Human thrombin variable region 1, including E39, is involved in interactions with α1-antitrypsin M358R and protein C

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Abstract **We used an antithrombin autoantibody (IgG D), the epitope of which encompasses ABE1 and amino acids located within variable region 1, to study thrombin interactions with R358 α1-AT and protein C. IgG D inhibited the thrombin interaction with R358 α1-AT, while hirugen had no effect, indicating that the interaction of R358 α1-AT with thrombin may involve the VR1 subsite. We also obtained evidence that VR1 may be involved in the activation of protein C by thrombin in the absence of thrombomodulin. Moreover, IgG D attenuated the inhibitory effect of calcium ions during protein C activation by thrombin, probably by masking E 39 within the VR1 site.**

Key words: Thrombin; Variable region; Autoantibody; Protein C; R358 α1-antitrypsin

1. Introduction

An antihuman thrombin autoantibody was discovered in a patient with recurrent unexplained thrombosis [1]. The purified antibody (IgG D) was found to inhibit thrombin binding to thrombomodulin, fibrinogen recognition, inhibition of thrombin by heparin cofactor II, and platelet and endothelial cell thrombin receptor activation [1,2]. These properties suggested that IgG D bound to an exosite known as anion binding exosite 1 (ABE1). Experiments with recombinant thrombin identified two critical domains for thrombin recognition by IgG D. One involves H71, R73 and R75 chymotrypsin numbering system), which are part of ABE1. A second set of amino acids, P37, Q38 and E39, are also part of the IgG D epitope [3]. This domain belongs to thrombin variable region 1 (VR1), the entire sequence of which (F34 to L40) is involved in substrate specificity and inhibitor (serpin) recognition. E39 (together with R35) forms part of a hydrophilic dipole known as the S3′ subsite, which is a putative specificity site C-terminal to the cleavage point (for a review see [4,5]).

E39 also has a negative influence on the cleavage of protein C by thrombin in the absence of thrombomodulin [6]. Recent data [7] show that E39 and E192 are involved in ionic interactions between thrombin and protein C. In three-dimensional models, E192, together with some other negatively charged amino acids, is located close to the thrombin active site, Hydrophilic residues such as E39 and E192 may be responsible for specific electrostatic interactions, that depend on the calcium ion concentration [8]. Moreover, E39 might be involved in repulsive charges between thrombin and the P3′ Asp residue of protein C, which could be responsible for inhibiting protein C activation by thrombin alone. This effect is reinforced by calcium ions and attenuated by the binding of thrombin to thrombomodulin [9–13].

We took advantage of the specificity of IgG D to determine the role of VR1 in the interaction of thrombin with a mutated α1-antitrypsin (M358 R) and with protein C.

2. Materials and methods

2.1. Reagents and proteins

The thrombin-selective synthetic substrates S 78, S 101, S 109, S 147, S 149, S 150 and S 157 were kindly provided by F. Nicham (Serbio Laboratories, Gennevilliers, France). The other synthetic substrates (S 2366 for activated protein C and S 2238 for thrombin) were from Chromogenix (Mölndal, Sweden). Chelating resin imminodiacetic acid was from Sigma (St. Louis, MO). The C-terminal hirudin (hirugen, NDGDFEEIPEEYLQ) was synthesized and purified by Dr. F. Troalen (Institut Gustave-Roussy, Villejuif, France). It was tested for its anticoagulant properties and found to increase the plasma thrombin time at concentrations above 1 μM.

Human thrombin and protein C were from Diagnostica Stago (Asnières, France). Antithrombin was purified as described elsewhere [14]. Human R358 α1-AT was isolated from the plasma of a 15-year-old boy [15].

Ig G from the patient (IgG D) and from normal control subjects were obtained through a three-step procedure involving ammonium sulfate precipitation, anion-exchange chromatography (Q-Sepharose, Pharmacia, Uppsala, Sweden) and protein G-Sepharose affinity as described in [2].

2.2. Effects of IgG D on the interaction between thrombin and R358 α1-antitrypsin (R358 α1-AT)

We tested the effects of IgG D on the pseudo first-order rate constant for thrombin inhibition by R358 α1-AT and by antithrombin. Thrombin (18 nM final) was prewarmed at 37°C and added with control IgGs and IgG D (0 to 1 μM final) in Tris/0.1 M NaCl buffer (pH 7.4) containing 0.5% (v/w) polyethylene glycol 6000. R358 α1-AT was immediately added (200 to 400 nM) for exactly 1 min at 37°C. Residual thrombin protease activity was quantified from the initial velocity (A) of 2 mM S 2238 substrate hydrolysis, monitored at 405 nm in an Eppendorf spectrophotometer (Roucaire, Vélizy, France). The observed pseudo first-order constant was calculated using the formula $k = \ln A_0 - \ln A/t$, where A_0 was measured in the presence of control IgGs.

The same experiment was performed using hirugen (0 to 20 μM) instead of IgG D as a competitor for the binding of R358 α1-AT to thrombin.

The interaction between human antithrombin (250 nM) and thrombin (25 nM) was tested as described elsewhere [1] in the presence of control IgGs and IgG D (0 to 1 μM). Residual thrombin activity was quantified as described for R358 α1-AT.

2.3. Effects of IgG D on protein C activation by thrombin

Protein C (0.56 μM) was added with 1 μM control IgGs or IgG D in 20 mM Tris/0.1 M NaCl buffer, pH 7.4, containing 0.1% bovine serum albumin (w/v) and 0 to 700 μM $CaCl_2$ at room temperature. The buffer was passed through a chelating resin column prior to the addition of calcium, to remove any contaminating divalent ions. Human thrombin (25 nM) was then added at 37°C. Protein C activation rates

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were determined by placing aliquots in a 96-well microplate after 2, 4 and 8 min of incubation, according to Zhang and Castellino [16]. The reaction was stopped by adding 4 μM antithrombin and 0.14 IU/ml heparin to each well. The amount of activated protein C was determined at 405 nm with 0.8 mM S 2366 substrate on a MR5000 microplate reader (Dynatech Laboratories, Saint Cloud, France). The graph of each protein C activation time (abcissa) versus the initial velocity of substrate hydrolysis is a straight line, the slope of which gives the activation rate of protein C (V_i, $\Delta A \cdot min^{-2}$).

2.4. Effect of IgG D on synthetic substrate hydrolysis by thrombin

Thrombin (10 to 60 nM final) was incubated for 10 min at room temperature with control IgGs or IgG D (1 μM final), then each substrate was added (0.3 mM) and the initial velocity of substrate hydrolysis by thrombin was measured at 405 nm.

3. Results

3.1. Effects of IgG D on the interaction of R358 α1-AT with human thrombin

IgG D competed with R358 α1-AT for binding to thrombin in experiments with three R358 α1-AT concentrations and increasing amounts of IgG D and this effect was not observed with control IgGs. IgG D produced a concentration-dependent inhibition of thrombin/R358 α1-AT complex formation. The pseudo first-order rate constant was calculated and the inhibition constant (K_i = 190 nM) was determined graphically (see Fig. 1).

Hirugen (0 to 20 μM) failed to displace R358 α1-AT from its binding site on thrombin, as shown in Fig. 2 fir three concentrations of R358 α1-AT.

3.2. Interaction of IgG D with antithrombin for binding to thrombin

As previously observed [1], thrombin binding to antithrombin was unaffected by IgG D. The observed pseudo first-order rate constants were 0.180, 0.235 and 0.240 min^{-1}, respectively, in the absence of IgGs, in the presence of control IgGs and in the presence of IgG D (560 nM) (not shown).

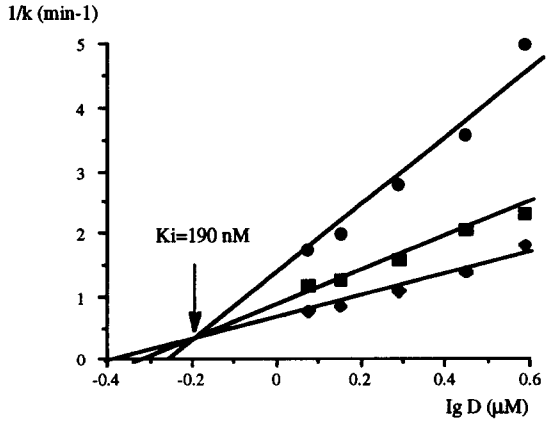

Fig. 1. Effect of IgG D on thrombin inhibition by R358 α1-antitrypsin. Thrombin (18 nM) was incubated with IgG D (0 to 0.6 μM) and R358 α1-AT was added for exactly one min at 37°C. Residual thrombin activity was quantified at 405 nm by the initial velocity of S 2238 substrate hydrolysis. The observed pseudo first-order constant was given by the formula $k = \ln A_0 - \ln A/t$, where A_0 was measured in the presence of control IgGs. The inhibition constant K_i was graphically determined according to the method of Dixon. The concentrations of R358 α1-AT were 200 nM (●), 300 nM (■), and 400 nM (◆).

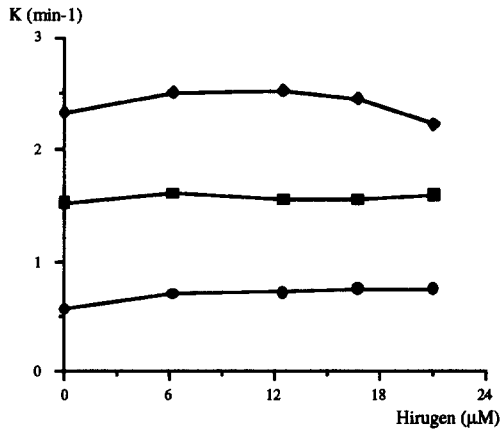

Fig. 2. Effect of hirugen on thrombin inhibition by R358 α1-antitrypsin. Thrombin (10 nM) was incubated with various concentrations of R358 α1-antitrypsin and 0 to 20 μM hirugen. Residual thrombin activity was quantified as described in Fig. 1. The concentrations of R358 α1-AT were 80 nM (●), 160 nM (■), and 240 nM (◆).

In the presence of increasing heparin concentrations (10^{-3} to 10^{-9} M), a bell-shaped curve was obtained by plotting the pseudo first-order rate constant against the heparin concentration. Control IgGs and IgG D had no influence (not shown).

3.3. Protein C activation

As shown in Fig. 3, the activation rate of protein C by thrombin was significantly reduced in the presence of 1 μM IgG D (V_i, 2.69 ± 0.50 $\Delta A \cdot min^{-2}$ versus 4.02 ± 0.28 $\Delta A \cdot min^{-2}$ in the presence of control IgGs).

As expected, calcium (0 to 700 μM) induced a concentration-dependent inhibition of protein C activation by thrombin (Fig. 4). The inhibition constant (K_i) for calcium ions was evaluated from the usual plots of the initial rate of protein C activation ($1/V_i$, arbitrary units) against the concentration of calcium chloride. As shown in Fig. 4 (inset), IgG D decreased the K_i (119 ± 23 μM in the presence of control IgGs and 48 ± 4 μM

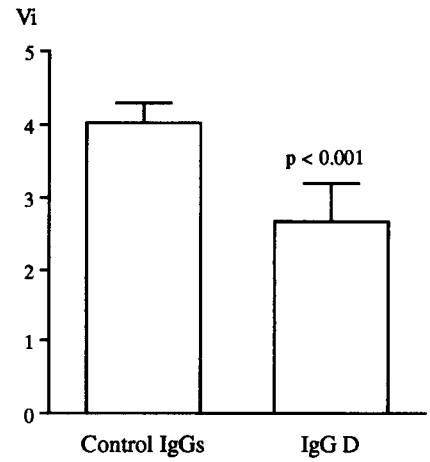

Fig. 3. Effect of IgG D on protein C activation by thrombin. Protein C (0.56 μM) was incubated with 25 nM human thrombin at 37°C. The reaction was stopped by adding 4 μM antithrombin and 0.14 IU/ml heparin. The histograms represent the V_i of the reaction, determined as described in Methods, in the presence of 1 μM control IgGs (8 determinations) or 1 μM IgG D (7 determinations).

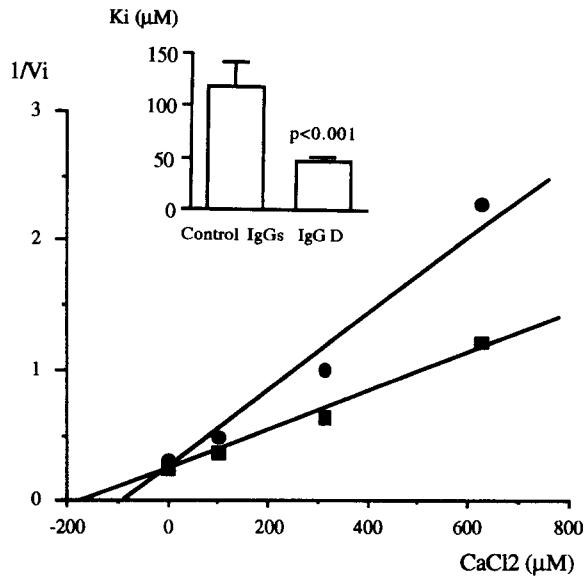

Fig. 4. Thrombin activation of protein C in the presence of IgG D and calcium ions. Protein C (0.56 μM) was incubated with 1 μM control IgGs (●) or IgG D (■) and 0 to 700 μM calcium chloride. Then 25 nM human thrombin was added and the activation rate of protein C (Vi) was determined after 2, 4 and 8 min as described in section 2. The K_i of calcium ions for the reaction was determined as the negative intercept of the abcissa. The inset shows the results of four determinations (mean K_i ± S.D.).

in the presence of IgG D, $P < 0.001$), thus attenuating the inhibitory effect of calcium ions on protein C activation by thrombin alone.

3.4. Synthetic substrates

Thrombin (10 and 60 nM final) was incubated for 10 min at 22°C with control IgGs or IgG D and quantified at 405 nm by the initial velocity of hydrolysis of seven synthetic substrates. As shown in Fig. 5, by comparison to control IgGs (mean of four determinations), IgG D did not inhibit substrate hydrolysis but induced a slight increase in the V_i of the reaction. These results are consistent with an alteration of the thrombin conformation induced by IgG D binding.

4. Discussion

An auto-antibody (IgG D) directed against human thrombin was isolated from a patient with recurrent arterial thrombosis. The IgG D recognized amino acids H71, R73 and R75 (located within ABE1) and inhibited all thrombin functions requiring the recognition of macromolecular substrates through ABE1 [2]. We now report that IgG D inhibits the thrombin interaction with a mutated serpin (R358 α1-AT) and also protein C activation in the absence of thrombomodulin. Since ABE1 is not involved in these reactions [4], the inhibitory effect of IgG D may be explained by an allosteric change subsequent to its binding to ABE1. The increased velocity of small-substrate hydrolysis indicates a thrombin more efficient catalytic site in the presence of IgG D and thus supports this hypothesis. Indeed, a similar effect was observed by Liu [17], who used a peptide from the thrombin receptor (residues 52–69) which bound to the ABE of thrombin and induced a conformational change in the active site. An alternative explanation is that thrombin interaction with R358 α1-AT and with protein C in the absence of thrombomodulin involves another site blocked by IgG D, the epitope of which comprises P37, Q38 and E39 [3]. Indeed, the domain emcompassing this sequence (VR1) was recently shown to be crucial for the interaction of the serpin PAI-1 with its target protease t-PA. The replacement of the thrombin VR1 subsite by the corresponding sequence of t-PA transforms PAI-1 into a potent thrombin inhibitor [18].

The role of VR1 as a subsite for the interaction of thrombin with R358 α1-AT could explain the strong inhibitory potency of the mutated α1-AT. The transformation of α1-AT into a thrombin inhibitor on substitution of M by R is explained by the importance of the P1 residue in recognition by the target protease, which has now been observed with other serpins [19]. However, this does not account for why R358 α1-AT inhibits thrombin more rapidly than does the natural inhibitor antithrombin. A role of ABE1 can be ruled out, as hirugen, a peptide binding specifically to this domain, did not influence thrombin inhibition by R358 α1-AT. The candidate domain is thus thrombin VR1, while the corresponding domain in R358 α1-AT remains to be determined. The P1′ to P5′ sequences are SIPPE, SLNPN and MAPPE in α1-AT, antithrombin and PAI-1, respectively. It is possible that the presence of the E in the P5′ position (the C-terminal part of the reactive loop) is required for the interaction with thrombin VR1. Indeed, IgG D does not affect thrombin inhibition by antithrombin (this study and [1]).

The interaction of IgG D with protein C activation is complex. We have previously shown that the antibody inhibits the cleavage of protein C by thrombin in the presence of thrombomodulin [2], probably through competition between IgG D and thrombomodulin for binding to thrombin ABE1. In the absence of thrombomodulin, IgG D also inhibits the activation of protein C by thrombin. This could result from the binding of the antibody to VR1, and suggests that this subsite plays a role in the recognition of the protein C cleavage site by thrombin. The P1′P5′ sequence of the cleavage site is LIDGK. The acidic residue in P3′ position is involved in the inhibition of the reaction by calcium ions, possibly through repulsive charges between thrombin E39 and protein C D172 (P3′). That IgG D attenuated the inhibition of protein C by calcium ions confirms

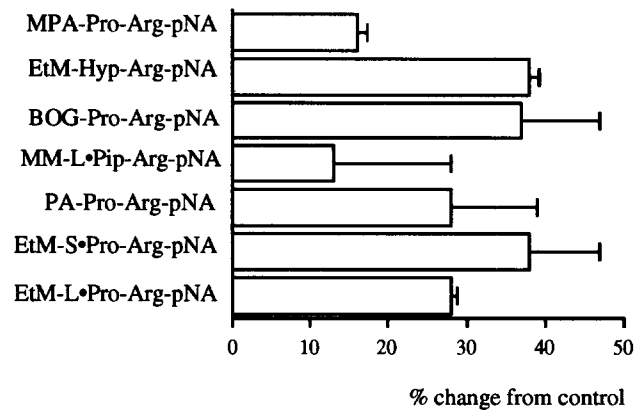

Fig. 5. Thrombin synthetic substrate hydrolysis in the presence of IgG D. Substrates were used at a concentration of 0.3 mM. The % change is the ratio of the initial velocity of substrate hydrolysis by thrombin (10 and 60 nM) in the presence of 1 μM IgG D to the rate in the presence of control IgGs × 100. Data are means (± S.D.) of four determinations.

this hypothesis and underlines the role of thrombin VR1 in protein C activation.

In conclusion, the thrombin VR1 subsite appears to be involved in the thrombin/R358 α1-AT and thrombin/protein C interactions. IgG D attenuated the inhibitory effect of calcium ions during protein C activation by thrombin, probably by masking E39 at the VR1 site.

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