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COMPARATIVE STUDIES ON THE MECHANISM OF ACTIVATION OF THE TWO HUMAN TRYPSINOGENS

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Summary

The activation of human trypsinogens 1 and 2 by porcine enterokinase at pH 5.6 shows that the two human zymogens are equivalent substrates for this enzyme and that both proteins are activated faster than the cationic bovine trypsinogen. At pH 8.0 and in the presence of 20 mM calcium the two human trypsinogens are activated by either human trypsin at the same rate but the affinity of both trypsins is higher for trypsinogen 1 than for trypsinogen 2. Two Ca^{2+} binding sites are identified in the two human zymogens and their $pK(Ca^{2+})$ values determined. For trypsinogen 1 the values are respectively of 2.8 and 3.3 for the primary and secondary Ca^{2+} binding sites, and for trypsinogen 2 of 3.4 and 2.7. These values are markedly different from those obtained for bovine cationic trypsinogen, especially in the case of trypsinogen 1. These results point out a different degree of saturation of the calcium binding sites of the 2 human zymogens that must exist in physiological conditions, suggesting different biological activities of the two trypsinogens.

Introduction

The presence of two trypsinogens in pancreatic secretion has been described in the literature for several species [1—5] including man [6]. Extensive studies of some trypsinogens [7—10] and many trypsins [11—14] do not permit a general idea to be formed explaining the reasons for these double zymogens or/and enzymes. It seems only that the presence of two trypsins in the duodenum is not necessary since the two enzymes have very similar specificities [14].

Abbreviation: Tos-Arg-OMe: *p*-tosyl L-arginine methyl ester.

The conversion of trypsinogen into trypsin by enterokinase is the first step of the cascaded activation sequence. The produced trypsin in turn catalyses the autoactivation of trypsinogens and the activation of other pancreatic zymogens. It was demonstrated that the peculiar sequence of the four aspartic residues of the activation peptide was a signal of recognition for enterokinase [15]. On the other hand, the same residues have a very negative effect on the activation of trypsinogen by trypsin and this effect is abolished by a high concentration of calcium which binds to the N-terminal aspartyl residues [16]. It was further proposed a loose arrangement of the N-terminal hexapeptide of the cationic bovine trypsinogen on the surface of the zymogen [8]. The comparison of the kinetic parameters of activation of the two bovine and the two porcine trypsinogens has shown that the anionic zymogens differed markedly from the cationic homologous protein since they were better substrates for enterokinase and trypsin than the cationic zymogens [10]. This difference suggests that the activation of trypsinogen into trypsin is governed by other still unknown factors.

The two human trypsinogens, like all mammalian trypsinogens, liberate on activation a peptide containing the characteristic sequence of the four aspartyl residues preceding the strategic Lys-Ile bond [17,18]. Previous studies have pointed out some major differences between autocatalytic activation of the two human zymogens [19].

In this paper we report a more detailed study of the activation of the two human trypsinogens by enterokinase and trypsin and we make an attempt to relate these differences to the conformational structure of the activation domain.

Materials and Methods

Material

Human trypsinogens 1 and 2 were purified as described previously [18] from pancreatic juice collected by catheterization of the main pancreatic duct from patients with normal pancreatic function. Cationic bovine trypsinogen was a commercial product from Worthington. The two human trypsins were prepared by controlled autoactivation of each zymogen in a 5 mM Tris-HCl buffer at pH 8.0 and in the presence of 20 mM calcium at 4°C. Porcine enterokinase was a kind gift of Dr. S. Maroux.

Methods

Determination of protein concentration. Enterokinase and trypsinogen concentrations were determined by measuring the absorbance at 280 nm, using the extinction coefficient $E^{1\%}_{1cm} = 15.2$ for trypsinogen 1, 15.1 for trypsinogen 2 [18], 14.4 for bovine cationic trypsinogen [20] and 8.6 for porcine enterokinase [21].

Conditions of trypsinogen activation. Trypsinogen activations were carried out at 4°C. Aliquots (0.1 ml) of incubation mixture were taken off periodically and trypsin activity was assayed. Trypsin activities were measured at 25°C pH 7.9 in a pH stat Radiometer using 100 mM NaOH. The assays were performed on 10 mM *p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe) in a 5 mM Tris-HCl buffer containing 20 mM $CaCl_2$ and 100 mM KCl. For each activation, a sample

of zymogen incubated without any activating enzyme was studied as control.

Trypsinogen activation by enterokinase was performed at pH 5.6 in a 28 mM succinate buffer [22]. Trypsinogen concentration was 33 μg/ml, enterokinase concentration was 0.33 μg/ml in the experiments in the absence of calcium and 5 μg/ml in the experiments in the presence of 1 mM calcium in order to distinguish initial rates of activation by enterokinase from autocatalytic activation.

Trypsinogen activation by human trypsin was performed at pH 8.0. Trypsinogen concentrations varied between 35 μg/ml and 65 μg/ml. Activating trypsin concentration varied from 1.2 μg/ml to 15 μg/ml. These activations were carried out in a 10 mM Tris-HCl buffer, 100 mM NaCl in the presence of different concentrations of calcium. The activation in the absence of calcium were performed in a 25 mM phosphate buffer containing 100 mM NaCl.

Results

(A) Trypsinogen activation by enterokinase

The kinetics of activation of the two human and the cationic bovine trypsinogens by porcine enterokinase at pH 5.6 with and without calcium are presented in Fig. 1.

Without calcium the two human trypsinogens were activated with the same initial rates of reaction whereas bovine trypsinogen was activated much more slowly (Fig. 1A).

In the presence of 1 mM calcium some differences appear between the kinetics of activation of the two human trypsinogens (Fig. 1B). The half-times of activation were 6 min for trypsinogen 1 and 14 min for trypsinogen 2.

As demonstrated in the same figure, in the presence of calcium and without added enterokinase the two trypsinogens are able to autoactivate but with a greater half-time of activation.

(B) Trypsinogen activation by human trypsins

(a) Comparison of the autoactivation of each trypsinogen by the two human trypsins. The kinetics of activation of trypsinogens 1 and 2 by human trypsins 1 and 2 were separately studied. Each trypsinogen is activated at a similar rate by either human trypsin.

(b) Comparison of the activation of trypsinogens 1 and 2 by human trypsin 1. In order to compare the two human trypsinogens as substrates for human trypsins, trypsinogens 1 and 2 at a concentration of 65 μg/ml were activated by 1.3 μg of trypsin 1. As shown in Fig. 2 trypsinogen 1 is a better substrate for trypsin 1 than trypsinogen 2 since the half-time of activation of trypsinogen 1 is equal to 33 min under these conditions, whereas the half-time of activation of trypsinogen 2 is equal to 98 min.

(C) Characterization of the two calcium ion binding sites of each trypsinogen

The effects of calcium concentration on the activation of trypsinogens 1 and 2 at pH 8.0 are plotted in Figs. 3 and 4. The graphs given at the top of the figure (log of percent of activity in relation with time), allows the determination of the constant k_1 of the hydrolysis of the Lys-Ile bond. In the absence of

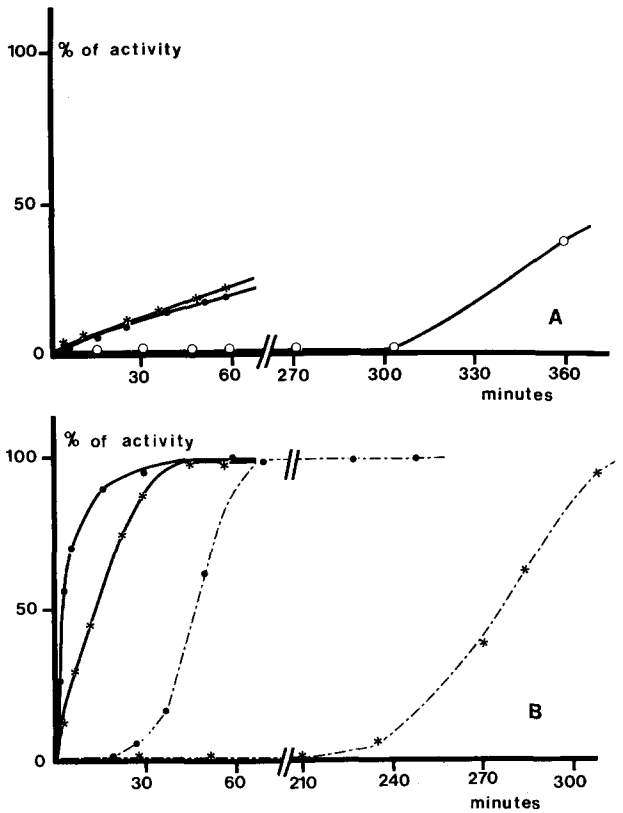

Fig. 1. Activation of human trypsinogens 1 and 2 and cationic bovine trypsinogen by porcine enterokinase at pH 5.6. (A) Human trypsinogen 1 (●———●), human trypsinogen 2 (*———*) and bovine cationic trypsinogen (○———○), each at a concentration of 33 μg/ml were activated by porcine enterokinase at a concentration of 0.33 μg/ml. Activations were carried out at 4°C in a 28 mM succinate buffer in the absence of calcium. (B) Trypsinogen 1 (●———●) and trypsinogen 2 (*———*) at a concentration of 35 μg/ml were activated at 4°C by porcine enterokinase (5 μg/ml) in 28 mM succinate buffer in the presence of 1 mM calcium. - — - — -, activation kinetics of trypsinogens 1 and 2 in the same conditions but without enterokinase.

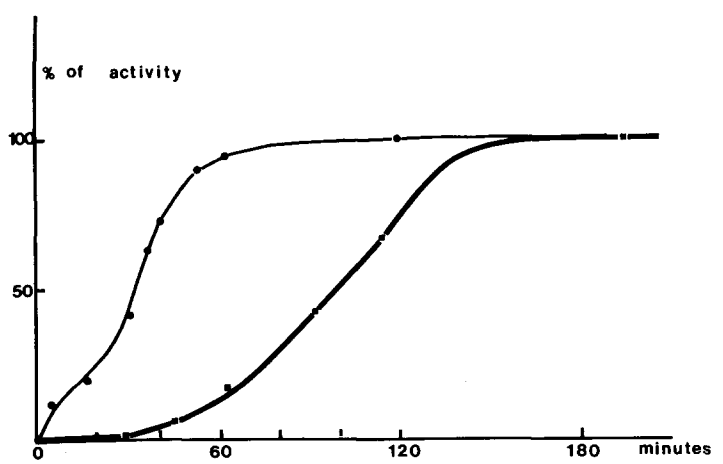

Fig. 2. Comparative activation of the two human trypsinogens by human trypsin 1. Trypsinogen 1 (●———●) and trypsinogen 2 (■———■) at a concentration of 65 μg/ml were activated by human trypsin 1 at a concentration of 1.2 μg/ml. Activation was carried out at 4°C in a 100 mM NaCl, 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$.

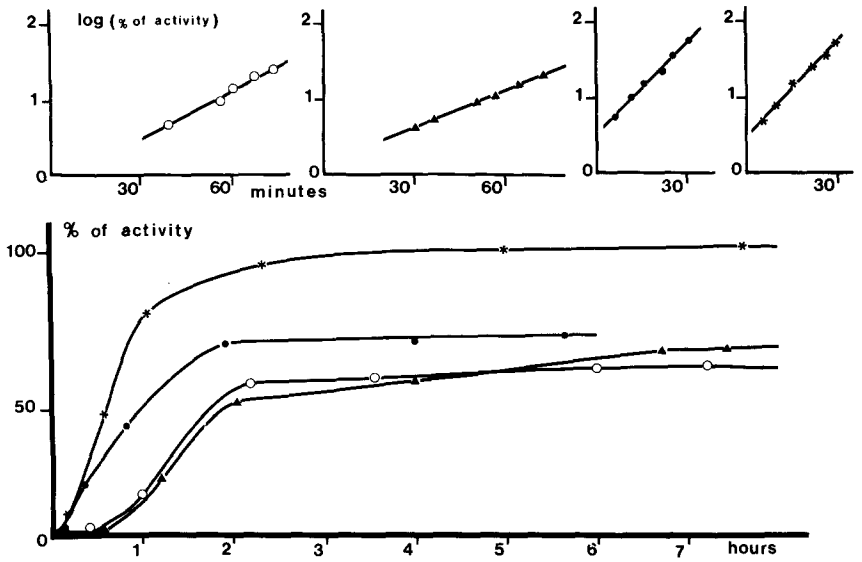

Fig. 3. Kinetics of activation of human trypsinogen 1 by trypsin 1 at pH 8.0 at different calcium concentrations. Concentration of trypsinogen 1: 35 μg/ml; concentration of trypsin 1: 5 μg/ml. Activations were performed in a 100 mM NaCl, 25 mM phosphate buffer, pH 8.0, without calcium (○———○); 100 mM NaCl, 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM Ca^{2+} (▲———▲); 1 mM Ca^{2+} (●———●); 20 mM Ca^{2+} (*———*).

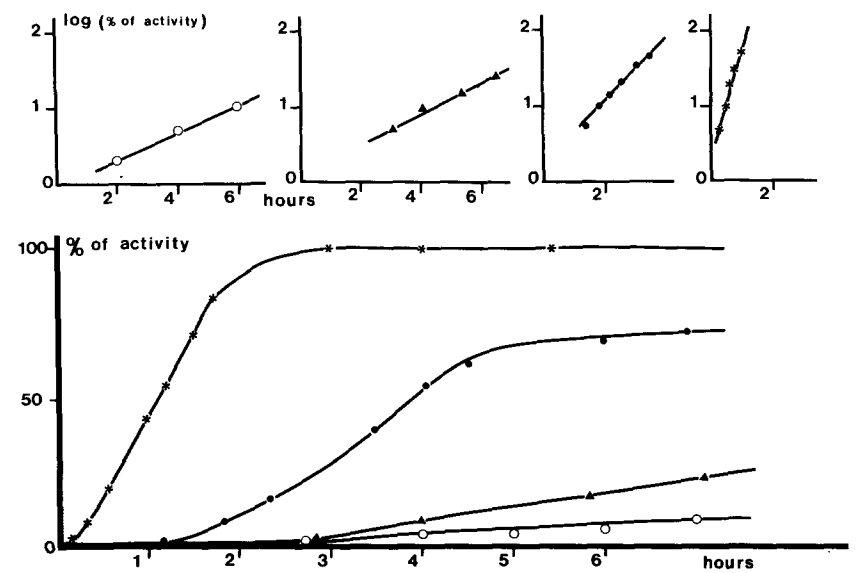

Fig. 4. Kinetics of activation of human trypsinogen 2 by trypsin 2, at pH 8.0 at different calcium concentrations. Concentration of trypsinogen 2: 35 μg/ml; concentration of trypsin 2: 5 μg/ml. The symbols are the same as in Fig. 3.

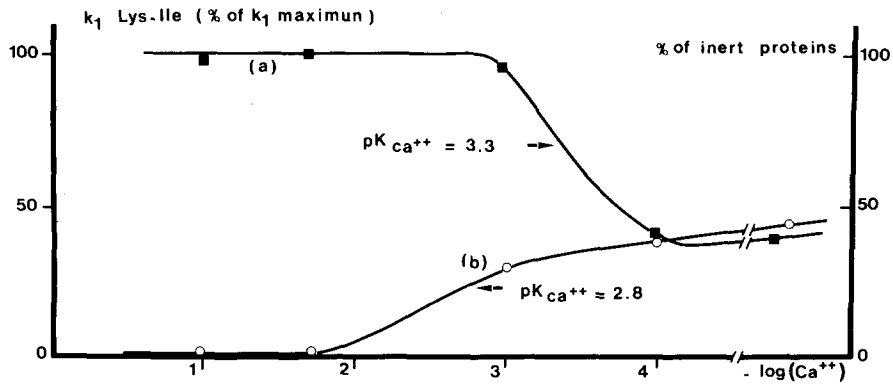

Fig. 5. Influence of Ca^{2+} concentration on the rate of hydrolysis, k_1 of the Lys-Ile bond (a) and on the percentage of inert proteins (b) in human trypsinogen 1.

calcium or in the presence of 0.1 mM calcium the initial rates of activation of each trypsinogen were unchanged. However a striking difference between the two zymogens appeared 2 h after the start of activation since trypsinogen 1 was transformed into active trypsin whereas trypsinogen 2 was not activated. The increase of calcium concentration from 0.1 mM to 1 mM leads to an increase of the initial rate of activation for both zymogens. In the presence of 20 mM calcium, the initial rate of activation of trypsinogen 1 was unchanged compared to 1 mM calcium whereas the initial rate of activation of trypsinogen 2 was twice the initial rate obtained with 1 mM calcium.

In Figs. 5 and 6 are reported the initial rates of trypsinogen activation and the formation of inert protein in relation to log Ca^{2+}. As demonstrated for bovine trypsinogen, the binding of Ca^{2+} to a primary site common to trypsinogen and trypsin prevents the formation of inert proteins. At higher Ca^{2+} concentrations, Ca^{2+} is also bound to a second site located on the four N-terminal aspartyl residues and obviously this binding accelerates the rate of hydrolysis

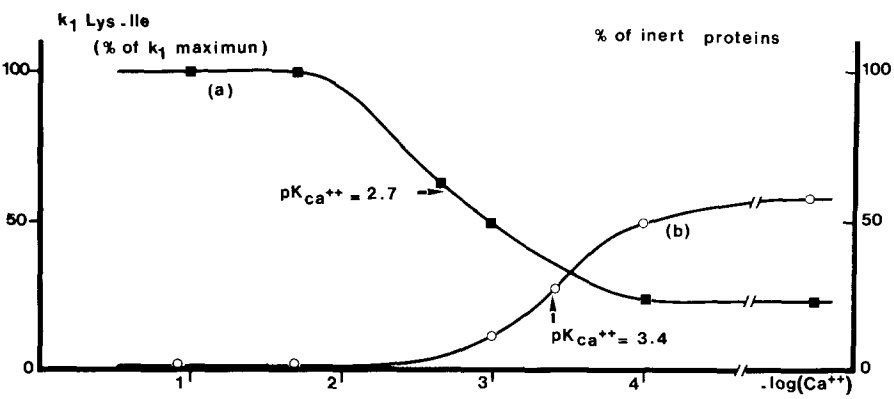

Fig. 6. Influence of Ca^{2+} concentration on the rate of hydrolysis, k_1 of the Lys-Ile bond (a) and on the percentage of inert proteins (b) in human trypsinogen 2.

of the Lys-Ile bond by trypsin. For human trypsinogen 1, the curve of inert protein formation in relation to $-\log[Ca^{2+}]$ gives the $pK(Ca^{2+})$ value of the primary Ca^{2+} binding site equal to 2.8 and the curve of k_1 Lys-Ile in relation to $-\log[Ca^{2+}]$ gives the $pK(Ca^{2+})$ value of the second calcium binding site equal to 3.3. These values are different from the values determined for trypsinogen 2 by the same methods and which are equal to 3.4 for the primary site and 2.7 for the second site.

Discussion

We previously described in human pancreatic secretion the presence of two anionic trypsinogens of which the major form, trypsinogen 1, is twice as abundant as trypsinogen 2 [18]. The presence of two different zymogens in the pancreatic secretions of man, as in other species, remains unexplained. Since we have demonstrated that the two human trypsins have very similar specificities, the study of the activation of their zymogens should be important in the understanding of their physiological role.

Comparison of the rates of activation of trypsinogens by enterokinase in the absence of Ca^{2+} shows that the two human zymogens are equivalent substrates for this enzyme. Moreover, both are activated faster than bovine cationic trypsinogen. The affinity of procine enterokinase for anionic trypsinogen is greater than the affinity for cationic trypsinogen in cattle and pigs [10]. In humans, the more anionic of the two trypsinogens (trypsinogen 2) is activated by enterokinase at the same rate as trypsinogen 1, suggesting that the anionic character of the zymogen is not a discriminatory factor. The faster activation of both human zymogens by porcine enterokinase compared to the bovine cationic zymogen contradicts the results recently reported by Geokas et al. for human cationic trypsinogen (i.e. trypsinogen 1) [23,24]. Since the trypsinogen studied by these authors was shown to be devoid of three of the four aspartyl residues of the N-terminal sequence, this discrepancy probably comes from this fundamental difference in the material studied.

The two human trypsins activate each human trypsinogen at a similar rate. These results confirm our previous data on the similar specificity of these two enzymes [14]. However, a strong difference appears between the two zymogens since trypsinogen 1 is a more specific substrate for either human trypsin than for trypsinogen 2. We have already observed that trypsinogen 1 autoactivates much more rapidly than trypsinogen 2 [19]. In presence of 1 mM calcium at pH 5.6, the autoactivation of trypsinogen 1 becomes predominant compared to enterokinase activation. This result demonstrates that in these conditions, which are close to the physiological in the duodenum [14,25], enterokinase is the starter of trypsinogen activation but the predominant subsequent mechanism becomes the activation of trypsinogens by trypsins.

Both human trypsinogens have two calcium binding sites as described before for bovine cationic zymogen [26], but their $pK(Ca^{2+})$ values are different. The calcium ion binding site common to trypsin and its parent zymogen and which protects the molecule against thermic denaturation and autocatalysis, has $pK(Ca^{2+})$ values of 2.8 and 3.4, respectively, for trypsinogens 1 and 2. The value found for human trypsinogen 2 is close to the value of 3.2 determined for

bovine cationic trypsinogen [26]. This result, associated with the strong resistance of trypsin 1 to autolysis and pH denaturation with regard to trypsin 2 [14], may be explained by a conformational difference between the two human trypsins. The second Ca^{2+} binding site which is present in trypsinogen only and which affects the rate of hydrolysis of the strategic Lys-Ile bond during trypsinogen activation by trypsin has $pK(Ca^{2+})$ values of 3.3 and 2.7 for trypsinogen 1 and trypsinogen 2. These values are both higher than that found for bovine cationic trypsinogen (1.7) and correspond to a calcium concentration necessary to obtain half-saturated sites of, respectively, $8 \cdot 10^{-4}$ M and $4 \cdot 10^{-3}$ M. Consequently, in physiological conditions this calcium site is unsaturated for human trypsinogen 2 (as well as for bovine cationic trypsinogen). This should be a means of protection against autoactivation of trypsinogen in pancreas. By contrast, the same Ca^{2+} binding site is probably saturated for human trypsinogen 1. The singular inhibitory effect of Ca^{2+} on the spontaneous activation of trypsinogen 1 previously observed [19] could be a way of preventing the activation in situ at pH 5.6.

All these results indicate a similar behaviour of human trypsinogen 2 and bovine cationic trypsinogen towards trypsin activation under different conditions and permit us to propose a loose arrangement of the N-terminal region of human trypsinogen 2 as described for the bovine zymogen [8]. However, it is difficult to explain all our results on human trypsinogen 1 in relation to the same N-terminal structure.

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