PRE-EXISTENCE OF THE ACTIVE SITE IN ZYMOGENS, THE INTERACTION OF TRYPSINOGEN WITH THE BASIC PANCREATIC TRYPSIN INHIBITOR (KUNITZ)

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1. Introduction

It has been shown in recent years that many zymogens including trypsinogen, chymotrypsinogen, procarboxypeptidase, prophospholipase [1–7], etc. display toward certain substrates or pseudo-substrates a weak intrinsic activity prior to activation. The same functional groups are involved in the catalysis by the enzymes and their parent zymogens and it seems that in most cases the much inferior activities of the zymogens are due primarily to an incompletely formed binding site and only secondarily to a less efficient catalytic apparatus [4,7].

The complex formed between trypsin and the basic pancreatic trypsin inhibitor (PTI) is presently the best known model of heterologous protein–protein interaction. Kinetic and thermodynamic properties of the association have been thoroughly studied [8] and X-ray crystallography has given a detailed description of the contact area of the two components in the complex [9]. The interaction between trypsin and PTI primarily involves the active site area of the enzyme [9]. Since it has been qualitatively shown that trypsinogen, similarly to trypsin, associates with PTI [10,11], we decided to study the effect of the rearrangement of the active site geometry, which occurs during the zymogenenzyme conversion, upon thermodynamics of binding to PTI.

2. Materials and methods

Bovine trypsinogen, trypsin and α-chymotrypsin were purified as previously described [12–14]. Pure bovine pancreatic trypsin inhibitor (Kunitz) was a gift from Choay Laboratories.

Activity measurements were carried out at 25°C, pH 8.0, in a pH-stat [14,15] with the following substrates: α-N-acetyl-L-tyrosine ethyl ester for chymotrypsin and α-N-benzoyl L-arginine ethyl ester for trypsin.

3. Results

3.1. *Identification of the complex formed between trypsinogen and PTI*

A chromatography of a mixture of trypsinogen and PTI through Sephadex G-75 (fig.1) at pH 8.0 separates two peaks. The first one contains both trypsinogen (mol. wt. 24 500) and the trypsinogen–PTI complex (mol. wt. 31 000), the second one contains free PTI (mol. wt. 6500). The first peak was pooled, lyophilized and dissolved in 10 mM HCl, at pH 2.0, to dissociate the fraction of trypsinogen associated in the complex from its inhibitor counterpart. A new chromatography on Sephadex G-75 equilibrated at pH 2.0 gave an easy separation of trypsinogen and PTI. The fraction of PTI bound to trypsinogen in the first peak of the first chromatography at pH 8.0 can then be easily determined. The peak contained an average concentration of 2 μM trypsinogen and 0.2 mol of PTI were found to be associated per mole of trypsinogen. Although no exact determination of the dissociation constant for the trypsinogen–PTI complex can be obtained from these results, it is easy to estimate that the dissociation constant ranges between 0.2 and 20 μM. If the value of the constant were 0.2 μM, i.e.

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10 times less than the trypsinogen concentration in the first peak, practically no dissociation would have occurred and a 1 : 1 trypsinogen–PTI complex would have been isolated. Conversely, if the value of the constant were 20 μM, i.e. 10 times higher than the zymogen concentration, practically no binding of PTI to trypsinogen would have been found. We have observed an intermediate situation with a non-stoichiometric association of 0.2 mol of PTI per mole of zymogen.

3.2. *Determination of the dissociation constant of the trypsinogen–PTI complex by competition experiments involving chymotrypsin (ChTi), trypsinogen (Tg) and PTI*

$$\begin{array}{l} \mathrm{Tg} \rightleftharpoons \mathrm{Tg{-}PTI} \\ + \\ \mathrm{PTI} \\ + \\ \mathrm{ChTi} \rightleftharpoons \mathrm{ChTi{-}PTI} \end{array}$$

$$\frac{K_d}{K'_d} = \frac{[\mathrm{ChTi{-}PTI}]}{[\mathrm{ChTi}]} \times$$

$$\frac{\{[\mathrm{Tg}]_0 - [\mathrm{PTI}]_0 + [\mathrm{ChTi{-}PTI}]\}\left(1 + \dfrac{K'_d}{[\mathrm{ChTi}]}\right)}{[\mathrm{PTI}]_0 - [\mathrm{ChTi{-}PTI}]\left(1 + \dfrac{K'_d}{[\mathrm{ChTi}]}\right)} \quad (1)$$

The three partners are incubated together until equilibrium is reached. The ratio K_d/K'_d can then be easily determined where K_d and K'_d represent dissociation constants of the trypsinogen–PTI and chymotrypsin–PTI complexes respectively. $[\mathrm{Tg}]_0$ and $[\mathrm{PTI}]_0$ are the total concentrations of trypsinogen and PTI. [ChTi] and [ChTi–PTI] are the concentrations of free and complexed chymotrypsin respectively. $[\mathrm{ChTi{-}PTI}] = [\mathrm{ChTi}]_0 - [\mathrm{ChTi}]$ where $[\mathrm{ChTi}]_0$ is the total chymotrypsin concentration. Since the value of K'_d is already known, $K'_d = 9.1 \times 10^{-9}$ M at pH 8.0, 25°C [14], K_d can be easily evaluated. Results in table 1 give K_d values obtained at different concentrations of trypsinogen, chymotrypsin and PTI. The dissociation constant of the trypsinogen-PTI complex at 25°C, pH 8.0 is $K_d = 1.8 \pm 0.3 \times 10^{-6}$ M. This value is in the range estimated from data in fig.1.

The same competition technique has been used to study (fig.2) the pH- and temperature dependences of the association constant ($K_a = 1/K_d$) of the trypsinogen–PTI complex (the pH- and temperature variation of K'_d for the chymotrypsin–PTI complex is known [14]). The stability of the trypsinogen–PTI complex decreases drastically below pH 8.0 and is very low at acidic pH. The essential ionizable group for the association of trypsinogen with PTI must be in the basic from and its pK value is 7.4. Similar properties have been found for the association of α-chymotrypsin with PTI (fig.2 A and [14]). A Van 't Hoff representation (fig.2 B) of the data obtained at different temperatures at pH 8.0 allows a determination of the enthalpy variation of the trypsinogen–PTI

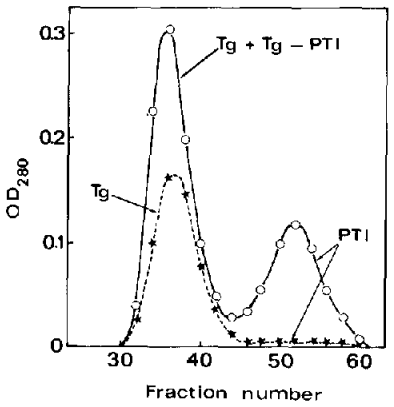

Fig.1. Evaluation of the dissociation constant of the trypsinogen–PTI complex by Sephadex G-75 chromatography. Trypsinogen (0.25 mM) was incubated during 20 min with PTI (1 mM) in 1.2 ml of an ammonium carbonate buffer (10 mM) at pH 8.0, 25°C. The mixture was then chromatographed in a Sephadex G-75 column (3 × 48 cm) equilibrated with the same buffer (full line). This chromatographic system separates well the trypsinogen–PTI complex (mol. wt. 31 000) from excess free PTI (mol. wt. 6500) but not from free trypsinogen (mol. wt.) (24 500). The peak which contained both the free trypsinogen and the complex was pooled and lyophilized. The lyophilisate was then dissolved in 2 ml of 10 mM HCl during 45 min at 25°C to dissociate the complex. A new chromatography on the same Sephadex G-75 column equilibrated at pH 2.0 with 10 mM HCl separated two peaks which contained trypsinogen and PTI respectively (broken line). The total amount of each partner under each peak was measured by spectrophotometry at 280 nm ($\epsilon^{\%}_{cm}$ = 13.9 and 8.29 for trypsinogen and PTI respectively). The amount of inhibitor was more precisely determined by measuring the inhibitory activity towards trypsin [8].

Table 1
Determination of the dissociation constant of the trypsinogen–PTI complex at 25°C, pH 8.0, by competition experiments involving trypsinogen, chymotrypsin and PTI

Total concentration of Chymotrypsin (M)	PTI (M)	Trypsinogen (M)	K_d/K'_d	K_d (M)
10^{-5}	4×10^{-5}	1.2×10^{-4}	197	1.8×10^{-6}
		2×10^{-4}	220	2×10^{-6}
		2.8×10^{-4}	156	1.4×10^{-6}
		3.5×10^{-4}	160	1.5×10^{-6}
2×10^{-6}	6×10^{-6}	1.1×10^{-4}	220	2×10^{-6}
		2.75×10^{-4}	208	1.9×10^{-6}
		4.4×10^{-4}	180	1.6×10^{-6}
5×10^{-7}	2.5×10^{-6}	2.6×10^{-5}	210	1.9×10^{-6}
		5.2×10^{-5}	220	2×10^{-6}

K_d and K'_d are dissociation constants of the trypsinogen–PTI and chymotrypsin–PTI complexes respectively. The competition between trypsinogen and chymotrypsin for complex formation with PTI was followed by measurement of the chymotrypsin activity in the pH-stat after equilibrium was reached. Results were corrected for the known residual activity of the chymotrypsin–PTI complex [14]. The ratio K_d/K'_d was calculated from equation (1) (see text).

association, $\Delta H^0_a = +3.8$ kcal × mole^{-1}. Since $\Delta G^0_a = -RT\log K_a = -7.8$ kcal × mole^{-1}, $\Delta S^0_a = 39$ cal × mole^{-1} × K^{-1}.

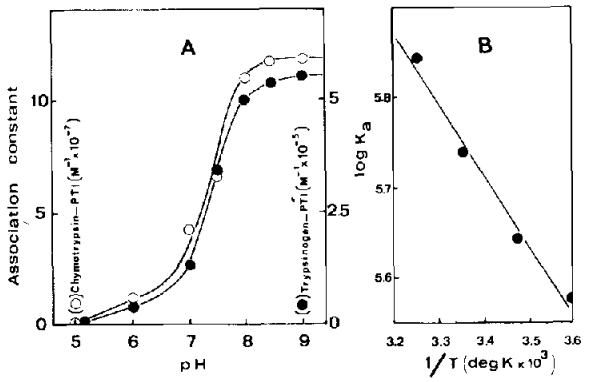

Fig.2. pH- and temperature dependence of the association constant of the trypsinogen–PTI complex. (A) Comparison between the pH dependence of association constants for the chymotrypsin-PTI (O) [14] and the trypsinogen-PTI (•) complexes at 25°C. (B) Van 't Hoff plot describing the temperature dependence of the association constant of the trypsinogen–PTI complex at pH 8.0.

4. Discussion

The association between trypsin and PTI is unusually strong. The dissociation constant of the 1 : 1 complex is 6×10^{-14} M at pH 8.0 and 25°C. One of the characteristics of the trypsin–PTI complex is its very low rate of dissociation, 6.6×10^{-8} sec^{-1}, which corresponds to a half-life of more than 4 months [8]. The contact area between trypsin and PTI mainly involves side-chains which belong to the active site of trypsin [9]: Asp-189* which bears the anionic binding site for the specific recognition of positively charged substrates, His-57 and Ser-195 which are partners in the charge relay system responsible for catalysis, Ser-214 which helps the carboxylate group of Asp-102 (the third partner in the charge relay system) to be rigidly positioned, Asp-194 which forms a salt bridge with the N-terminal Ile during zymogen activation, etc. [9,16]. The strategic interaction in the trypsin–PTI complex is the salt bridge which forms between Asp-189 of trypsin and Lys-15

* The chymotrypsinogen enumeration is used throughout [9].

Table 2
Comparison between kinetic and thermodynamic parameters for interactions between trypsinogen, trypsin or its derivatives and PTI at 25°C, pH 8.0

Complex	k_d^a (sec^{-1})	K_d (M)	$\Delta G'_a$ (kcal × $mole^{-1}$)	$\Delta H'_a$ (kcal × $mole^{-1}$)	ΔS°_a (e.u.)	Reference
Trypsin–PTI	6.6×10^{-8}	6×10^{-14}	−18.1	–	–	[8]
Pseudotrypsin–PTI	6.3×10^{-4}	9×10^{-9}	−11.0	–	–	[8]
Chymotrypsin–PTI	10^{-3}	9.1×10^{-9}	−11.0	+3.0	47	[14]
Trypsinogen–PTI	$\geq 4 \times 10^{-2}$	2×10^{-6}	−7.8	+3.8	39	this work

[a] K_d is the first-order rate constant for the dissociation of complexes.

of PTI. When this interaction is suppressed by disconnecting the carboxylate side-chain of Asp-189 (this new trypsin derivative, pseudotrypsin, is formed by autolytic cleavage of the Lys-188–Asp-189 bond in trypsin [17]) from the specificity site, the affinity of the enzyme for PTI drops considerably (table 2). The dissociation constant of the pseudotrypsin-PTI complex is 1.5×10^5 times higher than that of the trypsin–PTI complex; the first-order rate of dissociation is 10^4 times higher. Another way of estimating the importance of the ion-pair between Lys-15 (PTI) and Asp-189 (trypsin) is to substitute the trypsin partner by chymotrypsin. In that case, comparisons of the homologous sequences and of the three-dimensional structures of trypsin and chymotrypsin indicate that Asp-189 is replaced by a serine residue. Practically all other interactions which associate PTI to trypsin are preserved in the chymotrypsin–PTI complex [9]. Replacement of the essential carboxylate by a serine side-chain prevents salt-bridge formation and gives a dissociation constant for the chymotrypsin–PTI complex which is identical to that found for the pseudotrypsin–PTI association (table 2).

The association of trypsinogen with PTI has many features in common with the formation of the pseudotrypsin-PTI and chymotrypsin–PTI complexes: (i) the dissociation constant of the trypsinogen–PTI complex is very high as compared to that of the trypsin–PTI complex. Conformational changes in the region of the active site during trypsinogen activation increase the stability of the complex by a factor of 3.3×10^7. (ii) The trypsinogen–PTI complex dissociates very rapidly; displacement of trypsinogen by chymotrypsin from the trypsinogen–PTI complex is completed after 1 min, indicating a first-order rate of dissociation higher than 4×10^{-2} sec^{-1} (table 2). (iii) The association of trypsinogen with PTI is also dependent upon the ionization of a group with a pK of 7.4 which is presumably the imidazole sidechain of His-57 (fig.2 A). (iv) Similarly to the chymotrypsin–PTI interaction, the trypsinogen–PTI association is an entropy driven process (table 2). The high positive variation of ΔS°_a is due to expulsion of water molecules during complex formation, i.e. hydrophobic binding [9].

The enormous difference in affinity for PTI between trypsinogen and trypsin is most likely mainly due to the fact that Asp-189 is not available for salt-bridge formation with Lys-15 of PTI in the zymogen molecule in which the specificity site is incompletely formed. However, the chymotrypsin–PTI and pseudotrypsin–PTI complexes are still more stable than the trypsinogen–PTI complex by a factor of 200 (i.e. $\Delta(\Delta G^0_a) = 3.2$ kcal × $mole^{-1}$). The difference is probably explained by the absence of some hydrophobic interactions in the trypsinogen–PTI complex which exist in the chymotrypsin–PTI, pseudotrypsin–PTI and trypsin–PTI complexes. This explanation is strengthened by the fact that ΔS^0_a is higher for the chymotrypsin–PTI complex than it is for the trypsinogen–PTI complex (table 2). In summary, zymogen activation would both expose Asp-189, so that it becomes available for salt-bridge formation with the substrate, and increase the possibilities of hydrophobic interactions between substrate and the specificity site.

It is of interest to remark that whereas activation of trypsinogen provokes enormous variations of affinity for PTI, the activation of a prohormone such as proinsulin provokes much more limited changes of

affinity (a factor of 20) for the membrane receptor [18].

It may be worthwhile to mention at the end of this paper that 6-amino-hexanoic Sepharose-PTI columns quantitatively retain trypsinogen at pH 7–8. Trypsinogen is easily released from the affinity column at pH 2.0 or at pH 7–8 in the presence of excess free inhibitor. This technique could easily be used for the purification of zymogens of the numerous trypsin-like enzymes which are involved in blood coagulation [19] or in fertilization [20] for example.

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