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EFFECT OF α_2 MACROGLOBULIN ON SOME KINETIC PARAMETERS OF TRYPSIN

H. RINDERKNECHT, R.M. FLEMING and M.C. GEOKAS

Departments of Medicine, Veterans Administration Hospital, Sepulveda, Calif. 91343 and University of California, Los Angeles, School of Medicine, Los Angeles, Calif. 90024 (U.S.A.)

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Summary

1. Complex formation of trypsin with α_2 macroglobulin results in marked changes of the Michaelis—Menten constant, pH optimum and sensitivity to ionic strength in a system using *N*-carbobenzoxy-glycylglycyl—L-arginine-2-naphthylamide as substrate.

2. In contrast to the inhibition (50%) observed when α_2 macroglobulin-bound trypsin is assayed under conditions optimal for the free enzyme, there is minimal reduction of activity when determinations are performed at a substrate concentration and pH optimal for the bound enzyme.

3. The changes in substrate concentration and ionic environment required for maximum activity of α_2 macroglobulin-bound trypsin are similar to those observed with enzymes embedded in polyelectrolyte matrices and may reflect alterations in the microenvironment of the enzyme resulting from conformational changes of the macromolecule during interaction with trypsin.

4. Enzymatic activity of trypsin towards casein is greatly reduced by α_2 macroglobulin, even under assay conditions optimal for the bound enzyme, confirming previous findings that access to the active center for high-molecular weight substrates is sterically hindered by α_2 macroglobulin.

Introduction

Human α_2 macroglobulin (α_2 M) is capable of binding a considerable number of endopeptidases of different origin (pancreas, blood, bacteria, etc. [1—

Abbreviations: α_2M, α_2 macroglobulin; α_2M—trypsin, α_2 macroglobulin-bound trypsin; Bz—DL-Arg-NAn, *N*-benzoyl-DL-arginine *p*-nitroanilide; Z-Gly-Gly-Arg-NNap HCl, *N*-carbobenzoxy-glycyl-glycyl—L-arginine-2-naphthylamide hydrochloride.

7]). Such α_2 M—enzyme complexes characteristically retain most of the original esterase or amidase activity of the enzyme, but show an almost complete lack of activity towards native proteins. Thus, the enzymatic activity of α_2 macroglobulin-bound trypsin (α_2 M—trypsin) towards the low-molecular weight synthetic *N*-benzoyl-DL-arginine *p*-nitroanilide (Bz-DL-Arg-NAn) has been reported to be approximately 80% of that of the free enzyme whereas the activity towards globulin is zero. Denatured proteins and small peptides take an intermediate position on this scale [8]. This change in substrate specificity generally is attributed to steric hindrance of the active site of the enzyme by the macromolecule. There is a possibility, however, at least in respect to low-molecular weight substrates, that this effect may be due to other factors. The work presented here explores this hypothesis.

Materials and Methods

Trypsin. Trypsin (EC 3.4.21.4), twice crystallized, from Worthington Biochemical Corp., Freehold, N.J., was found to be 56% pure by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate according to Chase and Shaw [9]. The purity of other batches varied between 59 and 61%.

Trypsin assay. Trypsin activity was assayed with *N*-carbobenzoxy-glycyl-glycyl—L-arginine-2-naphthylamide hydrochloride (Z-Gly-Gly-Arg-NNap-HCl) from Bachem, Inc., Marina del Rey, Calif. Determinations were carried out, unless stated otherwise, by incubating samples containing trypsin with 0.5 ml of substrate solution (120 mg/100 ml water) and 0.05 M 2-amino-2-methyl-1-propanol buffer, pH 8.2 containing 0.15 mmole $CaCl_2$ /l in a volume of 1.8 ml for 15 min in a waterbath maintained at 25° C. The reaction was terminated by adding 0.2 ml 1 M citrate buffer, pH 4.5 and fluorescence was measured in an Aminco—Keirs spectrophotofluorometer (λ_{Ex}: 335 nm, λ_{Em}: 415 nm, uncorrected). A standard graph was constructed by determination of serial dilutions of trypsin. Other trypsin determinations were performed with Remazolbrilliant Blue-hide (Calbiochem, La Jolla, Calif.) as substrate [11], or casein (Hammersten) as outlined in a previous paper [8].

α_2 Macroglobulin. Plasma α_2 macroglobulin was prepared according to the method of Steinbuch [12] with minor modifications. α_2 Macroglobulin which precipitated unexpectedly in the first step of this procedure was redissolved in 0.9% NaCl and reprecipitated with half the concentration of Rivanol indicated in the original method. In view of the instability of α_2 macroglobulin at an acid pH, absorption with DEAE—cellulose at pH 5.0 was omitted. Instead the material was subjected in a final purification step to gel filtration on a Sephadex G-200 column as described previously [8]. The resulting product was concentrated by ultrafiltration and its purity ascertained by immunoelectrophoresis. A single precipitin line was observed. The concentration of α_2 M was determined by radial immunodiffusion assay. Immunoplates were supplied by Hyland Laboratories, Inc., Costa Mesa, Calif.

Trypsin-binding capacity. This parameter was determined as described in an earlier report [8]. Increasing amounts of trypsin (0.5—5 μg by active-site titration) were added to a solution of α_2 M (50 μg) and assayed with Remazol-brilliant-Blue hide [11]. Free trypsin is indicated by the appearance of a blue

color in the test mixture (sensitivity <5 ng). α_2 M-trypsin, on the other hand, is inactive towards Remazolbrilliant-Blue hide. In addition, trypsin binding capacity was estimated by incubation of α_2 M with excess trypsin for 10 min at 37° C in 0.005 M Tris buffer, pH 7.8 followed by addition of a 50% excess of soybean trypsin inhibitor (Calbiochem, La Jolla, Calif.), calculated on the basis of trypsin added, and continued incubation for 10 min. Trypsin activity was then determined with Z-Gly-Gly-Arg-NNap and the amount of trypsin bound by α_2 M calculated by taking into account the inhibition of trypsin activity (see below) due to complex formation with α_2 M. The amount of trypsin bound by the α_2 M preparation used in this work was 1.06 and 0.83 mole/mole of α_2 M respectively, as determined by the two methods. A large excess of soybean trypsin inhibitor resulted in considerably lower values [13].

Results

Influence of pH on the activity of free and α_2 M-bound trypsin. Fig. 1. illustrates the relationship between enzymatic activity and pH for free trypsin and an equimolar amount of α_2 M-bound trypsin. All assays were performed in equimolar (0.05 M) buffers with Z-Gly-Gly-Arg-NNap substrate as described above. It should be noted that trypsin and α_2 M—trypsin in equimolar quantities exhibited widely differing pH optima when assayed at their corresponding optimum substrate concentrations (below) but gave almost identical activity readings at their respective pH optima.

Kinetic studies. Fig. 2 illustrates the effect of increasing substrate concentrations on the activities of equimolar amounts of trypsin and α_2 M—trypsin at

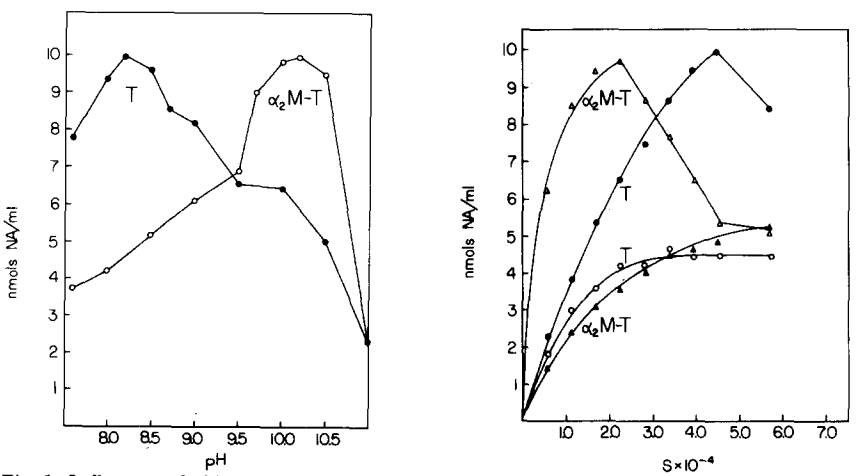

Fig. 1. Influence of pH on the activity of free (T) and α_2 macroglobulin-bound trypsin (α_2M—T). Equimolar amounts of trypsin (15.5 ng/ml by active site titration) and α_2M—trypsin were used in all experiments. Substrate concentrations $4.5 \cdot 10^{-4}$ M for free trypsin, $2.3 \cdot 10^{-4}$ M for bound trypsin. Buffers: 0.05 M 2-amino-2-methyl-1-propanol. NA, β-naphthylamine.

Fig. 2. Relationship between substrate concentration and activity determined with trypsin (T) and α_2 macroglobulin-bound trypsin (α_2M—T) at pH 8.2 and 10.0. Equimolar amounts of trypsin (15.5 ng/ml by active site titration) and α_2M—trypsin were used in all experiments. T at pH 8.2, ●———●; T at pH 10.0, ○———○; α_2M—T at pH 8.2, ▲———▲; α_2M—T at pH 10.0, △———△. NA, β-naphthylamine.

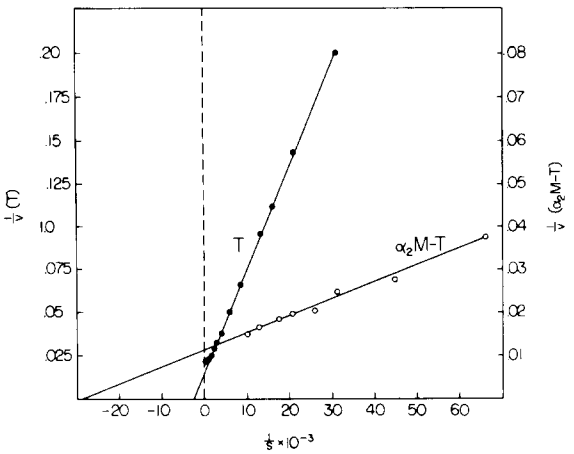

Fig. 3. Lineweaver—Burk plots of the activity of trypsin (T) and α_2 macroglobulin-bound trypsin (α_2 M—T) at their pH optima, pH 8.2 and 10.0 respectively.

pH 8.2 and 10.0. The optimum substrate concentrations for free and α_2 M-bound trypsin at the corresponding pH optima of the enzymes were $4.5 \cdot 10^{-4}$ M for the free enzyme and $2.3 \cdot 10^{-4}$ M for the α_2 M-bound enzyme. Further increases in substrate concentration resulted in inhibition of both, free and bound enzyme, indicating that in this region the Michaelis law is no longer obeyed. When the pH values of these test systems were reversed, the free enzyme appeared to be saturated at $3.3 \cdot 10^{-4}$ M Z-Gly-Gly-Arg-NNap. However, determination of the K_m (below) indicated a deviation from Michaelis conditions at this substrate concentration. Substrate concentrations greater than used in this experiment ($6.0 \cdot 10^{-4}$ M) apparently would be necessary to saturate the bound enzyme.

Michaelis constants. Fig. 3 presents Lineweaver—Burk plots of the action of trypsin and α_2 M—trypsin on Z-Gly-Gly-Arg-NNap at their respective pH optima. The constants derived from these plots (experiments independent of Fig. 2) and those obtained by linear regression analysis of the first 4 points of curve T, pH 10 and the first 9 points of curve α_2 M—T, pH 8.2 (Fig. 2) are given in Table I.

Influence of substrate concentration and pH on inhibition of amidase activity of trypsin by α_2M. For various reasons (insolubility, expense of substrate, etc.) trypsin assays are often carried out at substrate concentrations which do not saturate the enzyme. In the assay described by Buletza et al. [10] the substrate (Z-Gly-Gly-Arg-NNap) concentration is $1.7 \cdot 10^{-4}$ M which, on

TABLE I

	Michaelis constants	
	pH 8.2	pH 10.0
Trypsin	$4.18 \cdot 10^{-4}$ M	$1.62 \cdot 10^{-4}$ M
α_2M-bound trypsin	$2.22 \cdot 10^{-4}$ M	$3.51 \cdot 10^{-5}$ M

inspection of Fig. 2 (curve T, pH 8.2), is clearly suboptimal. This also applies to the assay of α_2 M—trypsin at this substrate concentration and pH 8.2 (Fig. 2, curves T, pH 8.2 and α_2 M—T, pH 8.2) indicated a 50.5% inhibition by α_2 M. In α_2 M-bound trypsin respectively in this system showed a 42.5% inhibition of trypsin activity by α_2 M. However, the same assays performed at optimum substrate concentrations for free trypsin (S = $4.5 \cdot 10^{-4}$ M) at pH 8.2 (Fig. 2, curves T, pH 8.2 and α_2 M—T, pH 8.2) indicated at 50.5% inhibition by α_2 M. In contrast, equimolar amounts of trypsin and α_2 M—trypsin each assayed under optimal substrate and pH conditions furnished almost identical readings. *V* for α_2 M—trypsin at pH 10 was about 90% of that observed with free trypsin at pH 8.

Inhibition of proteolytic activity of trypsin by α_2 M; influence of pH. It has been shown above that under optimum conditions trypsin activity of α_2 M—trypsin complex towards Z-Gly-Gly-Arg-NNap is close to that of free trypsin, but that the activity of α_2 M—trypsin is inhibited by about 50% if the pH and substrate concentrations chosen in the assay are optimal for free trypsin (Fig. 2). It was of interest therefore, to compare the proteolytic activity of α_2 M—trypsin towards casein with that of free trypsin at the pH optima for the free and bound enzymes respectively. Two sets of experiments with casein as substrate were carried out as described in an earlier report [8]. Controls containing no trypsin were run concurrently with each experiment. The results showed that inhibition of trypsin by α_2 M was 93.4% when the assay was carried out at the pH optimum (8.2) of free trypsin and 89.2% when the determination was performed at the pH optimum (10.0) of α_2 M—trypsin.

Effect of ionic strength on activity of free and α_2 M-bound trypsin. Fig. 4 illustrates the effect of ionic strength on the activity of free and α_2 M-bound trypsin assayed under substrate concentration and pH conditions optimal for free trypsin on one hand, and α_2 M—trypsin on the other. The activity of free trypsin was enhanced by about 5% in 50 mM NaCl when measured at optimum pH and substrate concentration. Further increases in salt concentration gradually diminished activity which was reduced by 40% in 1 M NaCl. In contrast, the

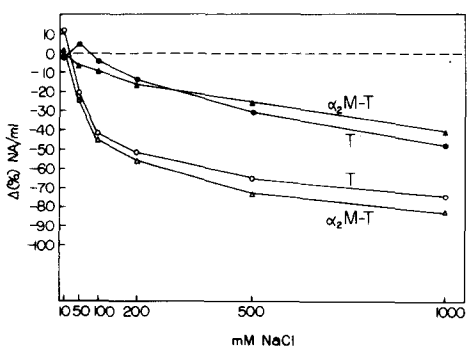

Fig. 4. Influence of ionic strength on the activity of trypsin (T) and $\alpha_2$macroglobulin-bound trypsin (α_2M—T) at pH 8.2 and 10.0. Equimolar amounts of free trypsin (15.5 ng/ml by active site titration) and α_2M—trypsin, and optimum substrate concentrations (see Fig. 2) were used in all experiments. Activity is plotted as % change in naphthylamine generated with increasing concentrations of NaCl relative to that in the absence of NaCl. T at pH 8.2, ●———●; T at pH 10.0, ○———○; α_2M—T at pH 8.2, ▲———▲; α_2M—T at pH 10.0, △———△. NA, β-naphthylamine.

activity of α_2 M—trypsin determined at optimum pH and substrate concentration was decreased precipitously by increasing concentrations of NaCl. It was inhibited by 50% in 0.15 M NaCl and 80% in 1 M NaCl. At a given pH and substrate concentration increases in ionic strength had a similar effect on both, free trypsin and α_2 M—trypsin. Very low salt concentrations (10—50 mM) had an anomalous influence on free trypsin, resulting in a small increase (5—10%) in activity irrespective of pH or substrate concentration.

Discussion

Recent investigations suggest that the physiological role of α_2 M is associated with the binding and specific function [8,14,15] of a number of enzymes and their removal from the circulation [16]. Human α_2 M has been reported to bind two molequivalents of trypsin [3,17]. More recently Saunders et al. [18] isolated five species of α_2 M which differed from one another in their trypsin-binding capacity which ranged from 0.14 to 1.9 and averaged 1.4 moles of trypsin/mole α_2 M. However, calculations were based on weight of 3 × crystallized trypsin (Worthington Biochem. Corp.). Other investigators [19—21] have found approximately equimolar binding ratios for trypsin with α_2 M. The trypsin-binding capacity of our α_2 M preparation averaged 1 mole of trypsin/mole α_2 M based on an active-site titrated trypsin standard [9] (2 × crystallized, Worthington Biochem. Corp.) of 56% purity and agrees with that reported by the latter authors.

There is ample evidence that the inhibitory effect of α_2 M on the activity of trypsin depends on the size of the substrate. In general the extent of inhibition is greater the higher the molecular weight of the substrate. It seems reasonable, therefore, to view the reduced enzymatic activity of the α_2 M—trypsin complex as a reflection of sterically hindered access to the active site. However, the work presented here indicates that other factors may contribute to the changes in enzymatic activity of the complex, at least towards low-molecular weight substrates. Harpel [22] recently has shown that the interaction of trypsin, chymotrypsin and other "serine proteases" with α_2 M results in the hydrolysis of a single peptide bond located within a disulfide-bridged loop of the macromolecule, but the mechanism underlying the binding and inhibition of enzymes has not been elucidated. Barret and Starkey [20] have suggested that such a cleavage may result in a conformational change in the α_2 M molecule that traps the enzyme. This concept is supported by the highly irreversible association between α_2 M and enzyme that has been observed by several authors [18,20]. The kinetic effects of entrapment of an enzyme within a macromolecule are likely to be complex and depend to a large degree on the microenvironment created by the composition and structure of the "trap". Studies on the behavior of enzymes entrapped within gels by covalent attachment to insoluble electrolyte or non-electrolyte polymers have shown that pH-dependence and apparent K_m are affected by the proximity of charged groups or by diffusional effects exhibited by the matrix toward substrate and products [23,24]. We have demonstrated that binding (trapping) of trypsin by α_2 M displaces the pH optimum of enzymatic activity towards more alkaline values (Fig. 1). According to Katchalski [23] a shift in the pH-activity profile of a polyelectrolyte—

enzyme derivative, for instance ethylene maleic acid copolymer—trypsin [24], may reflect changes in the values of the apparent acidic dissociation constants of the active site ionising group (His-57) affected by the polyelectrolyte microenvironment of the enzyme derivative. Although the microenvironment generated by the α_2 M—trypsin trap is unknown an increase in electronegativity of the α_2 M molecule has been observed after complex formation with trypsin [18] and plasmin [25] which — in analogy to the above model — might account for the shift of the pH optimum towards the alkaline region reported in this paper.

Changes in the values of the apparent K'_m of polyelectrolyte—enzyme derivatives have been related to an uneven distribution of substrate between the inside of the matrix and the outer solution and it has been shown that the substrate concentration in the domain of the polyelectrolyte—enzyme conjugate is greater than in the outer solution when the polyelectrolyte—enzyme conjugate and the substrate are of opposite charge [24]. By analogy, trypsin entrapped in α_2 M of increased electronegativity may attain the limiting rate V at a lower concentration of positively charged substrate (Fig. 2) than the native enzyme, and the apparent K'_m for the entrapped enzyme would be lower than that for free trypsin (Fig. 3). Our findings are compatible with this concept. In view of the decreased apparent K'_m for α_2 M-bound trypsin observed in this work diffusional effects by the trapping macromolecule would appear to be minimal for Z-Gly-Gly-Arg-NNap and other low molecular weight substrates. Decreased rates of diffusion of substrate and product in and out of the gel matrix increase rather than decrease the apparent K_m [24]. Although changes in the ionic strength might be expected to affect the bound enzyme more markedly than the free enzyme [26] increases in ionic strength had a similar effect on free and α_2 M-bound trypsin. The pH dependence of this effect is illustrated in Fig. 4.

The results of our work indicate that inhibition of proteolytic activity towards low molecular weight substrates by α_2 M may be attributed perhaps less to steric hindrance than to an altered microenvironment of the captive enzyme as reflected in changes of its apparent K_m and pH-activity profile. The kinetics of α_2 M-bound trypsin are similar to those observed when proteases are attached to a polyelectrolyte matrix and are compatible with the concept of entrapment of trypsin by the α_2 M molecule. The behavior of α_2 M-bound trypsin towards high molecular weight substrates as demonstrated in our experiments with casein is explained adequately on the basis of steric hindrance of access to the active site of the entrapped enzyme.

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