Allosteric activation of antithrombin is independent of charge neutralization or reversal in the heparin binding site

Jonathan Langdown, Wendy J. Carter, Trevor P. Baglin, James A. Huntington

University of Cambridge, Department of Haematology, Cambridge Institute for Medical Research, Division of Structural Medicine, Thrombosis Research Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, United Kingdom

Received 22 June 2006; revised 13 July 2006; accepted 18 July 2006

Available online 26 July 2006

Edited by Miguel De la Rosa

Abstract We investigate the hypothesis that heparin activates antithrombin (AT) by relieving electrostatic strain within helix D. Mutation of residues K125 and R129 to either Ala or Glu abrogated heparin binding, but did not activate AT towards inhibition of factors IXa or Xa. However, substitution of residues C-terminal to helix D (R132 and K133) to Ala had minimal effect on heparin affinity but resulted in appreciable activation. We conclude that charge neutralization or reversal in the heparin binding site does not drive the activating conformational change of AT, and that the role of helix D elongation is to stabilize the activated state.

© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Serpin; Protease; Allostery; Hemostasis; Thrombosis

1. Introduction

Antithrombin (AT) is the principal inhibitor of the coagulation proteases. It circulates at a high concentration in a state which is permissive of hemostasis (blood coagulation), and its activation by heparin-like glycosaminoglycans is critical for the cessation of clotting and the prevention of thrombosis. Thus, regulation of AT activity is central to the fine balance between bleeding and thrombosis (for review see [1]). The activation of AT towards factors IXa and Xa (fIXa and fXa) is significantly allosteric in nature, as binding of AT to a specific pentasaccharide sequence of heparin (H5) causes a two order of magnitude increase in rates of inhibition (for review see [2]). While biochemical [3–5] and structural studies [6,7] have demonstrated the nature of the native and activated states, how the conformational change is transduced from the heparin binding site to the distant reactive center loop (RCL) remains unresolved.

AT is a member of the serpin family of protease inhibitors (for review see [8]), and as such, inhibits serine proteases by incorporating its entire RCL into β-sheet A to trap the acyl-enzyme intermediate [9]. The structure of native AT differs from the prototypical serpin α1-antitrypsin in that the N-terminal portion of the RCL (the hinge region) is reversibly incorporated into β-sheet A (Fig. 1A, left). From kinetic studies it has been estimated that at least 99% of circulating AT is in this conformation, with less than 1% with a fully exposed RCL [5] (Fig. 1A, right). AT binds to its cofactor H5 by a two step mechanism summarized in Fig. 1A. Initial weak binding to form an intermediate complex (Fig. 1A, middle) is followed by a conformational change step which locks the pair in a tight-binding complex (Fig. 1A, right). This induced-fit mechanism [10] causes conformational changes in the heparin binding site that somehow promote the expulsion of the hinge region from β-sheet A. How this occurs is of great interest because it underlies the therapeutic anticoagulant effect of heparin, and also because AT is the only heparin binding protein known to utilize an induced-fit mechanism to confer specificity [11].

A hypothesis was put forward in 1994 to explain how H5 binding on helix D of AT was translated into hinge region expulsion [12]. The principle of this ‘electrostatic hypothesis’ is that elongation of helix D towards its C-terminus would engender electrostatic strain by lining up positively charged residues K125, R129, R132 and K133 on the same face of the helix. The strain in native AT prevents elongation of helix D, but when H5 interacts the electrostatic repulsion is relieved, thus promoting elongation. How helix D elongation might result in the expulsion of the hinge region of the RCL from β-sheet A was not suggested, but the coupling of the two events was supported by subsequent biochemical and structural studies [13–15]. Fig. 1B shows close-ups of the heparin binding region and β-sheet A which correspond to the structures shown in A. Several conformational changes in the heparin binding region are seen to occur upon interaction with H5, namely: the one-turn extension of helix A towards the N-terminus, the formation of a one-turn helix P just N-terminal to helix D, and the one-turn C-terminal extension of helix D (Fig. 1B right vs. left). However, helix A elongation and helix P formation are also observed in the intermediate state (Fig. 1B, middle), and also in the six-stranded latent form bound to H5 (Fig. 1C). Surprisingly, both structural changes were also observed in the recent structure of native monomeric AT (1T1F) (Fig. 1D). The only secondary structural change which is uniquely observed in the hinge region-expelled state is thus the C-terminal extension of helix D. These studies lend credence to the electrostatic hypothesis which we set out to investigate using a mutagenesis strategy aimed at both neutralizing the basic residues on and just C-terminal to helix D, and by mimicking H5 binding by reversing the charges within the

Abbreviations: AT, antithrombin; RCL, reactive center loop; fIXa, factor Xa; fIXa, factor IXa; H5, pentasaccharide; SI, stoichiometry of inhibition

Corresponding author. Fax: +44 1223 336827.
E-mail address: jah52@cam.ac.uk (J.A. Huntington).

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.fels.2006.07.057
We found that mutation of K125 and R129 does not activate AT, while mutation of the basic residues on the C-terminal loop following helix D appreciably activated AT towards both fIXa and fXa. The results demonstrate that neutralization or reversal of the charges within the H5 binding site does not drive the activating conformational change of AT. However, the composition of the loop following helix D is important, and its propensity to form an α-helix is integral to the allosteric activation of AT.

2. Materials and methods

2.1. Materials

Human α-thrombin and fIXa were obtained from Haematologic Technologies (Essex Junction, VT), and human fXa was obtained from Enzyme Research Laboratories (Swansea, UK). The pentasaccharide (fondaparinux, H5) was provided by Maurice Petitou (previously of Sanofi-Aventis), and the monoclonal antibody M27 [16] was the kind gift of Johan Stenflo.

![Diagram](image1)

Fig. 1. Pentasaccharide binding induces several conformational changes in AT. (A) AT is shown as a ribbon diagram in three crystallographically determined conformational states: native (left), intermediate (middle) and H5-activated (right, H5 as ball-and-stick). The orientation was chosen to highlight the important structural features of the RCL (yellow, with red ball-and-stick P1 residue), β-sheet A (blue) and the heparin binding site (helix A in green and D in cyan). In native and intermediate ATs the hinge region is partially inserted into β-sheet A (circled). The secondary structural changes in the heparin binding region are colored magenta. Helix A elongation and helix P formation appear to be associated with the initial binding state, and only helix D elongation is exclusively observed in the hinge region-expelled (activated) state. (B) Close-ups of the heparin binding site, the RCL and β-sheet A are shown under the structures to which they correspond, and the residues mutated in this study are indicated. H5 is shown as a transparent ball-and-stick. (C) The link between the state of β-sheet A and the C-terminal portion of helix D is strengthened by the observation that H5-bound latent AT has an elongated helix A and helix P, but no elongation of helix D. (D) A similar result was found for an unliganded monomeric structure of AT (1T1F).

![Diagram](image2)

Fig. 2. Pentasaccharide binding and activation of AT variants. (A) H5 binding is detected by monitoring intrinsic fluorescence enhancement (ΔF is fractional fluorescence change). Control AT (open circles) binds with high affinity and results in a ∼35% fluorescence enhancement. Similar results were found for the 132/133 Ala variant (open triangles), but the 125/129 Ala variant (open inverted triangles) showed little fluorescence enhancement, even at high H5 concentrations. (B) H5 activation of fXa inhibition was normal for the control and 132/133 AT variants, while no acceleration was observed for the 125/129 Ala variant, even with an excess of H5 (symbols as before). (C) However, at very high H5 concentrations some acceleration of fXa inhibition was seen for the 125/129 Ala variant, even with an excess of H5 (symbols as before). (C) The link between the state of β-sheet A and the C-terminal portion of helix D is strengthened by the observation that H5-bound latent AT has an elongated helix A and helix P, but no elongation of helix D. (D) A similar result was found for an unliganded monomeric structure of AT (1T1F).
2.2. AT expression and purification
AT variants K125E/R129E, K125A/R129A, R132A/K133A and K125A/R129A/R132A/K133A were made in a baculovirus expression system using the Bac-to-Bac system on the recombinant β-glycoform background of S137A. The mutations were created by site-directed mutagenesis of pFastBac1-AT plasmid using the Stratagene Quick-Change site-directed mutagenesis kit with the appropriate primers for the mutations. Invitrogen’s Bac-to-Bac procedures were followed and transfection of S9 cells was performed using Fugene (Roche Diagnostics Ltd) following the manufacturers instructions. Three rounds of viral amplification were performed in S9 cells after which Hi-five cells were infected (MOI of 0.2) and supernatant was harvested after 96 h. AT purification was achieved using a rabbit anti-human AT (Dako) conjugated Sepharose column (for 125/129 variants), or heparin–Sepharose (for control S137A, and R132A/K133A). Further purification was achieved by Q-Sepharose chromatography. A monoclonal antibody specific for relaxed AT (latent, cleaved and polymers) was pharose (for control S137A, and R132A/K133A). Further purification was achieved using a rabbit anti-human AT (Dako) were infected (MOI of 0.2) and supernatant was harvested after 96 h. by following change in intrinsic fluorescence with increasing H5 concentrations (Fig. 2A).

2.3. Rates of inhibition and H5 binding
Second order rate constants of protease inhibition (±H5), and equilibrium dissociation constants (Kd) for H5 binding were determined as previously [5]. Kd values for the AT variants containing the 125/129 Ala mutations were calculated from the slope of Kd vs. high H5 concentration (from Fig. 2C). An assumption that the same maximal rate would be achieved for the variant as for the control (at full H5 occupancy) allowed a calculation of the Kd from the following equation: Kd = k_{penta} \cdot [AT]_0/slope - [AT]_0 (k_{penta} is maximal second-order rate constant and [AT]_0 is total AT concentration).

3. Results
3.1. Pentasaccharide binding
By following change in intrinsic fluorescence with increasing concentration of H5 we determined Kd values for the control and 132/133 AT variants (Table 1, and Fig. 2A). It is known that 132 and 133 do not interact directly with H5, and accordingly only a small 2.4-fold decrease in affinity was observed. For the other variants, involving mutation of residues 125 and 129, no binding was detected by the fluorescence method even at high H5 concentrations (Fig. 2A).

3.2. Rates of protease inhibition
Mutations in AT often affect the partitioning between the substrate and inhibitory pathways, reflected by an increase in the apparent SI. For the control and all helix D variants, however, we obtained SIs indistinguishable from one (±H5, data not shown). Second-order rate constants of inhibition of thrombin, fXa and fIXa (±H5) are reported in Table 1. Rates of thrombin inhibition were normal for all AT variants. The rates of fXa and fIXa inhibition are highly sensitive to the conformation of AT, with H5 binding resulting in a 126-fold and a 233-fold increase k_{app} for control AT. The basal rates (no H5) of fXa and fIXa inhibition thus reflect the activating effects of the helix D mutations. We found that either neutralization or reversal of the charges within the H5 binding region of helix D (125/129) did not have any effect on rates of inhibition, but that neutralization of 132/133 resulted in a significant increase in rates of fXa (5.7-fold) and fIXa (7.9-fold) inhibition. The quadruple Ala variant was slightly activated towards fXa and fIXa (1.8 and 2.8-fold respectively), but less so than the 132/133 variant on its own.

Pentasaccharide activation for the 132/133 variant was normal for all three proteases, but could not be determined for the 125/129 variants due to the apparent loss of binding (Fig. 2B). However, very high concentrations H5 did increase the observed rate of inhibition of fXa for the two 125/129 Ala variants (slopes reported in Table 1), but no effect was seen under these conditions for the 125/129 Glu variant (Fig. 2C). These results suggest a finite H5 affinity of the 125/129 Ala variants. Assuming that upon saturation of H5 binding the rate of fXa inhibition would be equivalent for the 125/129 Ala variants and the control, a lower-limit Kd of ~1 mM can be estimated for the 125/129 Ala variants.

4. Discussion
This study was undertaken to test the long-standing electrostatic hypothesis of AT activation [12]. It was proposed

<table>
<thead>
<tr>
<th>Antithrombin</th>
<th>Penta binding*</th>
<th>Second-order rate constants of protease inhibition (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Penta</td>
<td>Thrombin</td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.3</td>
<td>9.8 ± 0.14 × 10³</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>1.4 ± 0.1 × 10⁴</td>
</tr>
<tr>
<td>R132A/K133A</td>
<td>6.1 ± 0.1</td>
<td>8.8 ± 0.10 × 10³</td>
</tr>
<tr>
<td></td>
<td>(2.4)</td>
<td>1.4 ± 0.06 × 10⁴</td>
</tr>
<tr>
<td>K125A/R129A</td>
<td>≥ 100 000 000</td>
<td>7.9 ± 0.02 × 10³</td>
</tr>
<tr>
<td></td>
<td>(≥ 40 000 000)</td>
<td>NC</td>
</tr>
<tr>
<td>Quad Ala</td>
<td>100 000 000</td>
<td>7.6 ± 0.40 × 10³</td>
</tr>
<tr>
<td></td>
<td>(≥ 400 000)</td>
<td>NC</td>
</tr>
<tr>
<td>K125E/K129E</td>
<td>UD</td>
<td>8.0 ± 0.30 × 10³</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>0</td>
</tr>
</tbody>
</table>

*Value in parentheses corresponds to magnitude of reduction in H5 affinity relative to control. UD indicates undetectable binding by either fluorescence or activity. NC means no change and ND is for not determined. Values in square brackets are the slopes from the graph shown in Fig. 2C, and the Kd values with an asterisk are calculated from the slopes according to the equation in Section 2.
that neutralization of basic residues along the face of helix D (i.e. K125 and R129) upon interaction with H5 would promote helix D elongation (including R132 and K133), and that helix D elongation provides the driving force for the AT conformational change which results in hinge region expulsion from β-sheet A (Fig. 1A and B). Subsequent structural and biochemical studies have demonstrated that helix D elongation is associated with, and energetically coupled to H5 binding, but the role of charge neutralization in propagating helix D elongation has not been investigated. We thus created variants which neutralized the charges within the H5 binding site on helix D (K125A/R129A) and in the following loop region (R132A/K133A). We also created a variant designed to mimic the effect of H5 binding by reversing the charges in the heparin binding site (K125E/R129E). These mutations were not predicted to alter the core structure of AT because the residues are found in different conformations in the many structures of unliganded AT, and in some cases their side chains were missing from electron density, indicating a high degree of flexibility. This was indeed borne out by the observation that the variants were fully active inhibitors of all three proteases tested, and thus any observed effect of the mutations derived from either local alterations in structure or electrostatic potential. Our results show that neutralization or reversal of the positive charges within the H5 binding site does not mimic the effect of H5 binding. Such an effect would have resulted in a significant increase in the rates of fIXa and Xa inhibition, and none was not observed. These results are consistent with data obtained from single mutations in the heparin binding region [17,18], and argue strongly against the electrostatic hypothesis.

However, neutralization of residues on the extended helix D (R132/K133) did marginally activate AT towards fIXa and fXa. This is likely to reflect a change in the equilibrium between the native hinge region-inserted form and the activated hinge region-expelled form. It is well established that the activating conformational change is the release hinge region from β-sheet A [5], thus, any observed increase in basal rate of fIXa or fXa inhibition is caused by an increase in population of the hinge region-expelled state. Factor IXa is particularly sensitive to the expulsion of the hinge region, as prevention of hinge region extension totally abrogates fIXa inhibition. Thus, the low basal rate of fIXa inhibition reflects the small fraction of AT in an activated state (assuming a rapid equilibrium). Based on rates of fIXa inhibition we estimate that about 0.4% of wild-type AT is in the activated state and neutralization of 132/133 results in about 3% in the activated state. How these mutations alter the equilibrium is unclear, however, it is unlikely to be the result of charge neutralization as several other mutations in this region have similar effects. Failure to glycosylate at Asn135 (plasma β-glycoform) increases heparin affinity and rates of protease inhibition, and recombinant β-forms created by mutating either Asn135 or Ser137 lead to an even greater increase [19–21]. These effects might be explained by an increase in the helical propensity of the C-terminal helix D loop, but the small increase in fraction in the activated state observed for the 132/133 Ala variant suggests that helix D elongation is not the conformational event which drives hinge region expulsion. Rather, helix D elongation is likely to occur after hinge region expulsion, perhaps, as previously suggested [15], to stabilize the activated state.

Acknowledgements: Funding is provided by the NIH (R01 HL68629), the MRC (UK) and the Tesny-Perry Trust.

References