AMIDASIC ACTIVITY OF Nε-ACETYLTRYPSIN FREE OR BOUND ON THE α_2 MACROGLOBULIN

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SUMMARY

Nε-acetyltrypsin (Tac) exhibits a higher activity than native trypsin towards an amide substrate, N^{α}-benzoyl dl-arginine p-nitroanilide (BAPA). Affinity constants for the substrate are not modified but the rate constant is increased five times.

A complex is formed between Tac and the α_2 macroglobulin (αM) in the same conditions as we have seen for native trypsin : 1 mole of αM binds to 2 moles of enzyme. The increase of Tac activity towards BAPA disappears once a stable complex is formed.

Possible explanations for these observations are discussed.

It has been previously shown that trypsin may bind α_2 macroglobulin (αM) to form a complex which retains the whole hydrolytic activity of the enzyme towards specific esters or amides but not to protein substrates (1). In the same way, only small molecular weight inhibitors can reach the binding site of the enzyme, while large ones like soya bean inhibitor (STI) are ineffective. The fixation of large molecules is probably prevented by the steric hindrance due to the α_2 macroglobulin. The basic pancreatic inhibitor which has a molecular weight three times smaller than STI inhibits trypsin in spite of the presence of the αM and the formation of a ternary complex has been shown in this case (2).

To ascertain which groups of the enzyme are involved in the binding on the macroglobulin, it was of interest to use a form of trypsin which could be sufficiently stable and homogeneous. The Nε-acetyltrypsin prepared by Labouesse and al. (3) fullfilled these properties and it has been already pointed out (4) that this compound has the same activity as native trypsin

towards specific substrates, BAEE, TAME or BAA*.

RESULTS.

The hydrolytic activity of the N$^{\epsilon}$-acetyltrypsin (Tac)** was investigated towards the N$^{\alpha}$-benzoyl dl-arginine p-nitroanilide (BAPA) by spectrophotometric titration, at 410 nm, of the liberated p-nitroaniline. In our method, the rate of hydrolysis is recorded with a Cary 15 spectrophotometer, just after the addition of the enzyme to the solution of substrate in tris buffer at pH 8,2.

The comparison between trypsin and Tac has shown a highly increased rate of the reaction in favor of Tac. The rate of hydrolysis measured at 25°C in these conditions was five times higher for Tac than for native trypsin.

With two other amide substrates, BAA and BANA, an increase in activity of the modified trypsin has not been found.

As it was shown by Erlanger (5), only the l form of BAPA is hydrolyzed by trypsin and the d form behaves as a competitive inhibitor ; it could be possible that the d-BAPA might have a peculiar behaviour with the acetyltrypsin. Thus, according to Erlanger's technic, pure d isomer was isolated from a trypsic digest of the racemic substrate so that, added to the dl-BAPA, the inhibitor concentration remains constant. The kinetic parameters were determined for trypsin and Tac at 30°C in the usual way from a Lineweaver-Burk plot (fig. 1).

It can be seen that the inhibition is competitive for both trypsins (fig. 1 A and B). The Ki of d-BAPA and the Km and kcat values of l-BAPA calculated from these curves are given in table 1. It is obvious that the affinities for the substrate and for the inhibitor are not significantly different for native and acetyltrypsin but the hydrolytic rate constant is very enhanced when trypsin is acetylated.

The increase in activity observed for Tac is difficult to explain. The acetylation of the 14 lysin résidues of trypsin modifies the electric charge of the molecule. For instance by electrophoresis at pH 7, the trypsin is migrating towards the cathode while Tac migrates towards the anode. The behaviour of

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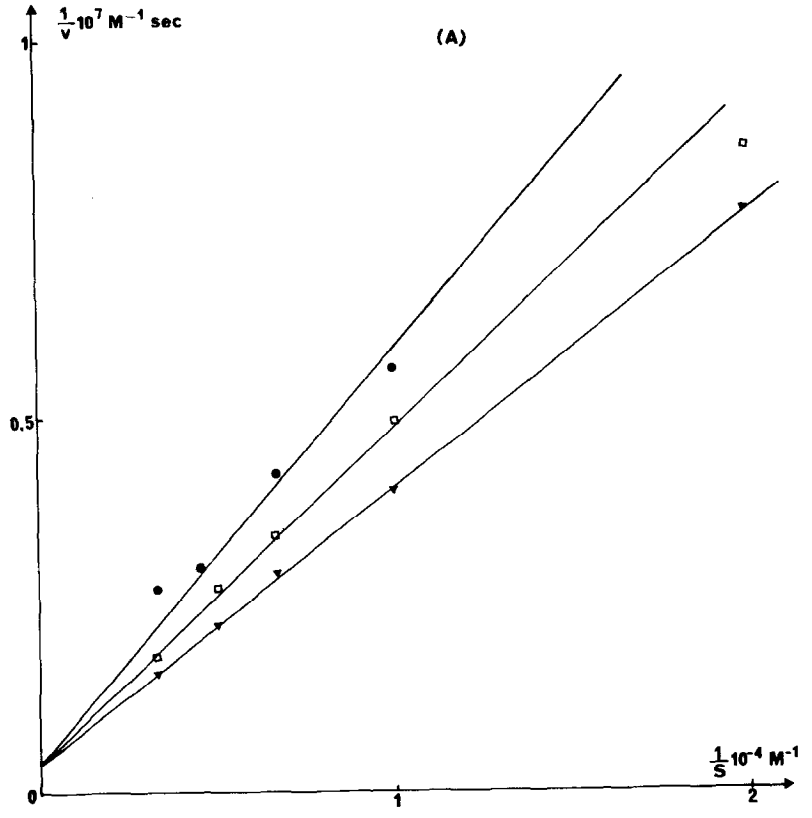
* Abbreviations : BAEE, Benzoyl l-arginine ethylester ; TAME, tosyl l-arginine methylester ; BAA, benzoyl l-arginine amide ; BANA, benzoyl dl-arginine naphtylamide.

** a gift from J. Chevallier (Lab. Enzymologie Physico-Chimique, Orsay)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(A)
1
0.5
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1
2
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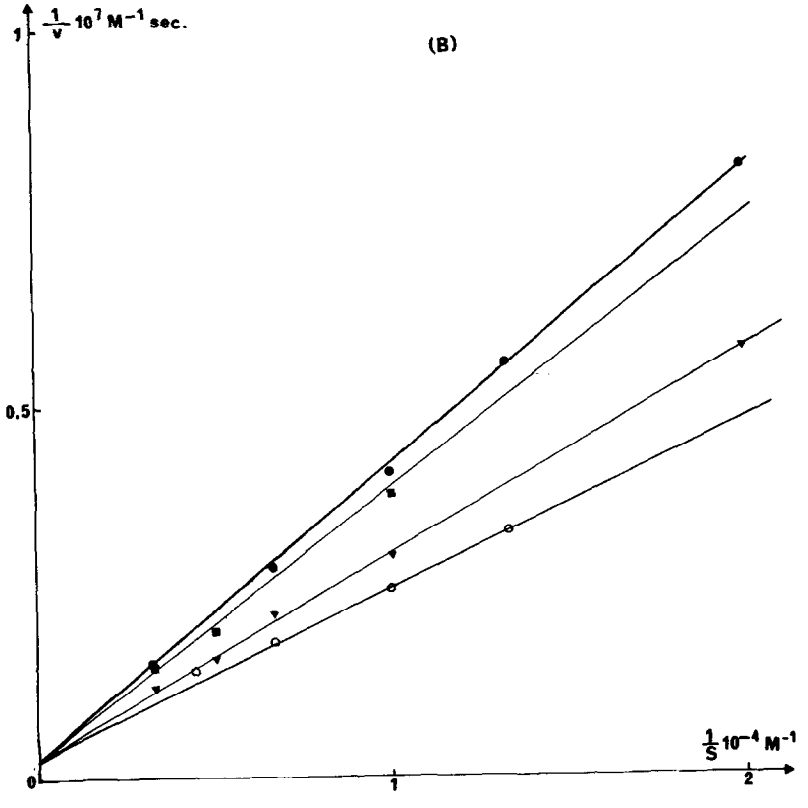
$\frac{1}{v}10^7\ M^{-1}\ sec.$
(B)
1
0.5
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2
$\frac{1}{S}10^{-4}\ M^{-1}$

Fig. 1 Relationship between hydrolysis rate of dl-BAPA in presence of known concentrations of d-BAPA : ○ 2,5. 10^{-4} M ; ▲ 3. 10^{-4} M ; □ 5. 10^{-4} M ; ■ 6. 10^{-4} M ; ● 7. 10^{-4} M. S = l-BAPA concentration; v = hydrolysis rate.
A) trypsin, 1,25. 10^{-6} M . B) acetyltrypsin, 0,42. 10^{-6} M.

TABLE 1

Summary values of kinetic constants measured at 30°C, pH 8,2 .

	Km (l-BAPA) M 10^3	Ki (d-BAPA) M 10^3	kcat sec^{-1}
Trypsin	0,55	0,52	1,9
Tac	0,66	0,57	9

these two forms of enzyme with the classic inhibitors is different : furthermore chicken ovomucoid inhibits trypsin but is completely ineffective for Tac (6) ; soya bean factor inhibits instantaneously trypsin but needs a certain time to suppress Tac activity . In this last case, we calculated the rate constant from recorded hydrolysis curves and found the value of 1,5. 10^5 1 M^{-1} sec^{-1} for k. Another specific inhibitor, the basic pancreatic factor reacts with Tac at a rate 100 times faster than it does with trypsin. Green (7) ascribed this different rate of reaction to a diminution of the electrostatic repulsions in Tac. The presence of an electron-attracting nitroanilide function in the substrate could perhaps contribute to an increase in activity when the global charge of the trypsin is modified.

The formation of a Tac-αM active complex has been studied in a similar way as for trypsin (1). The binding of the macroglobulin on the enzyme was spectrophotometrically followed until the addition of STI does not modify anymore the hydrolysis rate of the substrate. But we noticed here that the strong activity observed with free Tac, decreased progressively in the binding of αM on the enzyme. When all the Tac was saturated , the activity was reduced to 33% of the initial value.

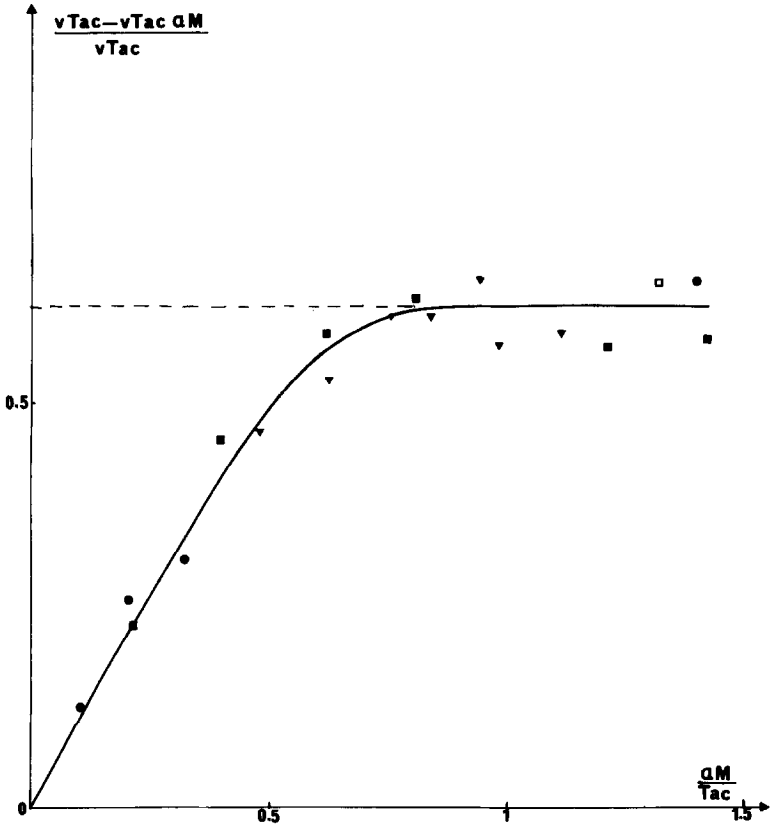

Fig. 2 Saturation curve of Nε -acetyltrypsin by α_2 macroglobulin. Variation of hydrolytic activity towards BAPA, versus the molecular ratio (αM) / (Tac).

v_{Tac} : hydrolysis rate of free Tac
$v_{Tac\ \alpha M}$: hydrolysis rate of Tac bound on αM

The Tac saturation curve (fig. 2) was obtained by plotting the relative variation of hydrolytic activity versus the ratio of the macroglobulin to the acetyltrypsin concentrations. The value of the limiting rate corresponding to the complex is calculated from the expression $v_{Tac} - v_{lim} / v_{Tac}$ obtained by extrapolating the horizontal part of the curve.

The number of sites implicated in the formation of the complex and the association constant of Tac and of αM is obtained from a Scatchard plot :

$$K_{ass} = \frac{(\text{fixed ligand})}{(\text{free ligand})(\text{free site})} \quad \text{which leads to} \quad \frac{1}{1-\varphi} = K_{ass}\frac{D}{\varphi} - nE$$

where D is the total concentration of ligand (αM) ; E the initial concentration

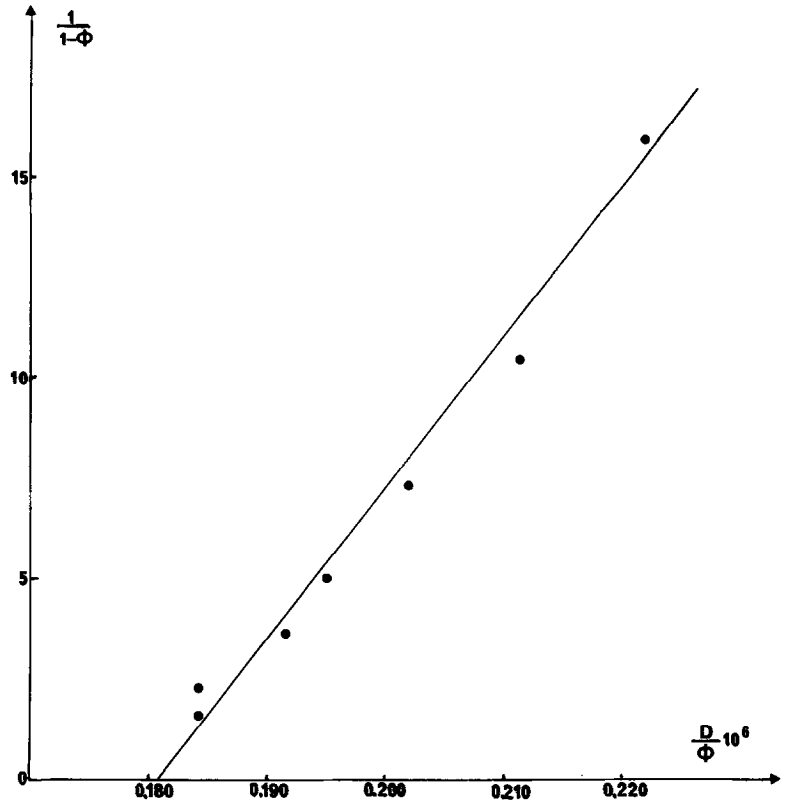

Fig. 3 Scatchard representation : $\frac{1}{1-\varphi} = K_{ass} \left(\frac{D}{\varphi} - nE \right)$ $E=0,348.10^{-6}M$. See text for full details.

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of enzyme ; φ the ratio between concentration of fixed ligand and total enzyme.

The intercept of the straight line with the abscissa (fig. 3) gives 0, 52 for the number of sites. This value is very close to the one obtained in the formation of the complex between native trypsin and αM where there are two sites, each one binding 1 mole of enzyme. The association constant calculated at 25°C is : $K_{ass} \simeq 3,7.10^8\ M^{-1}$. This value is also similar to the one determined for the binding of native trypsin on αM (1).

DISCUSSION.

The increase of hydrolytic activity of Nε-acetyltrypsin observed only towards the substrate : benzoyl dl-arginine p-nitroanilide could be clarified by the findings concerning the heterogeneity of commercial trypsin. Recently it was reported (8) that two chief components were separable from trypsin

by chromatography ; the β form with a single chain was shown 50% more active than the α form with an intra chain split between lysine-131 and serine-132. In the step of purification of N$^{\varepsilon}$-acetyltrypsin, the homogeneous compound, chromatographically selected, corresponds probably to the more active β form of the enzyme.

In order to explain the dicreasing of the activity when Tac binds αM, we have suggested the intervention of a subsidiary site which could be revealed on the enzyme molecule during the acetylation of lysine residues and then masked by the αM binding.

This suggestion led us to compare the presumable site implicated in the acetyltrypsin with the one involved in the activation of native trypsin by high substrate concentration (9, 10). But no modification in the kinetics was observed for Tac in a concentration range of BAPA from 10^{-3} to 10^{-5}M. Thus it is not possible to explain the increase of hydrolysis rate for Tac by an effect due to substrate concentration.

In any case the phenomenon of activation cannot be attributed to the presence of d form included in the racemic substrate since the Ki values of d-BAPA and Km values of l-BAPA are similar for both the enzymes , trypsin and Tac. Whether electrostatic attractions between nitro-group substrate and Tac are responsible for the increase of catalysis it would not be at the first step of the enzymatic reaction.

It has also been proved that an anionic protein, the N$^{\varepsilon}$-acetyltrypsin can bind the α_2 macroglobulin to form an active complex. This result does not agree with the suggestion of Boyde (11) who, investigating two aspartate aminotransferases, has shown that only the cationic isomer was able to bind the macroglobulin. The author thought that the formation of an αM-enzymatic complex was not possible with anionic proteins. Our data indicate that, in spite of their opposite electric charge, both the trypsin enzymes may bind α_2 macroglobulin.

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