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The covalent and non-covalent binding modes of elastase with α_2-macroglobulin influence the conformation of the protease

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1. INTRODUCTION

Human α_2-macroglobulin (α_2M) is a plasma glycoprotein composed of two non-covalently bound subunits, each of which is formed of two identical 180 kDa peptide chains linked together by disulfide bridges. This protein develops enzymatically active complexes with nearly all endoproteases, mainly with a 2:1 protease-α_2M stoichiometry. The following events accompany the formation of the 2:1 protease-α_2M complexes: (i) each of the four 180 kDa polypeptide chains are cleaved in the so-called bait region located near the middle of the peptide chain; (ii) four thiol groups are generated per α_2M molecule by disruption of the internal Glu-Cys thioester bonds of each peptide chain; (iii) the shape of the α_2M molecule undergoes a large conformational change (reviews [1,2]).

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Abbreviations: α_2M, α_2-macroglobulin; Pipes, piperazine-*N,N'*-bis(ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis

The newly generated Glu-X residues may form covalent bonds with free amino-Lys groups of the protease molecule [3]. This covalent binding mode can be inhibited by competing low-molecular-mass nucleophiles as hydroxylamine, the enzyme being always trapped by α_2M [4,5]. In the present paper, using measurements of energy transfer within the elastase molecule from a tryptophanyl residue to Tb^{3+} located at its calcium binding site [6,7], we investigate the conformational changes of this enzyme upon its α_2M interaction. The covalent and non-covalent binding modes of elastase with α_2M are first characterized by gel electrophoresis analysis of the complexes.

2. EXPERIMENTAL

2.1. *Materials*

Human α_2M was prepared by Zn^{2+} affinity chromatography as in [8] and filtered on Ultrogel ACA 22 as a final step of purification. Pancreatic elastase (from Biosys, France) was purified as follows: 1 ml of an enzyme solution (10–20 mg) in 10 mM Tris-HCl buffer, pH 8.0, was passed through a 1 × 5 cm DEAE Trysacryl column equilibrated with the same buffer. The enzyme-containing fractions were then filtered on a G 25

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Sephadex column equilibrated with 50 mM NaCl. The enzyme was stored in liquid nitrogen.

α_2M and elastase were labelled with ^{125}I- and [^{3}H]dansyl chloride as in [9], respectively.

2.2. *Methods*

The enzymatic activity of elastase was evaluated by the amidolytic activity of the enzyme, monitoring the hydrolysis rate of the chromogenic substrate Suc-Ala$_3$-pNA (from Serva) in 50 mM Hepes, 100 mM NaCl, pH 7.8, at 22°C.

The estimation of covalently bound elastase to α_2M was as follows: the [^{3}H]elastase-α_2M complexes were filtered under denaturing conditions [10] and the radioactive peak area corresponding to labelled elastase, comigrating with high-molecular-mass fragments of α_2M, estimated.

Electrophoresis was performed in 5% polyacrylamide gel (8 × 8 cm) containing 0.1% SDS, at pH 8.8. The samples were prepared by adding to 60 μl of 3 μM α_2M, 0–25 μl of 5×10^{-5} M elastase. After 10 min of incubation at room temperature, 5 μl of 0.5 M diisopropylfluorophosphate in dioxane were added, followed 15 min later by 6 μl of 10% SDS and 5 μl of mercaptoethanol. The mixture was then heated for 1 h at 45°C and submitted to electrophoresis. The gels were stained and cut in 2 mm-thick slices. The radioactivity was directly determined in a gamma counter (^{125}I) or the slices digested overnight at 60°C in the presence of a mixture of 0.8 ml of 30% H_2O_2 and 40 μl concentrated NH_4OH before adding scintillation liquid for ^{3}H estimation.

Fluorescence measurements were performed with a Kontron spectrofluorometer. The excitation and emission wavelengths were 285 and 540 nm, respectively. All the experiments were in 5 mM Pipes, 50 mM NaCl, pH 6.5, at 22°C. Luminescence was measured after successive additions of aliquots of $TbCl_3$.

Electron micrographs were performed as in [11].

3. RESULTS

3.1. *Interaction of elastase with α_2M*

The results of gel electrophoresis of α_2M-elastase complexes under denaturing and reducing conditions are shown in fig.1. The most conspicuous feature of the protein pattern is the conversion of the 180 kDa polypeptide chain of α_2M to 90 kDa fragments as a characteristic of the reaction of α_2M with proteases. Comparison of the stained protein material with [^{3}H]elastase incorporation indicates that the enzyme is not bound to these fragments but appears in two minor bands (a and b) whose apparent molecular masses are 215 and 115 kDa, respectively. We thus propose that these bands represent the covalent binding of the enzyme with two and one 90 kDa fragment, respectively. This attribution is strongly supported by the fact that the presence of hydroxylamine at a 20 mM concentration during the α_2M-elastase interaction completely inhibits the formation of these conjugates.

The extent of the bait region cleavage as a function of the elastase-α_2M stoichiometry [12] is not strongly different from that determined for trypsin [13,14] and chymotrypsin [10] but is nevertheless significantly greater. In fact, 2.6 α_2M peptide chains are cleaved upon elastase binding for a 1:1 elastase-α_2M molar ratio, while cleavage of 2.0 bait regions was expected. These data suggest that elastase does not covalently interact with α_2M with a 100% yield, i.e. a non-covalent binding mode of elastase also induces a proteolytic cleavage in both α_2M subunits as previously shown for the chymotrypsin-α_2M interaction [10]. We then determine, using filtration under denaturing conditions, the yield of elastase-α_2M covalent association. In the concentration range 0–1 elastase per α_2M the protease covalently binds with 70 ± 5% yield, whereas in the concentration range 1–2 elastases per α_2M covalent binding does not take place at all.

On the other hand, we find a quite linear relationship between the α_2M bait region cleavage and non-covalently bound elastase (the reaction is made in the presence of hydroxylamine) in the concentration range 0–1 elastase per α_2M; a single molecule of elastase is then able to cleave the four α_2M peptide chains as already observed for α_2M-chymotrypsin reaction [10].

Thus, several elastase-α_2M complexes, depending on the stoichiometry and on the binding mode of the protease, are prepared and tested for elastase-Tb^{3+} interaction.

3.2. *Measurements of the association constants of Tb^{3+} with α_2M-bound elastase*

The luminescence intensity (L) of mixtures of

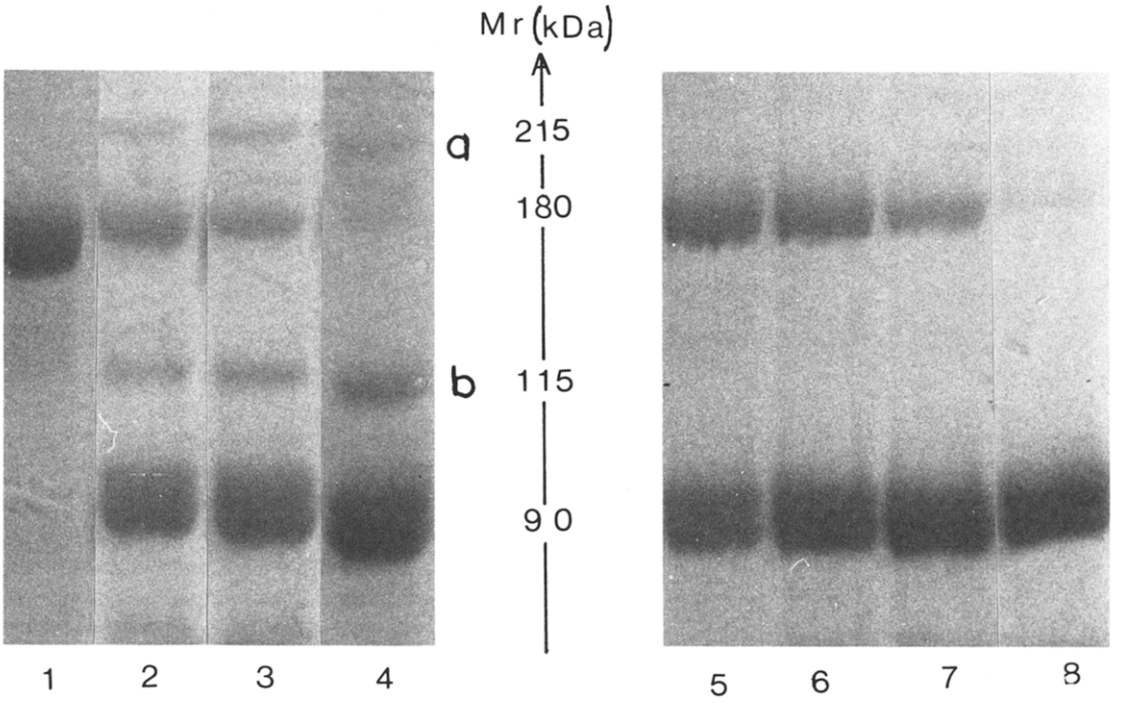

Fig.1. PAGE of α_2M-elastase complexes under denaturing and reducing conditions. The electrophoretic procedure and the preparation of the samples are described in the text. 1, unreacted α_2M; 2–4, elastase-α_2M complexes with molar ratios of 1.0, 1.5, 2.0, respectively; 5–8, elastase-α_2M complexes prepared in the presence of 20 mM hydroxylamine with molar ratios of 0.5, 0.6, 0.8, 1.0, respectively.

constant amounts of elastase (2×10^{-6} M) and increasing amounts of Tb^{3+} (from 8×10^{-6} to 8×10^{-5} M) are measured to determine the association constants (K_a) of Tb^{3+}-elastase complexes. The K_a values being sufficiently low, we used double reciprocal plots according to

$$\frac{1}{L} = \frac{1}{L_m} + \frac{1}{L_m K_a(Tb^{3+}\ \text{total})}$$

where L_m is the maximum intensity extrapolated for about 3×10^{-4} M Tb^{3+} [7].

Furthermore, the observed luminescence intensities were corrected so as to take into account (i) the screening effect due to the absorption of α_2M according to [15] leading to a correction factor of 1.75 and 2.7 for the 1:1 and 1:2 α_2M-elastase complexes, respectively, (ii) the α_2M-Tb^{3+} luminescence which is subtracted from the total luminescence of the α_2M-elastase-Tb^{3+} complexes, (iii) the hydroxylamine-Tb^{3+} interaction: a correcting factor is calculated from the observed luminescences of elastase-Tb^{3+} and elastase-hydroxylamine-Tb^{3+} mixtures. Furthermore, in order to check the assumption that elastase, purified as described, has only one Tb^{3+} binding site, we estimated the Ca^{2+} binding curve by competition with Tb^{3+} [7] and found a titration profile compatible with that already observed [16].

Routinely an elastase-Tb^{3+} titration was performed and served as a reference. The titration profile of the 2:1 elastase-α_2M complexes presents no abnormality with respect to the 1:1 conjugate, i.e. an inflection point reflecting a different Tb^{3+} affinity of each elastase molecule is not observed as might be expected. We thus assume for K_a calculation that the two bound elastase molecules behave similarly for Tb^{3+} fixation. Results are summarized in table 1. It is noteworthy that, (i) whatever the elastase-α_2M stoichiometry, the non-covalent binding mode of the protease increases its affinity for Tb^{3+} and enhances the luminescence

Table 1

Effect of the stoichiometry and the binding mode of elastase with α_2M on the association constant K_a[a], the maximum luminescence intensity L_m[b] of the elastase-Tb^{3+} complex and the K_m, k_{cat} values of the enzyme towards Suc-Ala_3-pNA

	Covalence (%)	Bait region cleavage	$K_a \times 10^{-4}$ (M^{-1})	L_m	K_m (mM)	k_{cat} (s^{-1})	K_m/k_{cat} ($M^{-1} \cdot s^{-1}$)
Elastase	–	–	3 ± 0.5	1	0.8 ± 0.03	9.4 ± 2	11.8
Elastase-α_2M (1:1)	70	2.6	1.3 ± 0.2	1.1	1.0 ± 0.03	4.7 ± 1	4.7
Elastase-α_2M (1:1)	0	4	8 ± 1	1.4	4.0 ± 0.1	9.4 ± 2	2.3
Elastase-α_2M (2:1)	35	4	4 ± 0.5	2.2	1.0 ± 0.03	5.1 ± 1	5.1
Elastase-α_2M (2:1)	0	4	11 ± 2	2.5	1.0 ± 0.03	4.3 ± 1	4.3

[a] K_a and L_m values refer to several determinations
[b] Maximum luminescence values refer to that of elastase which was arbitrarily taken equal to 1

intensity emission of the tryptophan $\longrightarrow$ Tb^{3+} energy transfer, (ii) maximum L_m values are also modified by the stoichiometry of the complexes, indicating that both the binding mode and the molar ratio of elastase to α_2M influence the conformation of the enzyme within the complexes. As also determined, these two factors modify the K_m and the k_{cat} values of the enzyme towards Suc-Ala_3-pNA.

4. DISCUSSION

The interaction of proteases with α_2M is well known to decrease dramatically the affinity of the bound enzyme towards macromolecular substrates and inhibitors as a result of trapping of the enzyme [4]. But the results presented in this paper indicate that a reduced accessibility of the active center of elastase upon its α_2M binding [17] is not the only factor that may control the decrease in the catalytic activity of the enzyme towards low-molecular-mass substrates. Indeed, a conformational change of elastase as a result of its different binding modes with α_2M is clearly evidenced by the variation in transfer efficiencies within the elastase molecule. These variations are probably of small amplitude since they are very susceptible to the donor (tryptophan) $\longrightarrow$ acceptor (Tb^+) distance and are not detectable on electron micrographs of the different complexes; they could however be of importance for the catalytic behavior of the enzyme.

Considering the 1:1 elastase-α_2M complex, the role of covalent bonds involving structural constraints different from those involved in a non-covalent binding mode may be of importance to explain different L_m values. This however is not exclusive since at the same time the amount of α_2M bait region cleavage is modified. It is likely that these factors, elastase binding mode and extent of α_2M proteolysis, are both controlling the L_m variations. The stoichiometry of the elastase-α_2M interaction, due to the proximity of the two α_2M binding sites of the protease [18], may also affect L_m variations, as a result of elastase-elastase interactions.

In any case, L_m variations clearly demonstrate that conformational changes of the elastase molecule are associated with its α_2M binding mode and probably influence its catalytic activity towards low-molecular-mass substrates.

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