Evidence for multiple interacting binding sites in bovine tryptase

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Abstract The interaction of bovine pancreatic trypsin inhibitor (BPTI) and bovine tryptase, which are co-localized in the same granules of bovine mast cells, has been analyzed at 30°C in 0.1 M Tris-HCl, pH 8.0. The analysis has unravelled that the functional unit of bovine tryptase is formed of (at least) four binding sites for this inhibitor. These interaction sites display a simple binding behaviour for small inhibitors (and substrates), whereas heterogeneous properties have been observed in the binding of BPTI. Furthermore, in the presence of BPTI, a positive functional interaction can be detected among the binding sites also for a small synthetic inhibitor, like benzamidine. Such features indicate the existence of a complex functional interplay among the sites of the functional unit which is transmitted through the secondary specificity sites.

Key words: Tryptase; BPTI; Benzamidine; Cooperativity

1. Introduction

Tryptases are trypsin-like serine proteinases found in the granules of mast cells. Similar enzymes have been isolated and characterized from human, dog, rat and bovine mast cells [1–7].

DNA cloning and sequencing has unravelled the amino acid sequences of four human tryptases [8–10], one dog tryptase [11] and two mouse tryptases [12], showing that the coding regions of these enzymes are ~70% identical and exhibit the usual catalytic triad of serine proteases [13]. Human, dog and rat tryptase have a tetrameric structure made up of four identical subunits [1–6], while the bovine enzyme shows a complex assembly of a higher number of subunits, probably due to peculiar hydrophobic interactions between some surface features of the monomers [7]. Furthermore, while tryptases purified from human sources (as well as from dog mastocytoma), are stabilized by heparin [4,14], the enzymatic activity of rat and bovine tryptase is only slightly affected by this glycosaminoglycan [6,15]. The occurrence of a large variance for the structural and functional properties of tryptases obtained from different animal and tissue sources is further confirmed by the observation that, while human and dog tryptases do not form complexes with classical trypsin protein inhibitors [1–4], tryptase from rat peritoneal mast cells was co-purified with an endogenous Kunitz-type protease inhibitor referred to as trypstatin [5,16]. Moreover, we have recently found bovine tryptase and bovine pancreatic trypsin inhibitor in the same granules of bovine liver capsule mast cells, and a K_i value of 5.6 nM was measured for the BPTI–enzyme complex dissociation constant [15].

The last observation prompted us to investigate to a deeper detail the interaction mechanism between bovine tryptase and BPTI, mostly because of the probable physiological significance of this interaction, which might underly an in vivo modulation mechanism. In fact, the existence of multiple subunits in bovine tryptase raises the question of the occurrence of multiple catalytic sites, of how the subunits (and the likely related sites) interact and of what is the functional effect of this interaction on the substrate binding as well as on the catalytic mechanism of the enzyme.

2. Materials and methods

2.1. Bovine tryptase preparation

Bovine tryptase was purified from liver capsule as described in [7]. The tryptase content of the enzyme preparations, was estimated to be ~90% of total protein content as reported in [15]. The concentration of the active enzyme was determined by active site titration using [^{3}H]DFP as previously described [7].

2.2. Enzyme assay

Bovine tryptase activity was assayed in a Kontron SFM 25 spectrofluorimeter equipped with a thermostated cuvette holder. 5 μl of enzyme solution (5 nM active sites final concentration) was added to 2 ml of 0.1 M Tris-HCl, pH 8.0, containing different aliquots (from 2 to 200 μl) of 1.5 mM Boc-Phe-Ser-Arg-MCA (Sigma) dissolved in dimethylsulfoxide. The fluorescence of MCA released from the substrate was excited at 370 nm and the emission at 460 nm was monitored at 30°C for 3 min.

2.3. Binding assays

Purified tryptase (5 nM active sites final concentration) and increasing concentrations of BPTI (Trasylol, Bayer) were mixed in 2 ml of 0.1 M Tris-HCl, pH 8.0, and maintained at 30°C for 30 min. Then 20 μl of 1.5 mM Boc-Phe-Ser-Arg-MCA were added, and the residual activity was measured as described above by comparison with an identical enzyme incubation mixture containing no inhibitor.

In the experiments with benzamidine, tryptase (5 nM active sites final concentration) and increasing concentrations of benzamidine (Sigma) were mixed in 2 ml of Tris-HCl, pH 8.0, and maintained at 30°C for 5 min. The residual enzymatic activity was measured as described above. In another set of experiments, the same increasing concentrations of benzamidine were added to 2 ml of Tris-HCl, pH 8.0, containing tryptase (5 nM) and BPTI (100 nM, a concentration inhibiting half of the enzyme activity), preincubated at 30°C for 30 min. Incubation with benzamidine and measurement of the enzyme residual activity were performed as above.

2.4. Effect of tryptase on BPTI

Bovine tryptase (60 pmol active sites) and BPTI (0.15, 3, 30 nmol) were incubated in 0.6 ml of 0.1 M Tris-HCl, pH 8.0, at 30°C. At $t = 0$, 30 and 60 min, 200 μl of the reaction mixtures were loaded on RP-HPLC column pre-equilibrated in 0.1% TFA, after blocking the enzyme activity with acetic acid (10% final concentration). Elution was performed with a water/acetonitrile gradient in 0.1% TFA. Identical mixtures with no enzyme were incubated and analyzed.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; 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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2.5. Data analysis

The data analysis has been carried out using binding equations according to a linkage scheme [18], which has been adapted to the different experimental conditions. Therefore, in the case of BPTI and benzamidine binding (columns a and c in Table 1) the following general equation has been employed

$$\overline{Y} = \sum_{i=1}^{i=n} K_i x/(1 + K_i x) \cdot 1/n \quad (1)$$

where $\overline{Y}$ is the fraction of free binding sites in the enzyme, x is the concentration of free inhibitor and K_i is the equilibrium association constant to the i site. The digit n corresponds to the number of types of binding sites ($n = 4$ was used for BPTI binding and $n = 1$ for benzamidine binding). In addition, the possibility of a cooperative interaction has been taken into account for the binding of BPTI to bovine tryptase (see below), and in this case the following modified version of Eq. **(1)** has been employed (see column b in Table 1):

$$\overline{Y} = \frac{K_1 x + K_2 x + 2K_1K_2x^2}{2(1 + K_1 x + K_2 x + K_1K_2x^2)} \cdot \frac{2}{n} + \sum_{i=3}^{i=n} K_i/(1 + K_i x) \cdot 1/n \quad (1a)$$

where $n = 4$ and the other symbols have the same meaning as for Eq. **(1)**.

In the case of benzamidine binding to bovine tryptase wherefore the two higher affinity sites had been already bound to BPTI (see below and column d in Table 1) the following equation has been employed:

$$\overline{Y} = \frac{K_3 x + K_4 x + 2K_3K_4x^2}{2(1 + K_3 x + K_4 x + K_3K_4x^2)} \quad (1b)$$

where the symbols have the same meaning as for Eq. **(1)** and K_3 and K_4 have been used since indeed the binding constants refer to the sites 3 and 4 of the BPTI binding process. Analysis of the data was performed using a Marquardt algorithm.

3. Results and discussion

3.1. Binding of BPTI to bovine tryptase

Steady-state kinetic data on few synthetic substrates for bovine tryptase did not show any significant deviation from a Michaelis–Menten mechanism [7] and the corresponding data at 30°C for one of these substrates (i.e. Boc-Phe-Ser-Arg-MCA) are reported as a note in Table 1. However, it must be pointed out that synthetic substrates, which are usually employed for this type of measurements, may not be very suitable for detecting fine site–site interactions. In fact, their action is mostly influenced by the stereochemistry of the primary specificity site and by the efficiency of the charge-relay system in the catalytic triad, which, although crucially important for the enzyme action, represents only a limited portion of the protein surface which contributes to the in vivo interaction with macromolecular substrates or inhibitors. Therefore, the study of the functional interaction between subunits (and sites) of bovine tryptase has been undertaken investigating in a combined fashion the binding of small synthetic and of large macromolecular inhibitors, such as benzamidine and BPTI, the endogenous inhibitor co-existing with bovine tryptase in the mast cells granules. Our aim was to possibly uncover conformational changes of larger portions of the enzyme, even not immediately neighbouring the active catalytic site. In fact, the wider interaction surface of macromolecular inhibitors seems to be more susceptible of detecting, and of being influenced by, long range ligand-linked structural transitions of the subunits.

Table 1
Intrinsic association constants for BPTI and benzamidine binding to bovine tryptase[a]

Intrinsic association constant (M^{-1})*	Inhibitor			
	BPTI		Benzamidine	
	a	b	c	d
K_1	1.21×10^8	3.87×10^7	5.5×10^4	
K_2	1.21×10^8	3.53×10^8		
K_3	2.70×10^6	2.29×10^6		1.53×10^4
K_4	2.22×10^4	2.28×10^4		1.43×10^6

Residual activity was measured in all experiments at 30°C in 0.1 M Tris-HCl, pH 8.0.

[a]The steady state parameters for the interaction of Boc-Phe-Ser-Arg-MCA with bovine tryptase at 30°C, pH 8.0 are as follows: $K_m = 9.4 \times 10^{-5}$ M, $k_{cat} = 19.8$ s^{-1}, $k_{cat}/K_m = 2.11 \times 10^5$ $M^{-1} \cdot s^{-1}$.

*The intrinsic association constants were obtained by non linear least-squares curve fitting using Eq. **(1)** (column a, $n = 4$, and column c, $n = 1$), equation 1a (column b, $n = 4$) and equation 1b (column d). Other details are reported in section 2.5.

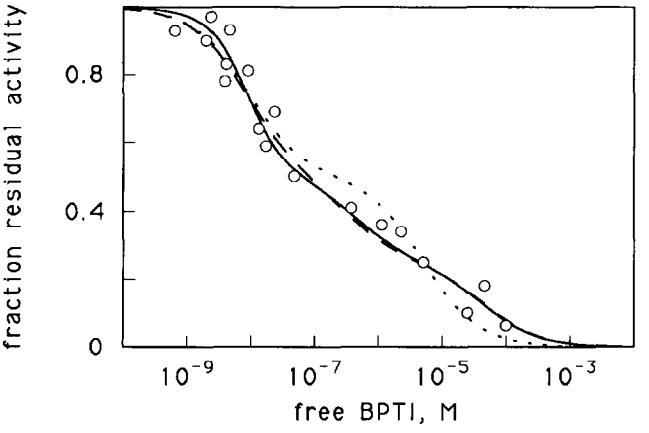

Fig. 1. BPTI binding to bovine tryptase. Binding was measured (open symbols) as described in section 2. The curves are non linear least-squares fits of equation 1 (---) and of equation 1a (—) to the experimental data ($n = 4$). The dotted line represents the fitting obtained when only two types of binding sites were considered (Eq. **(1)**, $n = 2$). Tryptase concentration was 5 nM active sites. Experiments were performed at 30°C, in 0.1 M Tris-HCl, pH 8.0. For details see section 2.5.

Fig. 1 displays the equilibrium binding of BPTI to tryptase at pH 8.0, 30°C, in 0.1 M Tris-HCl, a process which clearly appears multiphasic. Such a behaviour can be referred to either (i) the presence of multiple types of inhibitor binding sites with different intrinsic affinities; or (ii) the occurrence of a negative cooperative interaction between sites, such that, after few binding sites are occupied by the inhibitor, a conformational change takes place, which lowers the affinity of the ligand for the remaining binding sites. Indeed, a discrimination between case (i) and case (ii) is not easy, even though we can definitely rule out any marked intrinsic functional heterogeneity for the primary specificity site as well as for the catalytic site of tryptase. As a matter of fact, binding of a small synthetic inhibitor, such as benzamidine, which only interacts with Asp^{188} of tryptase through its amino group [13] and our own unpublished results), displays a simple binding behaviour (see open symbols in Fig. 2 and Table 1, column c), and the same seems to be true for the binding of a synthetic substrate (see note in Table 1). Therefore, the eventual applicability of case (i) should be referred only to variations among the secondary specificity sites concerning the widespread network of interactions present only when a large surface of the enzyme binds a macromolecular

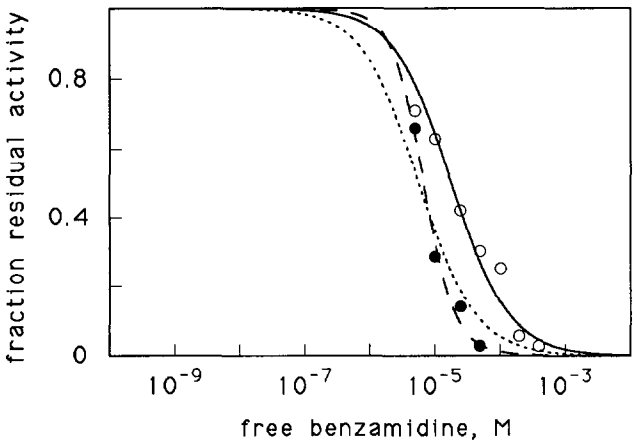

Fig. 2. Benzamidine binding to bovine tryptase. Binding was measured as described in section 2, in the absence (open symbols) and in the presence (filled symbols) of 100 nM BPTI (a concentration inhibiting half of the enzyme activity). The curves are non-linear least-squares fits of Eq. **(1)**, $n = 1$ (—) and of Eq. **(1b)** (---) to open and to filled symbols, respectively. The dotted line represents the curve fitting of Eq. **(1)** ($n = 1$) to the filled symbols. Tryptase concentration was 5 nM active sites. Experiments were performed at 30°C, in 0.1 M Tris-HCl, pH 8.0. For details see section 2.5.

inhibitor. Furthermore, a mechanism involving the degradation of inhibitor by tryptase during the process of inhibition can be ruled out. In fact, at various inhibitor/enzyme molar ratios, no cleavage of BPTI occurred, as we could evaluate from the HPLC profile of the incubation mixtures. Under the conditions used for the elution of the inhibitor, only one peak corresponding to unchanged BPTI was detected.

In view of these considerations, case (ii) may also include the possibility that the binding affinity decrease, observed only in the case of a large macromolecular inhibitor, might reflect some steric hindrance. Therefore, in such a case the close proximity of some of the binding sites and the occupancy of one of them impair (or weaken) the interaction of the neighbouring site with another large inhibitor molecule because of the partial surface overlapping of the secondary specificity site. It is interesting to recall that very recently Sommerhoff et al. [19] have shown that LDTI, a Kazal-type inhibitor (46 amino acid residues) isolated from the leech *Hirudo medicinalis*, inhibits only 50% of the proteolytic activity of human tryptase, up to a 40 nM inhibitor concentration. However, in the light of our data (Fig. 1), the leech inhibitor concentration used could be not sufficient to detect lower affinity sites in human tryptase.

A closer look of BPTI binding to bovine tryptase indicates that the complete description of the functional behaviour requires the presence of at least four binding sites, two of which show high affinity properties, whereas the other two display drastically different and much lower affinity constants. Thus, the overall curve cannot be satisfactorily fitted with only two types of binding sites (using Eq. **(1)**, see dotted curve in Fig. 1). As a consequence, the analysis of BPTI binding has been carried out employing Eq. **(1)** and **(1a)**, either (a) assuming the same value for the equilibrium constant of the two high affinity sites, or (b) leaving them free to vary (Table 1, column a and b). These two cases are reported in Fig. 1 (dashed line for case (a) and continuous line for case (b)), and it turns out that a somewhat better fitting can be obtained when some cooperativity between the two high affinity binding sites is introduced. An ~9-fold enhancement is found for the second binding constant, suggesting a possible interaction energy between the two binding sites of ~5.5 kJ/mol. However, it must be pointed out that the slight scattering of the data puts a fairly large error on this estimate, whereas their quality is good enough to confirm the occurrence of a four-site functional unit.

3.2. *Binding of benzamidine to bovine tryptase in the presence of BPTI*

The presence of binding sites with drastically different affinity constants in bovine tryptase offers the opportunity to investigate the functional properties of intermediate situations, when only a fraction of binding sites is occupied by the macromolecular inhibitor, such as to possibly detect the existence of conformational arrangements of the enzyme differing from the totally free and the fully saturated molecule. In this respect, we have carried out an investigation on the properties of tryptase in the presence of a concentration of BPTI, which was enough to occupy only the two high affinity binding sites. Strikingly, benzamidine binding to the two remaining unoccupied binding sites shows distinctly different interaction properties (see filled symbols in Fig. 2). As a matter of fact, an overall significant enhancement of the affinity constant for benzamidine can be immediately detected. Furthermore, the analysis of this binding behaviour unequivocally shows the occurrence of a very marked positive cooperativity between the two binding sites with an ~100-fold enhancement of the affinity constant for the binding of the second molecule of benzamidine, underlying an interaction energy between the two sites of ~11.4 kJ/mol (Table 1, column d).

3.3. *Conclusions*

A complete quantitative description of the whole behaviour described is not easy and likely impossible at this stage. However, we can try to give some hint of possible interaction networks operating in bovine tryptase, and to try some extrapolations to its in vivo role.

First of all, a feature which undoubtedly emerges from this functional study is the existence of a complex interaction mechanism within a functional unit which can be identified as formed by (at least) four binding sites. Within this framework, we can envisage the possible existence of a weak positive cooperativity between two sites which is transmitted only through a ligand-linked conformational change involving the secondary specificity site, since no evidence for such a phenomenon can be observed if the ligand binding occurs only at the primary specificity site (such as in the case of benzamidine binding). At this point, the occupancy of two sites creates a new structural situation, wherefore the binding of a third macromolecular inhibitor is strongly impaired by a decrease of ~12.6 kJ/mol of free energy. However, this effect is likely due to a steric hindrance of the secondary specificity site, since it can be observed only for a large inhibitor macromolecule, whereas the affinity of the primary specificity site for a small synthetic inhibitor, such as benzamidine, (even in the presence of two sites occupied by the macromolecular inhibitor) is only weakly affected. The existence of a new interaction network at intermediate ligand saturation degrees is further demonstrated by the evidence that in the presence of half of the sites occupied by the macromolecular inhibitor there is a strong positive interaction between

the two primary specificity sites, which is not present in the absence of BPTI.

Altogether, it appears that the ligand binding behaviour of bovine tryptase displays a complex interplay between positive and negative interaction effects, which becomes evident only in the presence of extended surface interaction, suggesting a possible relevant role in the in vivo interaction with macromolecular substrates. Therefore, a detailed quantitative description of this peculiar behaviour, even though it is clearly outside the purpose of this initial study, may contribute to a deeper comprehension of physiological role of mast cell tryptase.

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