

## Anniversary Issue Contribution

# Update on antithrombin I (fibrin)

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### Summary

Antithrombin I (fibrin) is an important inhibitor of thrombin generation that functions by sequestering thrombin in the forming fibrin clot, and also by reducing the catalytic activity of fibrin-bound thrombin. Thrombin binding to fibrin takes place at two classes of non-substrate sites: 1) in the fibrin E domain (two per molecule) through interaction with thrombin exosite 1; 2) at a single site on each  $\gamma'$  chain through interaction with thrombin

exosite 2. The latter reaction results in allosteric changes that down-regulate thrombin catalytic activity. Antithrombin I deficiency (afibrinogenemia), defective thrombin binding to fibrin (antithrombin I defect) found in certain dysfibrinogenemias (e.g. fibrinogen Naples 1), or a reduced plasma  $\gamma'$  chain content (reduced antithrombin I activity), predispose to intravascular thrombosis.

### Keywords

Fibrinogen, fibrin, thrombin, antithrombin I

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## Introduction

Thrombin binds to its substrate, fibrinogen, through an anion-binding site commonly referred to as 'exosite 1' (1, 2). Howell recognized nearly a century ago that the fibrin clot itself exhibits significant thrombin-binding potential (3). Subsequently, thrombin binding associated with fibrin formation in plasma was termed 'antithrombin I' by Seegers more than sixty years ago (4–6), and I continue to employ that term for this activity of fibrin. In recent years, recognition of the functional importance of antithrombin I for down-regulation of thrombin generation in plasma has brought a new perspective on its physiological role (7, 8). This present article provides an update on the constituents in fibrin that comprise antithrombin I, their mechanisms of action, and their physiological role.

## Antithrombin I activity

Antithrombin I activity is defined by two classes of non-substrate thrombin-binding sites in fibrin (9, 10), one of relatively low affinity in the E domain (~2 sites per molecule), and the other of 'high' affinity in D domains of fibrin(ogen) molecules containing a  $\gamma'$  chain variant termed  $\gamma'$  ( $\gamma'^{427L}$ ) (10) (Fig. 1). Altogether  $\gamma'$  chains comprise ~8% of the total  $\gamma$  chain population (11, 12). Virtually all  $\gamma'$  chains are found in a chromatographic subfraction termed 'fibrinogen 2', each molecule of which also contains a platelet-binding  $\gamma$  chain. 'Fibrinogen 1' is homodi-

meric with respect to its  $\gamma$  chains, and accounts for ~85% of human plasma fibrinogen.

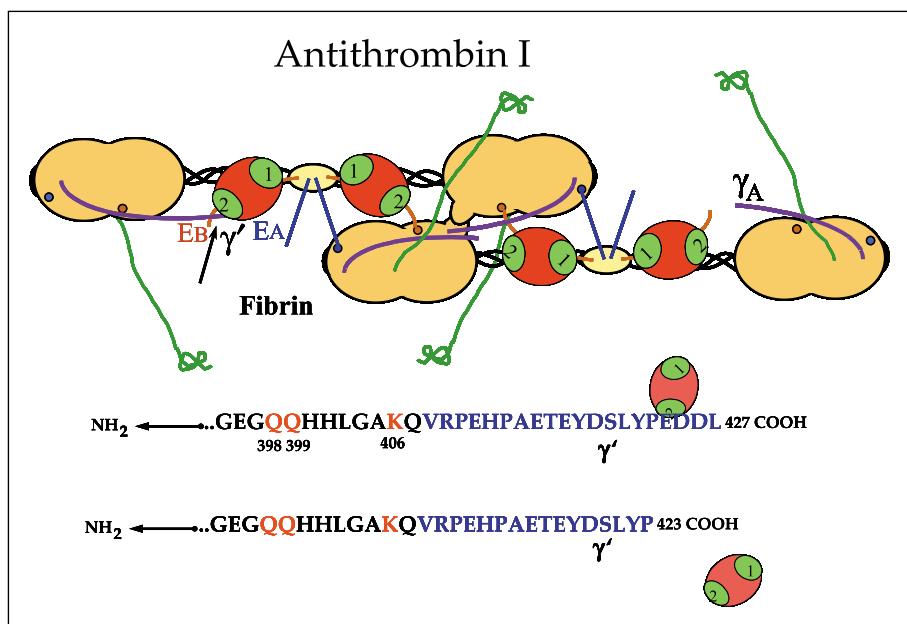
Low-affinity thrombin binding activity reflects thrombin exosite 1 binding in E domain of fibrin, as recently detailed by analyses of thrombin-fibrin fragment E crystals by Pechik et al. (13) (Fig. 2). In contrast, 'high affinity' thrombin binding to  $\gamma'$  chains takes place through exosite 2 (14–16) (Fig. 3). The  $\gamma'$  chain thrombin binding site is situated between residues 414 and 427, and tyrosine sulfation at  $\gamma'418$  and  $\gamma'422$  increases thrombin binding potential (17). The binding affinity of thrombin for  $\gamma'$ -containing fibrin molecules is increased by concomitant fibrin binding to thrombin exosite 1 (18) (Fig. 1).

The effect of  $\gamma'$  chain binding to thrombin exosite 2 is more complex than binding to thrombin exosite 1 at the fibrin E domain. The  $\gamma'$  chain-thrombin interaction induces non-competitive allosteric down-regulation of amidolytic activity at the thrombin catalytic site and consequently slowed release of fibrinopeptide A among other effects (19). This effect is independent of the slow-fast transition induced by  $\text{Na}^+$  binding (20) or the effect that is reflected in the slow cleavage of fibrinogen induced by thrombin binding to thrombomodulin (21). Because of delayed fibrinopeptide cleavage in  $\gamma'$  chain-containing fibrinogen 2, the fibrin that is produced has finer network fibers and contains more branches than does fibrin 1 (19, 22). This structural modification of matrix structure also results in delayed fibrinolysis (19). The down-regulating effect of the  $\gamma'$  peptide sequence on catalytic site activity is similar to that induced in

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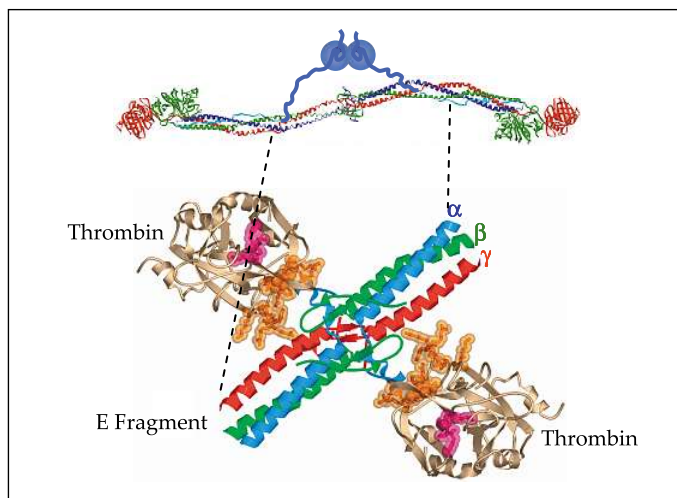
**Figure 1: Diagram of fibrin features that are relevant to antithrombin I, including details on the thrombin-binding  $\gamma'^{427L}$  chains and non-thrombin-binding  $\gamma'^{423P}$  chains.** The donor-acceptor glutamine (Q) and lysine (K) residues that are common to all  $\gamma'$  chains, and that become reciprocally cross-linked by factor XIII are shown in red. Residues that are unique to  $\gamma'$  chains are blue. Thrombin molecules are red and exosites 1 and 2 are green. The arrow indicates a thrombin molecule that is bound by both of its exosites to a  $\gamma'$  chain-containing fibrin molecule and to the E domain. Other thrombin molecules shown in this diagram are bound only by their exosites 1 to E domains. The EA and EB polymerization sites in the fibrin E domain that bind to complementary sites in neighboring D domains are indicated.

thrombin by other exosite 2-binding proteins like GP1b $\alpha$  or prothrombin fragment 2 (23–26), a monoclonal antibody directed against an epitope in thrombin exosite 2 (27), and DNA aptamer HD-22 (28). This suggests that thrombin exosite 2 binding interactions, for example with GP1b $\alpha$  or  $\gamma'$  chains, play a role *in vivo* in regulating thrombin generation. In addition to the effects of  $\gamma'$  chain binding on fibrin formation and lysis, fibrin-mediated enhancement of factor XIII activation (29–32) was slower in the presence of fibrinogen 2 compared with fibrinogen 1 (19). Thus,  $\gamma'$  chain-thrombin interactions play an important role in regulating factor XIII activation.

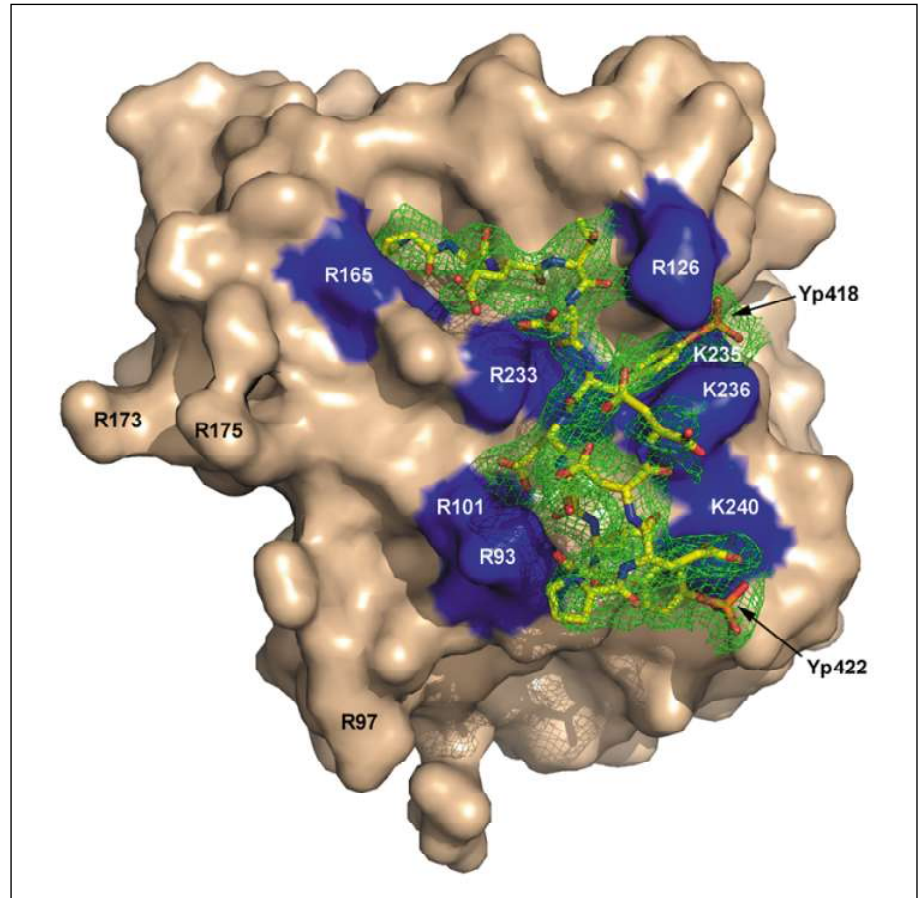
In addition to the full length  $\gamma'$  chains, a shortened version of this chain,  $\gamma'^{423P}$ , is present in most plasmas (33–36). We believe that  $\gamma'^{423P}$  chains arise by post-translational proteolytic processing of intact  $\gamma'^{427L}$  fibrinogen chains, but to date we have not been able to identify the basis for this occurrence. Since the ultimate C-terminal  $\gamma'$  424 to 427 sequence is required for thrombin binding at the  $\gamma'$  site (17),  $\gamma'^{423P}$  chains lack thrombin binding potential, and their formation would reduce antithrombin I activity at the expense of the  $\gamma'^{427L}$  chains.

## Antithrombin I and its relationship to thrombotic disease

The concept that antithrombin I is an important regulator of thrombin activity in clotting blood (8) is based upon a number of prior observations and reports: i) Fibrin from certain congenital dysfibrinogens, e.g. fibrinogen New York I (9) and fibrinogen Naples I (18, 37, 38), exhibit reduced thrombin binding capacity and are associated with marked venous or arterial thromboembolism. ii) Paradoxically, severe thromboembolic disease, both venous and arterial, occurs in afibrinogenemia and in hypofibrinogenemia (39–47) often in association with the infusion of fibrinogen. iii) Increased levels of prothrombin activation fragment F<sub>1+2</sub> (48, 49) or thrombin-antithrombin (TAT) complexes (47, 48) are found in afibrinogenemic plasma (i.e. congenital antithrombin I deficiency), and these abnormal levels can be normalized by fibrinogen infusions (47, 49), further suggesting that an underlying hypercoagulable state exists in this condition. iv) The report that an afibrinogenemic subject developed occlusive peripheral arterial thrombosis in the absence of a fibrinogen infusion (47) seems to be analogous to studies in ferric chloride-injured afibrinogenemic mice, which developed abundant intravascular thrombi at the site of injury that characteristically embolized downstream (50). v) The demonstration by Dupuy et al. (47) that increased thrombin generation in their patient's plas-



**Figure 2: Three-dimensional structure of two thrombin molecules bound to a fibrin E fragment that is projected from a ribbon diagram of a fibrin molecule.** The thrombin-fibrin complex is drawn as a ribbon diagram along a two-fold symmetry axis perpendicular to the plane of the page.  $\alpha$ ,  $\beta$ , and  $\gamma$  chain fragments are blue, green, and red, respectively. Thrombin molecules are in beige, and the residues included in exosite 1 are in orange. The PPACK inhibitor bound to the active site is in magenta. Adapted from Pechik et al. (13).



**Figure 3: Crystal structure of the  $\gamma'$  peptide ( $\gamma'$ 408–427) making contacts with basic residues (blue) in exosite 2 of thrombin.** From Pineda et al. (16). The  $\gamma'$  peptide interactions closely reproduce heparin binding at this site (56), thereby explaining why the binding of the  $\gamma'$  peptide and heparin are mutually exclusive and why thrombin bound to fibrin is resistant to inactivation by antithrombin and heparin cofactor II (14, 57).

ma was normalized by addition of fibrinogen underscored the thrombin inhibitory role of plasma fibrinogen. Demonstrating that fibrinogen 2 ( $\gamma A/\gamma'$ ) had a more profound effect in normalizing thrombin generation in afibrinogenemic plasma than did fibrinogen 1 ( $\gamma A/\gamma A$ ) (7), emphasized the dominant role of  $\gamma'$  chains in thrombin binding and inhibition by fibrin. Overall, these considerations indicate that antithrombin I is a major thrombin inhibitor.

In addition to evidence discussed above, there have been more recent reports suggesting that the content of  $\gamma'$ -containing fibrinogen in plasma has a relationship to the incidence of thrombotic disease (51–53). Uitte de Willige et al. (53) investigated the effect of  $\gamma'$ -fibrinogen/total fibrinogen ratios on the risk of venous thrombosis in the Leiden Thrombophilia Study (54). They demonstrated that reduced  $\gamma'$ -fibrinogen/total fibrinogen ratios were associated with an increased thrombosis risk and were correlated with a particular  $\gamma$  chain gene haplotype termed FGG-H2. The potentially relevant single nucleotide polymorphisms (SNPs) of that haplotype are located in intron 9 (9615 C/T) and just downstream from the polyadenylation site of exon 10 (10034 C/T). These may individually or collectively result in reduced production of  $\gamma'$  chain transcripts, though other explanations may exist.

On the other hand, Drouet et al. (51) suggested that subjects with elevated  $\gamma'$ -fibrinogen/total fibrinogen ratios correlated with a higher incidence of arterial thrombosis and Lovely et al. (52) reported an association between elevated levels of  $\gamma'$  chains and coronary artery disease, although this association did not hold with respect to the  $\gamma'$ -fibrinogen/total fibrinogen ratios. Significant elevations in  $\gamma'$  chain concentration were recently reported by Manilla et al. (55) in patients with myocardial infarction, although the differences were rather small (~10%), and no differences were found in the  $\gamma'$ -fibrinogen/total fibrinogen ratios. More studies will be required to place these several reports in the proper mechanistic perspective.

Finally, thrombotic microangiopathy (TMA) is a life-threatening syndrome with major forms that include thrombotic thrombocytopenic purpura (TTP) and haemolytic uremic syndrome (HUS), is characterized by microangiopathic haemolytic anaemia, thrombocytopenia, and microvascular thrombosis accompanied by varying degrees of tissue ischemia and infarction. We investigated a group of TMA subjects and found that there was an association between the syndrome and a lowered plasma  $\gamma'$  chain content (36). These observations suggest that low levels of antithrombin I activity may contribute to microvascular thrombosis in TMA.

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