Interaction of α_2 -macroglobulin-bound thrombin with hirudin

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The human thrombin bound to α_2 -macroglobulin (α_2 M) in a 1:1 stoichiometry is still able to interact with one of its specific inhibitors, hirudin. The dissociation constant of the complex hirudin- α_2 M-bound thrombin is 1×10^{-7} M, whatever the mode of thrombin binding, covalent or non-covalent.

a2-Macroglobulin Thrombin Hirudin

1. INTRODUCTION

Human α_2 -macroglobulin (α_2 M) is a plasma glycoprotein of M_r 725000 composed of two subunits, each containing two identical chains linked by disulfide bridges. α_2M forms enzymatically active complexes with nearly all endoproteases. The protease binding initiates a drastic conformational change of the α_2M molecule (reviews [1,2]). It has been proposed that the proteases are entrapped within the α_2 M molecule as they lose the ability to react with large substrates or inhibitors [3], although the active site is not involved by complex formation. However, several investigations [4,5] have shown that the inaccessibility of the active center of the bound enzymes by large molecules is a relative rather than an absolute restrictive phenomenon. Several observations thus argue against a molecular trap hypothesis as a proven fact [6].

However, we failed to observe a reaction of α_2 M-bound thrombin with antithrombin III [7]. Here, we investigate such an interaction using hirudin, an inhibitor of thrombin, with a lower molecular mass and an affinity constant for the free enzyme [8] greater than that of AT III [9].

2. EXPERIMENTAL

2.1. Materials

Human $\alpha_2 M$ was prepared by Zn^{2+} affinity chromatography as in [10]. Thrombin was obtained as in [11]; the enzyme had a specific activity of 3200 units/mg. Hirudin was from Serva and had a specific activity of 1500 units/mg.

2.2. Methods

The enzymatic activity of thrombin was evaluated by the amidolytic activity of the enzyme, monitoring on a Cary 118 C at 25°C the hydrolysis rate of the chromogenic substrate H.D.Pip.Arg. pNA (Kabi Kiagnostica, Sweden).

The activity of the hirudin preparation was determined according to [8]. Thrombin was labelled by fluorescein isothiocyanate as in [7].

The α_2 M-thrombin complexes were prepared by adding the protease at a 1×10^{-5} M α_2 M preparation in 0.1 M NaCl, 0.05 M phosphate (pH 7.5). The reaction was sometimes performed in the presence of 0.1 M hydroxylamine [11]. The complexes were isolated by Sephacryl S 200 filtration in the same buffer.

3. RESULTS

The specific and stoichiometric reaction of hirudin (M_r 10800) with human thrombin achieves inactivation of the clotting activity of the protease as well as of its amidolytic activity on synthetic substrates [8]. The association rate is so fast that, even in the nanomolar range, the rate association constant cannot be accurately determined. On the other hand, a 100-fold molar excess of $\alpha_2 M$ is unable to dissociate the complex even after a 48 h incubation at 25°C. Indeed, no amidolytic activity corresponding eventually to an formed α_2 M-thrombin complex can be detected.

As shown in fig.1, the rate of inhibition of the enzymatic activity of the α_2 M-bound thrombin is very low compared to that of the free enzyme, equilibrium only being reached after several hours of incubation, even in the presence of a large excess of inhibitor. The inset of the figure is a plot of the thrombin activity as a function of hirudin concentration after equilibrium has been reached. From these data, a dissociation constant is calculated, $K_i = 1 \times 10^{-7}$ M. The value of this constant is not significantly different whatever the

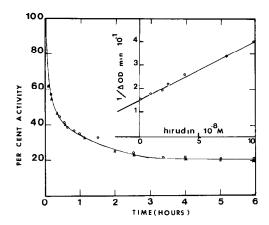


Fig. 1. Time dependency of the inhibition of α_2 M-bound thrombin by hirudin at pH 7.5 and 25°C. Hirudin at a final concentration of 5×10^{-7} M is incubated with the α_2 M-thrombin complex at 1×10^{-8} M. The activity of thrombin is tested at the indicated times. (0---0) α_2 M covalently bound thrombin, (Δ --- Δ) α_2 M noncovalently bound thrombin. The inset is a Dixon plot of the residual activity of the α_2 M-thrombin complex for different concentrations of hirudin after equilibrium has been reached (5 h at 25°C).

binding mode of thrombin to α_2M , covalent or non-covalent.

To exclude the possibility of the formation of an inactive thrombin-hirudin complex after the noncovalently bound thrombin has been displaced from its $\alpha_2 M$ complex by hirudin, thrombin was first labelled by fluorescein isothiocyanate. Then the protease is reacted with $\alpha_2 M$ and the complex incubated with a 100-fold molar excess of hirudin for 5 h and the reaction mixture chromatographed on Sephacryl S 200 (fig.2). As expected, labelled thrombin is eluted associated with the α_2M bulk using the covalent α_2 M-thrombin complex. whereas only a few percent of the thrombin molecules are eluted as an inactive thrombinhirudin conjugate, using the non-covalent α_2 M-protease complex obtained in the presence of hydroxylamine. Then, the observed dissociation constant is really that of the ternary complex α_2 Mbound thrombin-hirudin.

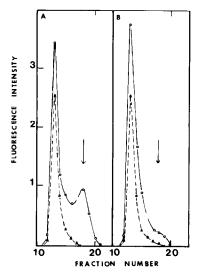


Fig.2. Elution profiles of α_2 M-bound thrombin—hirudin complexes. The α_2 M—thrombin complexes are incubated at 25°C for 10 h with a 100-fold molar excess of hirudin and chromatographed on Sephacryl S-200. The arrows correspond to the expected elution peak of the hirudin—thrombin complex. (A) and (B) are the non-covalent and covalent α_2 M—thrombin complexes, respectively. The elution profiles are performed using the fluorescence of fluorescein-labelled thrombin (\bigcirc — \bigcirc); the residual activity of the bound protease is also tested (\triangle --- \triangle).

4. DISCUSSION

The interaction of hirudin with the α_2M thrombin complex is very much slower than with the free enzyme. This difference in reaction rate probably did not allow authors in [12] to observe this interaction. Furthermore, a 5-fold molar excess of hirudin is necessary to detect a significant inhibition. The factors causing this drastic reduction rate may reflect the limit of accessibility of α_2 M-bound proteases. Indeed, AT III, with a molecular mass nearly 6-fold greater, does not react with the conjugated thrombin even in the presence of heparin [11]. But other factors may be involved, for instance, the value of the affinity constant of the inhibitor for the protease: that of hirudin being considerably greater than that of AT III for free thrombin [8,9]. Furthermore, a conformational modification of the surrounding of the active center of the protease upon $\alpha_2 M$ binding cannot be excluded.

It may also be pointed out that, whatever the binding mode of thrombin to $\alpha_2 M$, covalent or not, the K_i for the hirudin—thrombin interaction is identical: the accessibility of the bound protease and/or its affinity constant for hirudin are not modified, supporting the idea that the absence of covalent bonds does not modify the flexibility of the $\alpha_2 M$ -thrombin complex and, in any case, pro-

vides additional support for identical $\alpha_2 M$ protease binding sites [7].

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