} is the observed catalytic rate constant, $k_{cat}^{\varnothing}$, k_{cat}^{1}, k_{cat}^{2} are the catalytic rate constants for the unprotonated, single-protonated and double-protonated form of the ES complex, K_1 and K_2 are the protonation constants for the same complex and p is the

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; BABIM, bis-(5-amidino-2-benzimidazolyl)-methane; DAPP, 1,3-bis(*p*-amidinophenoxy)-propane; Boc-Phe-Ser-Arg-MCA, *t*-butyloxy-carbonyl-Phe-Ser-Arg-7-amido-4-methyl-coumarin; MCA, 7-amino-4-methyl-coumarin

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binding polynomial for two protons, i.e.

$$p = 1 + K_1[H^+] + K_1K_2[H^+]^2 \qquad (1A)$$

Data analysis for the pH dependence of substrate binding (i.e. K_m) was performed using the following equation

$$K_m^{obs} = K_m^{\varnothing}(1 + K_{UNL}[H^+]/1 + K_{LIG}[H^+]) \qquad (2)$$

where $K_m{}^{obs}$ is the observed Michaelis–Menten equilibrium constant, $K_m{}^{\varnothing}$ is the same parameter in the unprotonated form, K_{UNL} and K_{LIG} are the protonation equilibrium constants in the free and substrate-bound enzyme, respectively.

For the pH dependence of DAPI binding to bovine tryptase, the following equation was used:

$$K_a^{obs} = K_a^{\varnothing}((1 + K_{LIG}^1[H^+] + K_{LIG}^1K_{LIG}^2[H^+]^2)/(1 + K_{UNL}^1[H^+] + K_{UNL}^1K_{UNL}^2[H^+]^2)) \qquad (3)$$

where $K_a{}^{obs}$ is the observed equilibrium association constant for the binding of DAPI, $K_a{}^{\varnothing}$ is the equilibrium association constant for the binding of DAPI to the unprotonated enzyme, $K_{UNL}{}^1$, $K_{UNL}{}^2$, $K_{LIG}{}^1$ and $K_{LIG}{}^2$ are the first and the second protonation constants for the free and inhibitor-bound enzyme, respectively.

3. Results and discussion

In a previous paper, we described the binding behaviour of benzamidine to bovine tryptase and we found that this molecule behaves as an inhibitor with an equilibrium association constant ($K_a = 5.5 \times 10^4$ M^{-1} at pH 8.0) [17] which compares very well with the equilibrium dissociation constants reported for the inhibition of human tryptase (K_i = 12–25 μM [13,14]) and trypsin (K_i = 35 μM [14]). While benzamidine carries only one positively charged group, diamidines, such as DAPP [19], DAPI [16] (see Fig. 1) and BABIM [14], show two positive charges located at opposite ends of the molecule. As a matter of fact, these molecules have been shown to be better inhibitors than benzamidine, even though an affinity enhancement related only to the second positive charge, such as in the case of DAPP, has been observed only for human α-thrombin and porcine pancreatic β-kallikrein-B and not for β-trypsin [19]. The marked increase of DAPI affinity, measured for the same enzymes [16], has been proposed to arise mostly from the more extended contact surface achieved in the enzyme-binding cleft, with respect to benzamidine, upon burying of the indole moiety of DAPI. The same phenomenon may be responsible for the observation that DAPI binds bovine mast cell tryptase with an equilibrium association constant ($K_a = 1.5 \times 10^7$ M^{-1} at pH 8.0) substantially higher than that of benzamidine

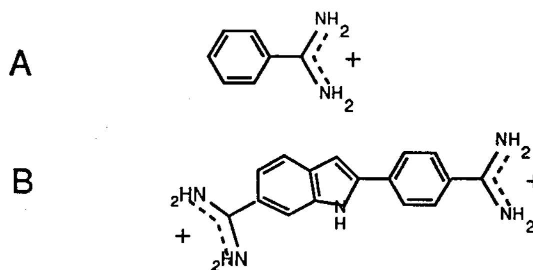

Fig. 1. Chemical structure of benzamidine (A) and DAPI (B).

($K_a = 5.5 \times 10^4$ M^{-1} at pH 8.0 [17]), with a 1:1 stoichiometry for the complex formation of both inhibitor/bovine tryptase complexes. DAPI inhibition constant for bovine tryptase is comparable to that of BABIM for dog tryptase [14] and it may be worth pointing out that bovine and dog enzymes are both sensitive to inhibition by BPTI [1,17], at variance with the human enzyme that was found to be resistant to this protein inhibitor [2–4].

In order to gain insight into the role of different functional groups responsible for substrate/inhibitor interactions with bovine tryptase, we analysed the pH dependence of (1) the hydrolysis of Boc-Phe-Ser-Arg-MCA and (2) the inhibition constant of DAPI.

For the substrate examined, the dependence of the initial velocity of hydrolysis on substrate concentration and the time course of the hydrolysis reaction conform to simple Michaelis–Menten kinetics, under all the conditions used. Over the explored pH range (6.0–9.0), values of k_{cat} for the tryptase-catalyzed hydrolysis of the ES complex seem to be affected by two ionizing groups with pK of 8.4 (±0.3) and 6.3 (±0.2), respectively (see Eq. 1 and Fig. 2A). The lower pK value suggests the involvement of a His residue (likely that involved in the catalytic triad) in the regulation of k_{cat} while the higher pK value may be attributable to the protonation of the N-terminus of the enzyme, suggesting a functional modulation operated by its partecipation in an ion pair, as suggested by Schechter [20], in a specific activation pocket. Values of K_m for the formation of the enzyme–substrate complex, over the same pH range, indicate that upon substrate binding only one group shifts the pK from ≈7.5 (±0.2) in the free enzyme to pK ≈6.8 (±0.2) in the substrate-bound molecule (see Eq. 2 and Fig. 2B). This pK change indeed may be related to the protonation of an His residue but this is not necessarily the catalytic one since the pK_{LIG} (6.8) for the substrate-bound enzyme is different from the lower pK value (6.3) derived from the pH dependence of k_{cat} (see above). The pK values are summarized in Table 1.

The pH dependence of the association equilibrium constants for DAPI binding to tryptase (i.e. K_a values) in going from pH 6.0 to 9.0 (Fig. 3A) reflects a shift in the pK of two groups involved in DAPI binding as calculated from Eq. 3 which was used to generate the unbroken line shown in Fig. 3B. Values of pK_{UNL} and pK_{LIG} are reported in Table 1 and they show that the proton-linked modulation of DAPI interaction with bovine tryptase corresponds to the pK shift upon DAPI binding of one group from 7.5 (±0.2) to 6.8 (±0.2) and of another one from pK 9.9 (±0.3) to 8.9 (±0.3). The first group seems to correspond to the same residue involved in the modulation of K_m (see Fig. 2B) and, thus, of substrate

Table 1
Values of pK, pK_{UNL} and pK_{LIG} for Boc-Phe-Ser-Arg-MCA substrate and DAPI binding to bovine tryptase at 25°C

	Substrate	DAPI
pK	8.4	–
	6.3	–
pK_{UNL}	7.5	7.5
		9.9
pK_{LIG}	6.8	6.8
		8.9

Values of pK (from the pH dependence of k_{cat}) were determined by curve-fitting from Eq. 1 (see Fig. 2A). Values of pK_{UNL} and pK_{LIG} for the substrate (from the pH dependence of K_m) were determined by curve-fitting from Eq. 2 (see Fig. 2B). Values of pK_{UNL} and pK_{LIG} for DAPI binding (from the pH dependence of K_a) were determined by curve-fitting from Eq. 3 (see Fig. 3B). A mean error value never exceeding ±10% was estimated for all the values reported.

binding while the second group appears more specifically related to the interaction of DAPI with bovine tryptase. However, in both cases, it turns out that the interaction of these groups with DAPI brings about a decrease of their pK values, suggesting that the inhibitor binds better when the residues are unprotonated which does not necessarely imply that the interaction is better when the residue is uncharged. Thus, it is important to note that, although the pK of the second group in the substrate-bound enzyme (=8.9) is closely similar to that observed for k_{cat} (see Table 1), it does not mean that the same group is involved since the potential role of other ionizable group(s) (e.g. Tyr^{227} [21]) in the binding-site area may be proposed on the basis of molecular modeling analysis of bovine tryptase (our unpublished results).

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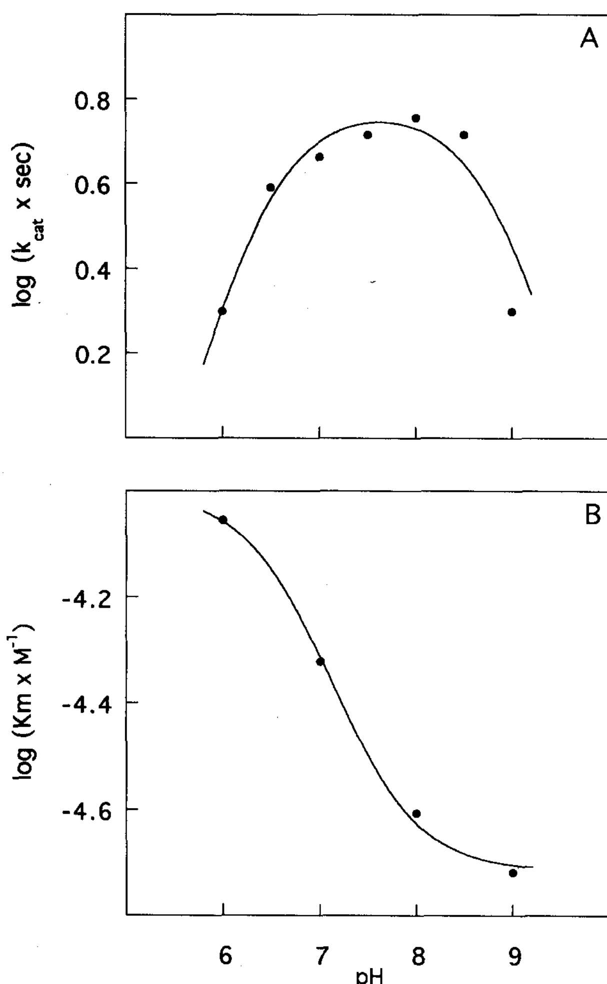

Fig. 2. pH dependence of k_{cat} (panel A) and K_m (panel B) for the hydrolysis of Boc-Phe-Ser-Arg-MCA catalyzed by bovine tryptase at 25°C. The solid lines are the theoretical curves calculated (panel A) from Eq. 1 for two ionizing group(s) and (panel B) from Eq. 2 for one ionizing group. pK (from k_{cat}), pK_{UNL} and pK_{LIG} (from K_m) values are reported in Table 1.

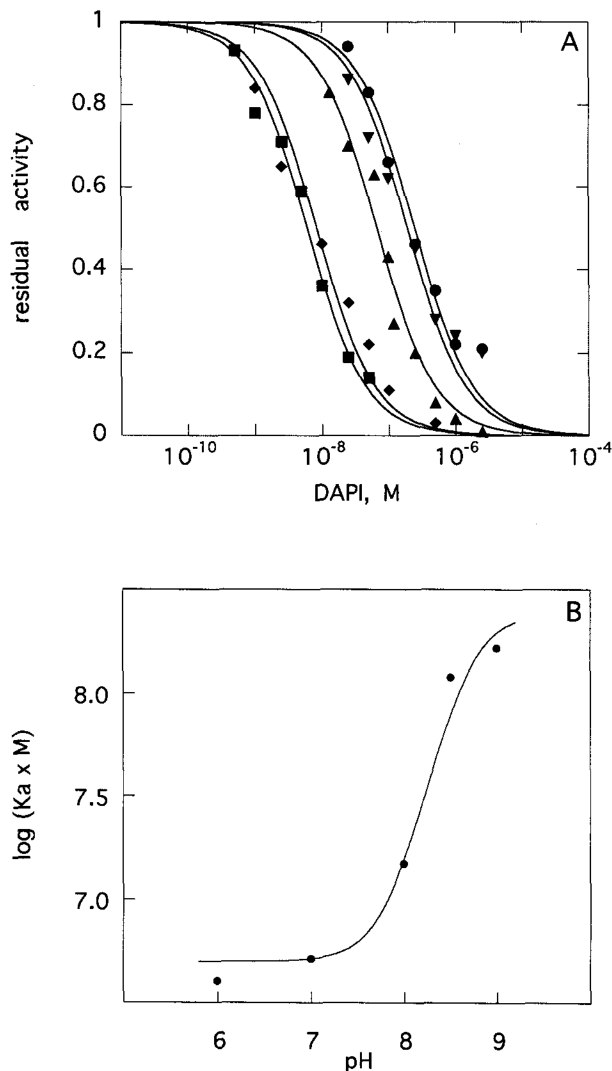

Fig. 3. Panel A: DAPI binding to bovine tryptase at different pH values and 25°C. pH 6.0 (●), pH 7.0 (▼), pH 8.0 (▲), pH 8.5 (♦) pH 9.0 (■). The curves are generated by fitting experimental data as reported in [17]. Panel B: pH dependence of K_a for DAPI binding to tryptase at 25°C. The solid line is generated according to Eq. 3 for two ionizing groups. The pK_{UNL}, pK_{LIG} values are reported in Table 1.

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