Regulation of Blood Coagulation by the Serpin, Antithrombin

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Hemostasis:
Balance between procoagulant factors and anticoagulant factors which maintain blood in a fluid state and protect against blood loss resulting from injury

Activation of Procoagulant Factors by Injury

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Anticoagulant Factors

- **Protein C anticoagulant system**
  - Activated protein C shuts down the coagulation cascade by proteolytically inactivating the procoagulant factors VIIIa and Va.

- **Fibrinolytic system**
  - Tissue plasminogen activator and plasmin act in concert to dissolve a fibrin clot once it has formed.

- **Antithrombin/heparan sulfate anticoagulant system**
  - Antithrombin and its glycosaminoglycan activator, heparan sulfate, localize blood clotting to an injury site by inhibiting procoagulant proteases which escape from the site.

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**Regulation of Blood Coagulation by Antithrombin and Heparan Sulfate**

- **Antithrombin**
  - 58 kDa glycoprotein of the serpin (serine protease inhibitor) superfamily which circulates in blood plasma at ~2 µM.
  - Key inhibitor of the procoagulant proteases, thrombin, factor Xa, and factor IXa. Inhibits proteases by forming stable 1:1 complexes which block the protease active site.
  - Requires activation by heparan sulfate or heparin-type glycosaminoglycans to inhibit target proteases at a rapid physiologic rate.
  - Basis for widespread clinical use of heparin for anticoagulant therapy.
  - Two circulating glycoforms, α (~90%) and β (~10%) which differ in their glycosylation of Asn135 and their affinity for heparin.
### Inherited Deficiencies of Antithrombin

- Acquired or inherited deficiencies of antithrombin are an established cause of thrombophilia.
- Type 1 inherited deficiencies result from mutations which inhibit the expression of antithrombin due to frameshift or premature stop codon mutations or due to reduced synthesis.
- Type 2 inherited deficiencies result from the normal expression of a dysfunctional mutant antithrombin usually due to a single amino acid substitution.

### Natural Mutations Causing Antithrombin Dysfunction

- Mutant antithrombins may exhibit dysfunctions in protease binding, heparin binding or both.
- Mutants with protease binding dysfunctions are associated with thrombophilia in heterozygotes; Homozygotes are unknown.
- Mutants with heparin binding dysfunctions are associated with thrombophilia only in heterozygotes and not homozygotes.
- Loss of both functions may result from antithrombin mutations which cause conformational instability and conversion to an inactive conformational state.

### Serpins Regulate Most of the Proteolytic Enzymes of Blood Coagulation

<table>
<thead>
<tr>
<th>Serpin</th>
<th>Target protease(s)</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin</td>
<td>Thrombin, FXa, FIXa</td>
<td>Thrombosis</td>
</tr>
<tr>
<td>Heparin cofactor II</td>
<td>Thrombin</td>
<td>Thrombosis (?)</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activators</td>
<td>Bleeding</td>
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<tr>
<td>α2-antiplasmin</td>
<td>Plasmin</td>
<td>Bleeding</td>
</tr>
<tr>
<td>Protein C inhibitor</td>
<td>Activated protein C</td>
<td>Bleeding (?)</td>
</tr>
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Serpins
- Globular proteins with a conserved 350 amino acid core domain
- Distributed widely in eukaryotes, prokaryotes and viruses

- Latent Native
  - Metastable fold: melting T~60°C
- Cleaved
  - melting T~80°C
  - melting T~100°C

Lock-and-Key-Type
Protein Protease Inhibitors

Serpin-type protein protease inhibitors

Dementiev et al., 2003; Huntington et al., 2000; Dementiev et al., 2005;
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Antithrombin inhibits its target proteases at nonphysiologic rates in the absence of heparin, i.e., with association rate constants \( k_{\text{ass}} \) corresponding to half-lives of minutes to hours. Heparin accelerates these reactions from 1,000-100,000-fold to achieve diffusion-limited association rate constants with half-lives of msec.

\[ \text{AT} + \text{E} \rightarrow \text{AT-E} \]

\( \text{AT} = \) antithrombin
\( \text{E} = \) thrombin, factor Xa, factor IXa

Heparin/Heparan Sulfate Structure

- Polymer of repeating uronic acid-glucosamine disaccharide units
- Uronic acid either glucuronate or iduronate
- Variably sulfated at 2-, 3-, and 6-positions of glucosamine and 2-position of iduronate
- Heparin more highly sulfated and has more iduronate than heparan sulfate

A Specific Pentasaccharide Sequence

In Heparin and Heparan Sulfate Mediates the Binding and Activation of Antithrombin

Positioning of six sulfates and two carboxylates, and in particular four critical sulfates (*), in this sequence are required for high-affinity binding and anticoagulant activation of antithrombin.

A 3-O-sulfate in saccharide F provides a signature of the sequence and is essential for pentasaccharide binding and activation.
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X-Ray Structure of Antithrombin Complexed with the Heparin Pentasaccharide

Jin et al., 1997

Close-Up of the Heparin Binding Site of Antithrombin

Lys 114, Lys 125 and Arg 129 of Antithrombin Are "Hotspots" for Binding the Pentasaccharide

Titration of the binding of heparin to antithrombin monitored by protein fluorescence changes

Effect of mutating antithrombin basic residues to Ala on the Kd for heparin pentasaccharide binding

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Heparin binds to antithrombin in a two-step process in which an initial low heparin affinity binding induces an antithrombin conformational change leading to high affinity binding.

Saturable dependence of the binding rate constant on heparin concentration.

Wild-type Mutant Mutant Mutant Mutant

Heparin binds to antithrombin in a two-step process in which an initial low heparin affinity binding induces an antithrombin conformational change leading to high affinity binding.

Rate of heparin pentasaccharide binding to antithrombin monitored by protein fluorescence changes.

Deletion of the nonreducing terminal saccharide D, the reducing terminal GH disaccharide or the 3-O-sulfate on the central saccharide F causes large decreases in pentasaccharide affinity for antithrombin.

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- GH binds only after conformational activation and serves to lock antithrombin in the activated state.
- DEF is sufficient to induce conformational activation of antithrombin without the GH disaccharide.
- GH binds only after conformational activation and serves to lock antithrombin in the activated state.

Preequilibrium Binding Mechanism

- Elimination of saccharide D alters the heparin binding mechanism by favoring binding to a preequilibrium fraction of activated antithrombin.
- Saccharide D contributes substantial binding energy to the initial low-heparin affinity binding step.

Allosteric Activation of Antithrombin by the Heparin Pentasaccharide Occurs by an Induced-Fit Binding Mechanism

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Heparin pentasaccharide enhances $k_{\text{ass}}$ for antithrombin inhibition of factors Xa and IXa several hundred-fold, but minimally enhances $k_{\text{ass}}$ for antithrombin inhibition of thrombin.

Full-length heparin chains with at least 16-18 saccharides and which contain the pentasaccharide are required to enhance antithrombin reactivity with thrombin several-thousand-fold.

Full-length heparins produce further increases in $k_{\text{ass}}$ for antithrombin reactions with factors Xa and IXa beyond those produced by the pentasaccharide.

These rate enhancements require physiologic levels of calcium ions.

Heparin Activates Antithrombin by Both Conformational Activation and Bridging Mechanisms.

Heparin binding exosite

Pentasaccharide

Reactive loop

Heparin

Binding exosite

Thrombin

FXa/FIXa

Bridging Mechanism

X-Ray Structures of the Antithrombin-Thrombin-Heparin Michaelis Complex provide proof of the bridging mechanism.

Dementiev et al., 2004

Li et al., 2004

Antithrombin

Thrombin

Reactive loop-active site interaction

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Native low activity state of antithrombin: reactive loop hinge buried in sheet A

Heparin-activated state of antithrombin: reactive loop hinge expelled from sheet A

Blocking the insertion of the reactive loop hinge into sheet A by mutations of the P14 serine residue to charged and bulky residues cause antithrombin to be activated without heparin

Huntington et al., 1996; Futamura et al., 2000; Langdown et al., 2004

Native antithrombin

Antithrombin-hexa- and pyranosyl-D-gluco-hepta-saccharide complex

Heparin Pentasaccharide

binding to helix D induces reactive loop expulsion from sheet A by shortening of the loop connecting helix D to strand 2 of sheet A

Meagher et al., 2000; Belzar et al., 2002

• Hypothesis: the antithrombin reactive loop contains a substrate recognition sequence for factors Xα and IXα but not for thrombin and heparin activation is required to make this sequence accessible to these proteases

• Test of hypothesis: mutate the P6-P3 reactive loop sequence to decrease recognition by factors Xα and IXα and increase recognition by thrombin

P₁ P₂ P₃ P₄ P₅ P₆ P₇
W V I A S P R S N
V V I A S E L L H P₁
W V I A S E L L H P₇
W V I A S E L L H P₇
V I A S E L L H P₇
V I A S E L L H P₇
V I A S E L L H P₇
V I A S E L L H P₇
A L I A R S P

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Reactive loop mutations alter the reactivity of unactivated and pentasaccharide-activated antithrombins to similar extents with any protease
Reactive loop mutations minimally affect the pentasaccharide enhancement of antithrombin reactivity with any protease indicating that the determinants of the enhanced reactivity are not in the reactive loop

Chuang et al., 2001

Modified Conformational Activation Mechanism
Conformational activation of antithrombin by the heparin pentasaccharide enhances antithrombin reactivity with factors Xa and IXa by generating new interaction sites (exosites) outside the reactive loop

Mapping the Antithrombin Exosite by a Chimeric Approach: substitute six regions circumscribing the reactive loop with the homologous regions from the nonheparin-activatable serpin a1-proteinase inhibitor (a1PI)
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Antithrombin chimeras show normal pentasaccharide-enhanced reactivities with factor Xa and factor IXa except for the chimera in which strand 3 of sheet C is replaced.

A factor Xa/IXa-specific interaction exosite resides in antithrombin strand 3C.

Target three strand 3C residues, Tyr253, Glu255 and Lys257, which are conserved in the thirteen vertebrate antithrombins which have been sequenced, for mutation.

Strand 3 of β-sheet C

Mutation of two conserved strand 3C residues, Tyr253 and Glu255, is sufficient to reduce the pentasaccharide enhancement in antithrombin reactivity with factor Xa to a level comparable to that of the strand 3C chimera. The strand 3C mutations produce parallel losses in the reactivity of pentasaccharide-activated antithrombin with factor IXa.

Tyr253 and Glu255 are key determinants of the factor Xa/IXa exosite in antithrombin.

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- Both conformational activation and bridging mechanisms of heparin activation of antithrombin involve the generation of new exosites to enhance the interaction with target proteases.
- Exosites are generated on antithrombin by conformational activation or provided by bridging full-length heparins.

Reactive Loop Sequence

Only P2-P1' residues in the reactive loop sequences of vertebrate antithrombins are conserved, consistent with this sequence providing only the minimal determinants for recognition of target proteases.
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The antithrombin reactive loop sequence may have evolved to limit the reactivity of the serpin with the anticoagulant protease, activated protein C. Activated protein C has a similar trypsin-like specificity for PI Arg substrates as does thrombin factor Xa and factor IXa. Yet, heparin-activated antithrombin inhibits activated protein C at a physiologically insignificant rate, i.e., 10-million-fold slower than that for thrombin factor Xa and factor IXa! Antithrombin

Negative Features of Heparin Anticoagulant Therapy

- Heparin interacts nonspecifically with plasma proteins, platelets and blood vessel wall
- Poor bioavailability and rapid clearance
- Patient-to-patient variability in dosage
- Risk of bleeding

Improved heparin-based anticoagulants

- Low molecular weight heparins
- Synthetic heparin pentasaccharide (fondaparinux)
- Provide a more predictable anticoagulant effect with less bleeding risk due to fewer nonspecific interactions and better targeting to antithrombin
Reference (1)
Reference (2)
- Olson, S.T., Swanson, R., Coleman, S.W., Olson, S.T. (2001) "Heparin enhances the specificity of antithrombin for thrombin and factor Xa independent of the reactive center loop sequence," J. Biol. Chem. 276:15661

Reference (3)
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