INTERACTIONS BETWEEN TRYPSIN, α_2 MACROGLOBULIN AND SOYBEAN TRYPSIN INHIBITOR

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Summary

The present study reveals that a trypsin-α_2 macroglobulin complex cannot be dissociated by the intervention of soybean trypsin inhibitor even after a 60 hour-incubation time. If the inhibitor is first bound to trypsin, the addition of α_2 macroglobulin restores about 60% of the enzyme activity. These results from kinetic measurements have been confirmed by absorption difference spectra. The values of the association constants of trypsin with these two proteins being of the same order, we think that, when trypsin is surrounded by the large molecule of α_2 macroglobulin, the binding site of the inhibitor is blocked by steric hindrance while a first association of soybean trypsin inhibitor with the enzyme does not implicate the same aminoacids which interact with α_2 macroglobulin.

As to the undissociable inhibitor, it might be strongly bound to trypsin by an acyl covalent bond.

Studies on the formation of a stable complex between trypsin and α_2 macroglobulin have been the subject of many communications (1, 2, 3). It was shown that 2 molecules of trypsin can bind to 1 molecule of $\alpha_2 M$* with a very high association constant. In this enzymatic complex, hydrolytic power towards small synthetic substrates is preservated but not towards large proteinic ones. In the same way, inhibitors of high molecular weight are uneffective in stopping the activity of " complexed trypsin ".

In order to understand the mechanism of trypsin binding to these proteins, we had compared the behaviour of two inhibitors of different size : the basic pancreatic inhibitor and the soybean trypsin inhibitor. It resulted from that study (4) that pancreatic inhibitor can progressively reach the

* Abbreviations : $\alpha_2 M$, α_2 macroglobulin ; STI, soybean trypsin inhibitor ; T , trypsin ; ZAPNA, Nα carbobenzoyl L arginin p-nitroanilide; PNA, p-nitroaniline.

binding site on trypsin by forming a ternary complex which has lost a great part of its hydrolytic power while STI is completely unable to inhibit any activity of the T-α_2M complex.

In the present paper, we report the results of experiments on the interaction between T, α_2M and STI which depends on the processus of addition of these proteins. The evolution of the system as a function of incubation mixture is investigated by two methods : kinetic measurements of amidase hydrolysis and absorption difference spectra. Actually, the results obtained by adding either α_2M to the T-STI complex or STI to the T-α_2M complex are very different although the association constants values of trypsin to both of these proteins are similar. The addition of α_2M to the inhibited trypsin restores a great part of the enzyme activity while STI cannot inhibit any activity in T-α_2M whatever long the incubation time may be.

A comparison of the results in both situations from kinetics and from variations in absorption difference spectra leads to the same conclusions.

Materials and Methods

-α_2M isolated from porcine serum in our laboratory (5) is stored at 4°C in phosphate buffer pH 7.3

- porcine trypsin is purchased from Miles Seravac and further purified by chromatography on SE-sephadex according to Schroeder and Shaw (6).

-STI obtained three times crystallized from Sigma, is further purified according to Yamamoto and Ikenaka (7), on DEAE cellulose pH 5.3 with a salt gradient of 0.2 to 0.5M acetate buffer.

Protein concentrations are determined by absorbance at 280 nm, using $E_{cm}^{1\%}$ = 10.2 for α_2M (8), 14.2 for porcine trypsin (9) and 8.9 for STI (not yet published). The molecular weights are assumed to be respectively : 960,000 , 24,000 and 20,000.

Preparation of protein mixtures Different types of mixtures of trypsin, α_2M and STI, prepared as indicated in the following , are incubated at 25° before testing the activity (fig. 1).

The first complex (T-α_2M or T-STI) is obtained by incubating both proteins, in stoechiometric proportions, 10 minutes at pH 7.3 . The zero time of incubation starts when the third protein is added to mixtures 1 and 2

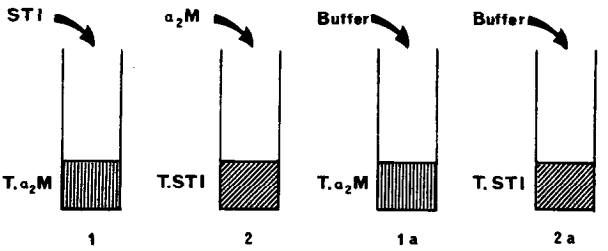

Fig. 1 Diagram representing different types of mixtures.

(protein is replaced by buffer in mixtures 1_a and 2_a for control). The total volume of each mixture is calculated to allow 6 assays on a 50 µl volume solution containing 10 µg trypsin, 300 µg α_2M and 12 µg STI.

- Amidasic activity of trypsin in 50 µl incubation mixture is determined at pH 8.2 by measuring the hydrolysis rate of ZAPNA * as an absorbance increase at 410 nm (9).
- Absorption difference spectra are recorded in a Cary 15 at 25° in double compartments cells with 0.436 cm path lengths, recordings taken from 310 to 260 nm on the expanded scale (full scale 0.1 absorbance unit). The spectra are recorded on mixtures after different times of incubation as for kinetic determinations.

Results

Kinetic measurements show that :

In mixture 1, STI is unable to inhibit trypsin from T-α_2M complex even after a long time of incubation (until 60 hours) : the rate of hydrolysis of the amide substrate remains constant for each mixture prelevement (fig. 2 curve a) and is the same as for mixture 1_a (Table I, 1 and 1_a).

In mixture 2, α_2M added to T-STI complex can progressively bind to trypsin and a great part of the enzyme hydrolytic power is restored (fig. 2_b) but recovering of activity is never complete (no more than 60% after 24 hours of incubation and next no change). We were able to prove that there is no formation of a ternary complex between T-α_2M and STI as it had been found with basic pancreatic inhibitor (5) and with STI when elastase takes the place of trypsin in the enzyme -α_2M complex (10). Thus, if we add a dose of trypsin

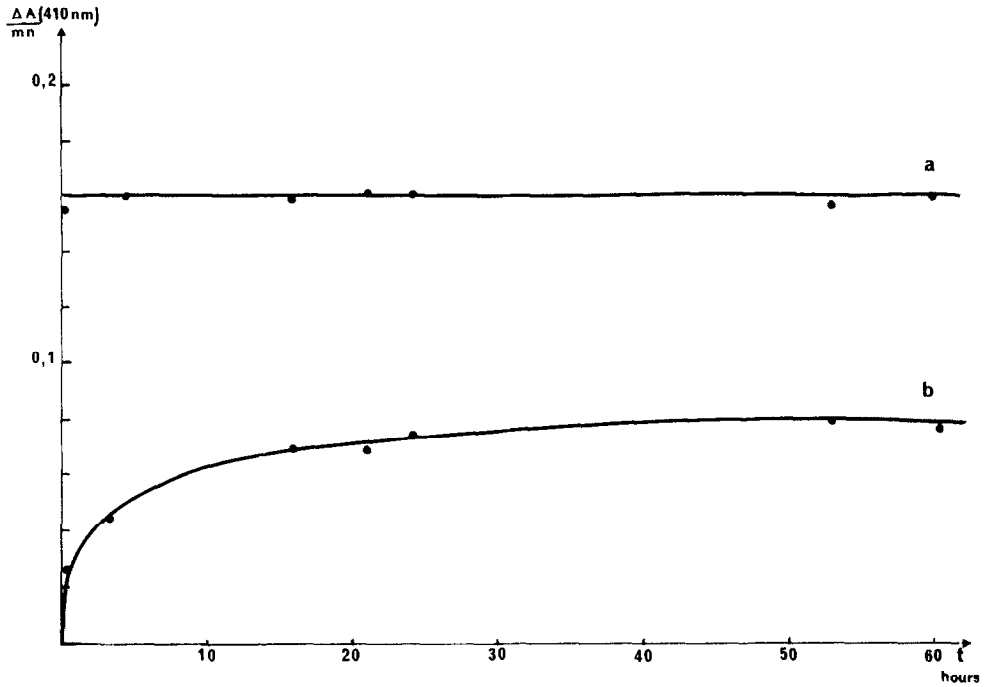

Fig. 2 Hydrolysis rate of ZAPNA measured at 25°, pH 8.2
Variation of PNA absorbance at 410 nm versus time of incubation for two types of mixture :
a) trypsin-α_2 macroglobulin complex + STI
b) trypsin-soybean trypsin inhibitor complex + α_2M

in the spectrophotometric cuvette during kinetics recording on 21 hour-incubation mixtures, we observe :

- in the case of mixure 1, no increase in the hydrolysis rate ; which proves the presence of unfixed STI in its native form able to inhibit the newly added trypsin (Table I, 1).

- in the case of mixture 2, an increase in the hydrolysis rate corresponding only to the part of trypsin uninhibited because the concentration of free STI is not sufficient. The trypsin in such a fraction has immediately bound to free α_2M as it was revealed by an ulterior addition of STI which does not bring out any inhibition (Table I, 2). A same value of hydrolysis rate is obtained when STI is added to T-α_2M (mixture 1_a), this complex being not susceptible to inhibition (Table I, 1_a). A control measure shows that T-STI complex (mixture 2_a) is never dissociated in our experimental conditions (Table I, 2_a).

TABLE I

Incubation time	mn	hours					
	5	3	16	21	24	53	60
(1) T-α_2M + STI	0,155	0,160	0,160	0,160	0,160	0,155	0,160
+ T				0,160	0,165	0,160	-
(2) T-STI + α_2M	0,030	0,045	0,070	0,070	0,075	0,080	0,075
+ T				0,160	0,155	0,160	-
+ STI				0,155	0,150	0,160	-
(1_a) T-α_2M	0,160	0,160	0,155	0,160	0,155	0,160	-
+ STI	0,160	0,155	0,155	0,160	-	-	-
(2_a) T-STI	0,003	0,003	0,002	-	-	-	-

Table I. Activity of trypsin expressed by ZAPNA hydrolysis rate in 4 types of mixture as a function of incubation time.

The progressive dissociation of T-STI complex when α_2M is added has been confirmed by absorption difference spectra (fig. 2). Curve (a) reports the absorption difference spectrum of T-α_2M versus free T and free α_2M and is used as a basis for the following measurements. Curves (b) and (c) report the absorption difference spectra of T-STI (in one compartment of cell I) , T-α_2M (in the other compartment of cell I) versus T-STI +α_2M mixture (in one compartment of cell II) , T (in the other compartment of cell II) respectively after a 4-hour and 43-hour incubation time. In this last cell, trypsin is introduced to ensure the same concentration of protein in the two cells.

At zero time, T-STI complex is not dissociated and there is no T-α_2M present in the cell II ; this state can be represented by curve (a). Then dissociation of T-STI starts and T-α_2M appears in the mixture with free STI : the absorption difference spectrum is lowered as a function of time (curves(b) and (c)).

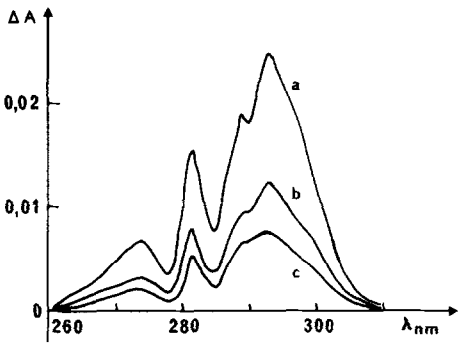

Fig. 3 Absorption difference spectra
curve (a) T-α_2M complex versus free T and free α_2M.
curves(b) and (c) T-α_2M and T-STI versus T-STI + α_2M mixture and free T, after respectively 4 and 43 hours of incubation.

If dissociation of T-STI were complete the final recorded spectrum would be only the spectrum of T-STI against free T and free STI. The influence of this spectrum has been taken into account but it should be noticed that the perturbation is not very important because weight-volume concentrations of T and STI are weak in comparison with the α_2M one's (less than 5%). Calculations based on the variations of absorbance at 292 or 282 nm lead to a value for T-α_2M complex which is very close to the one calculated from kinetic measurements (50 to 60%). However a precision of 10% on this evaluation is the best expected.

Discussion

Although the association constant determined by Lebowitz and Laskovski (11) for the T-STI complex ($K = 6.10^9 M^{-1}$) is higher than the association constant for T-α_2M complex (2) ($K = 3.10^8 M^{-1}$) the inhibitor cannot bind to trypsin when α_2M is present. If we admit that there is an equilibrium reaction :

$$T\text{-}\alpha_2M + STI \underset{2}{\overset{1}{\rightleftharpoons}} T\text{-}STI + \alpha_2M$$

In step 1 of the reaction the energy of activation barrier could be too high to permit the removal of α_2M . We can think that trypsin is surrounded by α_2M molecule when the complex is formed as it was obvious from electronic microscopy (12) so that the access of the binding site would be blocked. This hypothesis implies the intervention of an important number of aminoacids in

the contact region of the proteins; which is consistant with the results of Barrett and Starkey (13) who considered that in T-α_2M complex and more generally in all proteinase - α_2 M complexes the enzyme is " trapped " inside the α_2M molecule. In this way the reaction would be irreversible and the complex undissociable.

In step 2 of the reaction T-STI complex is partially dissociated by α_2M binding . In order to understand why a part of STI is made free from the inhibited-complex by α_2M intervention while another part of STI remains strongly bound to trypsin, we report first to Haynes and Feeney (14) in their study on protease inhibitors and trypsin association. These authors propose an initial Michaelis-type complex made up by the binding of inhibitors amino acids chain to the substrate binding site of trypsin (Arg in the case of STI). Subsequently a conformational change and the formation of additional non-covalent bonds would result in the stabilization of the inactive enzyme-inhibitor complex. According to the results of Chepyzheva et al. (15) based on tritium hydrogen exchange kinetics, 10 to 35 aminoacids residues of trypsin should be in contact with pancreatic inhibitor (mol. wt. 6,500) and 25 to 50 residues with ovomucoïd (mol. wt. 28,000). So, for STI (mol. wt. 20,000) we can expect that about 20 to 40 aminoacids residues interfere in the association with trypsin. Papaioannou and Liener (16), Delarco and Liener (17) have established that the contact region between trypsin and STI implicated four tyrosines and five aminogroups among which Arg 55 of trypsin and Arg 63 of STI. In our experiments (mixture 2) α_2M could interact with aminogroups of trypsin non implicated in the T-STI complex and induce a conformational change of the enzyme resulting in a decreased interaction between T and STI, a release of the inhibitor and the formation of T-α_2M complex. This explanation does not agree with Barrett and Starkey (13) hypothesis. According to these authors, the enzyme-α_2M complex cannot be obtained with catalytically inactive enzyme because its " trapping " by α_2M would need a preliminary proteolysis of the macroglobulin.

As to the part of STI which cannot be dissociated from the T-α_2M complex it is tempting to suggest the mechanism of proteolysis of the inhibitor proposed by Osawa and Laskovski (18). These investigators present evidence that a long interaction of trypsin with STI results in a splitting of the Arg^{63}-Ile^{64} bond in the peptidic chain of STI. The formation of an acylbond between the active serine of trypsin and the sensitive arginine of the inhibitor could result in a

covalent complex. Thus α_2M could no more interact with trypsin to drive away the inhibitor.

In conclusion, our results prove, with quantitative data, that STI cannot bind on trypsin when the enzyme is first complexed to α_2M , even after a long time incubation. Although the aminoacids implicated in the binding of STI or α_2M are not the same, the large molecule of α_2M , in surrounding the trypsin molecule, probably blocks the access to the binding site of STI.

Conversely we have shown that α_2M is able to remove a part of STI from the inhibited-trypsin and form a stable complex with free trypsin. As observed with T-α_2M the restored activity is limited to small substrates.

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