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Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases. a paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors

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Abstract

Serpin family protein proteinase inhibitors regulate the activity of serine and cysteine proteinases by a novel conformational trapping mechanism that may itself be regulated by cofactors to provide a finely-tuned time and location-dependent control of proteinase activity. The serpin, antithrombin, together with its cofactors, heparin and heparan sulfate, perform a critical anticoagulant function by preventing the activation of blood clotting proteinases except when needed at the site of a vascular injury. Here, we review the detailed molecular understanding of this regulatory mechanism that has emerged from numerous X-ray crystal structures of antithrombin and its complexes with heparin and target proteinases together with mutagenesis and functional studies of heparin-antithrombin-proteinase interactions in solution. Like other serpins, antithrombin achieves specificity for its target blood clotting proteinases by presenting recognition determinants in an exposed reactive center loop as well as in exosites outside the loop. Antithrombin reactivity is repressed in the absence of its activator because of unfavorable interactions that diminish the favorable RCL and exosite interactions with proteinases. Binding of a specific heparin or heparan sulfate pentasaccharide to antithrombin induces allosteric activating changes that mitigate the unfavorable interactions and promote template bridging of the serpin and proteinase. Antithrombin has thus evolved a sophisticated means of regulating the activity of blood clotting proteinases in a time and location-dependent manner that exploits the multiple conformational states of the serpin and their differential stabilization by glycosaminoglycan cofactors.

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Protein proteinase inhibitors of the serpin superfamily play a critical role in regulating proteinases in diverse physiologic processes such as development, wound healing and the immune response [1,2]. The family name is an acronym for serine proteinase inhibitor, but it is now well established that serpins inhibit proteinases of both the serine and cysteine mechanistic classes [3]. This unusual "cross-class" inhibition results from serpins utilizing a novel conformational trapping mechanism to inhibit proteinases which exploits the two-stage proteolysis mechanism of serine and cysteine proteinases. Serpins also frequently utilize cofactors as a means of regulating their own activity. Together, these features set the serpins apart from the simpler lock-and-key type proteinase inhibitors such as those of the Kunitz and Kazal families.

One of the better known and studied members of the serpin superfamily is antithrombin, a serpin that regulates the serine proteinases of the blood clotting cascade [4,5]. Antithrombin plays a key anticoagulant role by preventing the activation of procoagulant proteinases except at a site of injury, as is borne out by the well-established association of inherited or acquired deficiencies of this serpin in humans with an increased risk of developing abnormal blood clots or thrombosis [6]. More recent studies have further demonstrated the paramount importance of this serpin for survival by showing that complete antithrombin deficiency in mice results in embryonic lethality at a time that coincides with vascular development and that is a consequence of extensive fibrin deposition in the heart and liver and a consumptive coagulopathy [7]. An important aspect of antithrombin anticoagulant function is that the activity of the serpin is itself regulated by the glycosaminoglycan cofactors, heparin and heparan sulfate. We review here the current understanding of the molecular mechanisms by which antithrombin regulates blood clotting proteinases since it provides a useful paradigm for understanding the complex modes by which serpins regulate proteinase activity. We will first consider the means by which antithrombin achieves specificity for its target proteinases and second discuss how heparin brings about activation of the serpin.

Mechanism of proteinase inhibition

Antithrombin inhibits its target proteinases by an unusual branched pathway suicide substrate mechanism that is shared by other serpin family inhibitors of proteinases (Fig. 1) [1]. Notable among the serpins is their use of both a substrate recognition sequence contained within an exposed reactive center loop (RCL) as well as exosite recognition determinants outside the RCL to lure the proteinase to form an initial Michaelis-like complex (Fig. 2) [8]. In the case of antithrombin, a P1 Arg and flanking residues provide the "bait" substrate sequence recognized by the trypsin-like serine proteinases of the blood clotting cascade. Additionally, attractive and repulsive exosites exist outside this sequence whose differential accessibility in repressed and glycosaminoglycan-activated states of the serpin allows regulation of proteinase binding [9]. Once the proteinase is bound, its catalytic serine proceeds to attack the reactive P1 Arg bond in the serpin RCL, resulting in the formation of an acyl-intermediate complex in which the RCL has been cleaved but remains covalently linked to the catalytic serine through the P1 residue. The cleavage triggers the suicide mechanism by freeing the metastable serpin to undergo a massive conformational change in which the cleaved RCL inserts into the center of a 5-stranded β-sheet, the A sheet, causing the tethered proteinase to be dragged to the opposite end of the serpin and inactivated through conformational deformation (Figs. 1 & 2) [10-12]. The conformational trapping of the acyl-intermediate complex competes with a normal deacylation of the intermediate to yield free proteinase and cleaved serpin, thus allowing in some cases a fraction of the proteinase to escape trapping.

Regulation of antithrombin reactivity by glycosaminoglycans

At concentrations present in blood, antithrombin inhibits blood clotting proteinases unusually slowly because it exists in a repressed reactivity state. Heparin or heparan sulfate-type glycosaminoglycan cofactors transform the serpin into a fast inhibitor of blood clotting proteinases by relieving this repression. This transformation is the basis for the widespread clinical use of heparin as an anticoagulant drug. The heparin effect appears to be important for the physiological function of antithrombin, since natural mutations in the heparin binding site of the serpin that reduce heparin activation are associated with an increased risk of thrombosis [6]. The heparan sulfate molecules that line the surface of blood vessel endothelial cells are thought to activate circulating antithrombin and thereby to be responsible for the unusual nonthrombogenic properties of the vessel wall [13,14]. Since blood clotting is activated and localized at the site of an injured vessel, luminal and subluminal endothelial cell heparan sulfate molecules are thought to activate antithrombin anticoagulant function at such sites. Heparin released from extravascular mast cells closely associated with blood vessels may additionally activate antithrombin at these sites. This would allow antithrombin to rapidly inactivate blood clotting proteinases that escape from an injury site and prevent the dissemination of blood clots away from the injury.

Heparin and heparan sulfate allosterically activate antithrombin by binding the serpin through a common sequence-specific pentasaccharide and inducing activating conformational changes in the protein (Figs. 3-5) [15,16]. The binding is of high affinity, with a dissociation constant of ~50 nM at physiologic pH and ionic strength [17]. The pentasaccharide is responsible for the bulk of the binding energy of full-length heparin and heparan sulfate chains, the longer glycosaminoglycans binding antithrombin with ~3-fold higher affinity than the pentasaccharide. The full-length glycosaminoglycans may additionally activate antithrombin as bridging cofactors by binding both the serpin and proteinase and promoting their interaction in a ternary complex (Fig. 3) [18]. While allosteric activation by the sequence-specific heparin pentasaccharide selectively enhances antithrombin reactivity with factors Xa and IXa, the bridging effect of full-length glycosaminoglycan chains augments antithrombin reactivity with factors Xa and IXa and is solely responsible for upregulating antithrombin reactivity with another important target proteinase, thrombin [17,19].

Conformational changes induced in antithrombin by allosteric activation

The molecular details of the allosteric activation mechanism have been suggested by X-ray crystal structures of antithrombin free and complexed with the heparin pentasaccharide (Fig. 5) [20,21]. The heparin binding site on antithrombin is comprised of three distinct regions of the protein that come together in the tertiary structure, namely, the N-terminal region, the N-terminal end of helix A and all of helix D together with its N-terminal loop (Fig. 6). Positively charged basic residues in each of these regions, including Lys11, Arg13, Arg46, Arg47, Lys114, Lys125 and Arg129, interact through ionic and hydrogen bonds with negatively charged sulfate and carboxylate groups on the heparin pentasaccharide.

Comparison of free and heparin complexed antithrombin structures further reveals significant conformational changes in the protein induced by heparin binding (Figs. 5 & 6) [21]. These conformational changes are more limited than those induced by proteinase reaction but nevertheless involve significant structural rearrangements both in the heparin binding site and in the region surrounding the RCL where the proteinase binds. In the heparin binding site, the N-terminus and helix A undergo large reorientations to widen the binding site and position basic residues for interaction with pentasaccharide groups. Additionally, helix D rotates, the C-terminal end of helix D extends and a new P helix is formed in the loop preceding this helix, resulting in the repositioning of basic residues in these regions for interaction with

pentasaccharide groups. In the proteinase binding RCL region, a major structural change occurs in which the RCL, initially buried through its N-terminal hinge in a partially open sheet A, is expelled from the sheet and extended away from the serpin body. The hydrogen bonding network and hence the surface electrostatics in the region surrounding the RCL is further altered [22]. These structural changes are responsible for the allosteric activation of antithrombin reactivity toward factors Xa and IXa by mechanisms discussed further below. The expulsion of the RCL from sheet A appears to be driven by a closing of the gap in sheet A that is in turn linked to the C-terminal extension of helix D in the heparin binding site [23,24]. Helix D extension thus produces a shortening of the loop that connects the helix to strand 2 of sheet A as well as a compaction of the hydrophobic core to cause the contraction of the sheet. In this way, the conformational changes in the heparin binding site are transmitted to the proteinase binding site to induce activation of the serpin.

Mechanism by which allosteric activation enhances antithrombin reactivity

How the conformational changes induced in the proteinase binding region by the heparin pentasaccharide activate antithrombin reactivity with proteinases has been the subject of numerous studies. It was initially thought that RCL expulsion from sheet A made the RCL more accessible to target proteinases and that this increased accessibility accounted for the activating effect of heparin on antithrombin reactivity [25]. However, such a mechanism failed to explain why heparin allosteric activation was selective in enhancing antithrombin reactivity with two target proteinases, factor Xa and factor IXa, and minimally enhanced antithrombin reactivity with thrombin. That the RCL did not contain the determinants responsible for heparin activation of antithrombin reactivity with proteinases was shown by experiments in which the RCL bait sequence was altered by mutagenesis to create an optimal thrombin recognition sequence [19,26]. This had the expected result of greatly increasing antithrombin reactivity with thrombin and decreasing reactivity with factors Xa and IXa, but unexpectedly, such changes affected basal antithrombin reactivity and had no effect on the selectivity or magnitude of heparin pentasaccharide enhancements of antithrombin reactivity with the three proteinases. Another series of experiments showed that changing the P1 Arg bait residue greatly reduced antithrombin reactivity with all target proteinases, but again had no effect on the differential heparin enhancements of antithrombin reactivity with the proteinases [27]. Such studies revealed the importance of exosite determinants outside the RCL in mediating heparin activation of antithrombin [28].

Identification of exosites that specifically interacted with factors Xa and IXa was accomplished by engineering chimeric antithrombins in which secondary structural regions that define the surface of the serpin from which the RCL protrudes were swapped with those of α_1-proteinase inhibitor, a serpin that is not activated by heparin [29]. Of the six chimeras prepared, only one, in which strand 3 of sheet C was substituted, showed a marked loss in the ability of heparin pentasaccharide to enhance antithrombin reactivity with factors Xa and factor IXa without affecting its reactivity with thrombin. Intriguingly, site-specific mutations in this region showed complex effects implying the existence of both favorable and unfavorable exosites in this region whose expression depended on heparin activation [30]. Notably, mutating two residues in strand 3C, Tyr253 and Glu255 that are highly conserved in vertebrate antithrombins but not in other serpins, closely duplicated the strand 3C chimera defect in factor Xa reactivity that was strictly dependent on heparin activation. However, mutating Tyr253 alone caused a comparable large loss in factor Xa reactivity but this loss was independent of heparin allosteric activation. These strand 3C mutations produced a similar pattern of losses in antithrombin reactivity with factor IXa that were dependent on heparin activation for the strand 3C chimera but did not depend on activation for single Tyr253 and Glu255 mutations. Such results have suggested a model of allosteric activation in which Tyr253 constitutes a key exosite residue that specifically interacts with factor Xa and factor IXa in both native and heparin-activated

states (Fig. 7) [9]. However, other residues in strand 3C produce unfavorable interactions that attenuate the favorable Tyr253 interaction in native antithrombin. According to this model, heparin activates antithrombin reactivity by relieving the unfavorable interactions. That Tyr253 and Glu255 cooperate as positive factor Xa and IXa-specific interaction exosites in heparin-activated antithrombin is supported by the finding that changing the strand 3C homologues of these residues in a P1 Arg variant of α_1-proteinase inhibitor to their antithrombin counterparts specifically enhances α_1-proteinase inhibitor reactivity with factors Xa and IXa, but not with thrombin [31].

Recent X-ray structures of Michaelis complexes of heparin-activated antithrombin with catalytic Ser-inactivated variants of factor Xa [32], factor IXa [33] and thrombin [34,35] support the importance of favorable exosite interactions identified by mutagenesis studies in mediating the enhanced reactivity of allosterically-activated antithrombin with factor Xa and factor IXa but not with thrombin (Fig. 7). These structures clearly show that factors Xa and IXa bound to the antithrombin RCL are able to engage the exosites on strand 3 of sheet C surrounding the RCL that were identified by mutagenesis studies. The complementary exosites on the proteinases involve a critical conserved Arg 150 residue in the autolysis loop that binds in a pocket formed by the antithrombin exosites on strand 3C [36,37]. Thrombin lacks the conserved Arg and therefore is not able to interact with the antithrombin exosites. Heparin allosteric activation thus minimally affects antithrombin reactivity with thrombin and the rate-enhancing effect of heparin is solely due to the ternary complex bridging mechanism.

Based on the X-ray structures of the Michaelis complexes showing the expected expulsion and extension of the RCL away from sheet A that is characteristic of heparin-activated antithrombin, it has been proposed that RCL expulsion is essential for bound factors Xa and IXa to engage the strand 3C exosites and that this is the key activating event required for allosteric activation [32,33]. One problem with this proposal is that it fails to account for the repulsive determinants in strand 3C that are inferred to repress antithrombin reactivity in the absence of heparin activation. The model of allosteric activation discussed above that accounts for this and much other data suggests instead that favorable exosite interactions are already engaged in native antithrombin but are diminished by unfavorable interactions. Activation in this case involves the mitigation of the unfavorable interactions, with RCL expulsion contributing to but not being required for such activation (Fig. 8). Much other data favor this latter activation model over the former. One key observation is that Cα-Cα distances between P1Arg and the Tyr253 exosite in free antithrombin are no greater than those in the heparin-antithrombin-factor Xa Michaelis complex, implying that factor Xa bound to the RCL is just as accessible to the strand 3C exosites in the RCL-inserted native state as it is in the RCL-expelled activated state of the serpin. Additionally, the X-ray structure of a fluorescein-derivatized RCL hinge variant of antithrombin that is activated without the need for heparin shows the RCL backbone inserted into sheet A as in native antithrombin, implying that RCL expulsion and extension away from sheet A is not a requirement for activation and that alteration of the surface electrostatics may be the dominant activation mechanism [38]. Recent evidence provides additional support for the model by showing that mutation of a buried Tyr131 in the C-terminal loop of helix D to Leu results in a pentasaccharide-independent partial activation of antithrombin reactivity with factor Xa without inducing the spectroscopic signature of RCL expulsion from sheet A, presumably through rearrangements in the hydrophobic core [39]. Moreover, a Lys133 to Pro mutation that blocks helix D extension shows a pentasaccharide-dependent partial allosteric activation of antithrombin without the spectroscopic changes that report RCL expulsion [24]. Together, these findings support the model in which factor Xa and factor IXa interact with both the RCL and the strand 3C exosites in native antithrombin, but unfavorable interactions result in a repressed reactivity with these proteinases. Allosteric activation by heparin pentasaccharide enhances reactivity by changing the surface electrostatics so as to mitigate the unfavorable interactions and retain the favorable

RCL and exosite interactions. Although recent reports show that blocking RCL expulsion from sheet A by an engineered disulfide abrogates heparin pentasaccharide activation of antithrombin [40], the disulfide may have global effects that rigidify the allosteric core and block the activating changes observed in the Tyr131 and Lys133 mutants. Further reconciliation of these models of allosteric activation will require X-ray structures of unactivated antithrombin-factor Xa/IXa Michaelis complexes as well as mutagenesis studies that identify those antithrombin residues responsible for the proposed unfavorable interactions with factors Xa and IXa.

Antithrombin residues involved in binding the heparin pentasaccharide

Other studies have focused on delineating the mechanism of heparin pentasaccharide binding to antithrombin and the activating conformational changes induced in the heparin binding site by this binding. Notably, mutagenesis of the antithrombin basic residues implicated in pentasaccharide binding by the X-ray structure have revealed that three of these residues, Lys125 and Arg129 in helix D and Lys114 in the P helix preceding helix D, represent binding hotspots since mutation of any one of these residues causes major losses in binding energy much greater than would be expected from loss of a single ionic interaction (Fig. 6) [41-43]. By contrast, Lys11 and Arg13 in the N-terminal region and Arg46 and Arg47 in helix A, make lesser contributions to binding affinity since mutation of any one of these residues causes a loss of binding energy that in most cases approximates the loss of a single ionic interaction [44,45]. The three binding hotspot residues must therefore bind the pentasaccharide cooperatively. Recent work examining the binding energy losses resulting from single, double or triple mutations of the binding hotspot residues suggest a strong interdependence of Lys125 and Arg129 interactions that accounts for their cooperativity but an independence of Lys114 interactions implying that Lys114 cooperates with other basic residues [46]. Interestingly, all but Arg46, whose mutation causes the smallest decrease in heparin binding affinity, are highly conserved in the thirteen vertebrate sequences of antithrombin that are known, representing all five vertebrate families [47].

Analysis of the ionic strength dependence of heparin pentasaccharide binding to antithrombin reveals that not only ionic interactions but also significant nonionic interactions make important contributions to the binding affinity [17]. A conserved triplet of Phe residues in helix D appears to be responsible for much of the nonionic binding energy due to the indirect role of at least two of these residues in stabilizing the activated conformation [48]. Proton NMR spectroscopic changes accompanying pentasaccharide activation of antithrombin support the idea that these and other aromatic residues comprise an allosteric communication network in the hydrophobic core through which structural changes in the heparin binding site are transmitted to the proteinase binding region [49]. Arg24 in the N-terminal region and Trp49 in helix A play similar indirect roles in heparin activation by making critical new intramolecular interactions in the activated state that stabilize this state [45,50].

Induced-fit model of allosteric activation

Insight into the dynamics of heparin pentasaccharide binding and allosteric activation of antithrombin have come from rapid kinetic studies of the binding interaction [17,51]. Such studies have shown that binding is a two-step process with an initial weak binding of heparin to antithrombin followed by the activating conformational changes in the protein that enhance binding affinity ~1000-fold and that are reported by CD, near UV and tryptophan fluorescence changes. Notably, only Lys125, and to a lesser extent Lys11, contribute to the first weak heparin binding step whereas all other residues, including the key Lys114 and Arg129 binding residues, interact only after the second conformational activation step [16]. The interactions of Lys125 and Lys11 are also augmented after conformational activation. These findings indicate that

heparin pentasaccharide binding and allosteric activation of antithrombin is driven by an induced-fit mechanism wherein activation produces a complementary fit between antithrombin and the pentasaccharide that greatly enhances pentasaccharide binding affinity and overcomes the energetic barrier for transforming antithrombin into the less favored activated conformation (Fig. 9). Notably, an alternative mechanism suggesting that activation is driven by heparin neutralizing positive charge in the heparin binding site has been refuted by the observation that replacing the positively charged residues with neutral or negatively charged residues fails to activate antithrombin basal reactivity with factor Xa or factor IXa [52].

Parallel studies of the interaction of truncated pentasaccharide variants with antithrombin have confirmed and extended the induced-fit model of allosteric activation of the serpin [53]. Such studies have revealed that the nonreducing end trisaccharide of the pentasaccharide is the key unit that functions to recognize and bind native antithrombin and produce an induced-fit conformational activation of the serpin (Fig. 9). However, the trisaccharide shows a greatly reduced ability to stabilize the induced-fit activated state, implying that the reducing end disaccharide provides much of the binding energy for this stabilization. By contrast, removal of a single saccharide from the nonreducing end of the pentasaccharide blocks the induced-fit pathway of antithrombin activation but allows conformational activation through an alternative less efficient pre-equilibrium pathway. In this pathway the saccharide selectively binds a minor pre-equilibrium fraction of activated antithrombin and shifts the pre-equilibrium toward the activated conformation at a rate limited by the slow conversion of the native serpin to the activated form. The pentasaccharide thus contains structural features that allow recognition and binding of both native and activated states but with a considerable binding preference for the activated state so as to favor the induced-fit pathway of activation.

More recent studies have highlighted the role of a unique 3-O-sulfate group on the central saccharide of the pentasaccharide sequence that is a hallmark of this sequence in heparin and heparan sulfate chains [54]. Remarkably, deletion of the 3-O-sulfate results in a massive binding energy loss of ~60% that is greater or comparable to the losses produced by deleting either the reducing end disaccharide or the single nonreducing end saccharide. Nevertheless, the 3-O-desulfated pentasaccharide is able to fully activate antithrombin at saturation and thus retains a preference for binding activated over native antithrombin although this preference is modest. The 3-O-sulfate interacts with Lys114 in the X-ray structure of the antithrombin-pentasaccharide complex (Fig. 6) [21], implying that this interaction plays a pivotal role in heparin binding and allosteric activation. While it might be expected that loss of either partner of this interaction would yield similar binding and activation defects, rapid kinetic studies show that this is not the case. The 3-O-sulfate but not Lys114 thus makes an important contribution to the first step binding of the pentasaccharide to native antithrombin, whereas Lys114 is critical for inducing pentasaccharide-bound native antithrombin into the activated state without the need for the 3-O-sulfate. Formation of the P helix in which Lys114 resides is likely to be the critical structural change that drives this activation. Once the P helix is formed, Lys114 can engage the 3-O-sulfate and other sulfate/carboxylate groups to stabilize the activated antithrombin conformation. This engagement appears critical for additional stabilizing interactions to be made between the reducing end disaccharide and the basic residues, Arg13, Arg46, and Arg47, since elimination of either the Lys114-3-O-sulfate interaction or the reducing end disaccharide causes comparable losses in the binding energy that stabilizes the activated conformation. Arg13, Arg46 and Arg47 interactions with the pentasaccharide thus appear dependent on Lys114 interactions and explain the cooperativity between these interactions.

The finding that the 3-O-sulfate is not essential for conformational activation has suggested that the impaired conformational activation of antithrombin by low-affinity heparins lacking the 3-O-sulfate must arise from some other structural difference. This difference may be the

rare glucuronate residue of the minimal trisaccharide activating unit of the pentasaccharide, since replacing the glucuronate with the more common iduronate-2-O-sulfate found in heparin replicates the impaired activating effect of low-affinity heparins lacking the pentasaccharide sequence (Fig. 4) [53].

Evidence for a 3-step mechanism of allosteric activation

Further molecular dissection of the allosteric activation mechanism has come from recent studies of heparin pentasaccharide binding to conformationally altered latent and cleaved forms of antithrombin that have reduced affinity for the pentasaccharide [55]. Both latent and cleaved forms have undergone the massive conformational change induced by proteinase reaction in which the RCL has fully inserted into sheet A, the latent form differing from the cleaved form in having undergone this conformational change spontaneously without RCL proteolysis because of the greater thermodynamic stability of the RCL-inserted state [56]. Surprisingly, the kinetics of heparin pentasaccharide binding to the latent and cleaved antithrombin forms reveal a two-step induced-fit binding mechanism that is strikingly similar to the mechanism of pentasaccharide binding to native antithrombin. Thus, the affinity of the initial binding step and rate constant for the subsequent induced-fit conformational activation step are indistinguishable for pentasaccharide binding to native and latent/cleaved forms of antithrombin. The differences in the binding kinetics arise solely from large differences in the rate constant for reversal of the induced-fit conformational change, this rate constant reflecting the off-rate constant for pentasaccharide dissociation from activated antithrombin.

That a set of shared induced-fit structural changes accompany pentasaccharide binding to native and latent/cleaved antithrombin forms is supported by X-ray structures of free and heparin pentasaccharide complexes of latent antithrombin (Fig. 10) [20,21]. These structures were obtained from the corresponding crystal structures of native antithrombin since antithrombin crystallizes as a dimer between native and latent forms. In the pentasaccharide complex structures, latent antithrombin is bound to the pentasaccharide at the same site and with similar contacts as native antithrombin. Moreover, many of the induced-fit conformational changes in the heparin binding site of native antithrombin are paralleled by similar changes in latent antithrombin, including rotation of helix D, formation of the P helix at the N-terminal end of helix D and the repositioning of basic residues for interaction with pentasaccharide groups. Thus, the pentasaccharide produces a similar induced-fit when it binds to both native and latent antithrombin structures and this presumably accounts for the observed similarity of the induced-fit kinetic mechanisms of pentasaccharide binding to both antithrombin forms. Absent from the latent antithrombin structure are the long-range conformational changes induced by pentasaccharide binding to native antithrombin including sheet A contraction and RCL expulsion from the sheet.

An additional important difference between the native and latent structures within the heparin binding site is that pentasaccharide binding induces the C-terminal extension of helix D in native antithrombin but not in latent antithrombin. This difference presumably reflects the linkage between helix D extension and the contraction of sheet A in native antithrombin that is responsible for RCL expulsion from the sheet. No contraction of sheet A or expulsion of the RCL is possible in latent antithrombin because the RCL is fully inserted into the sheet and this insertion is essentially irreversible. The inability of latent antithrombin to undergo helix D extension and its correlation with a faster off-rate constant for pentasaccharide dissociation from antithrombin implies that helix extension produces a better induced-fit of the pentasaccharide with native antithrombin than is possible with the latent form. This is supported by the finding that the bonding distances for Lys125 and Arg129 in the latent antithrombin complex are several Ångströms longer than they are in the native antithrombin complex [57]. Newer X-ray structures of the native antithrombin-pentasaccharide complex provide additional

support for this view by showing that the complex may exist in an intermediate state in which the structural changes in the heparin binding site have occurred except for helix D extension and the proteinase binding site changes involving the closing of sheet A and RCL expulsion from the sheet have not taken place [40,58], i.e., the changes resemble those observed in the latent antithrombin-pentasaccharide complex (Fig. 10). Interestingly, the mutant antithrombin in which Lys133 in the C-terminal extension of helix D is replaced by a Pro to block helix D extension shows an affinity and kinetics of pentasaccharide binding that differ from native antithrombin only in the off-rate constant in exactly the same manner as the interaction with latent antithrombin [24]. Unlike the latent antithrombin interaction, the Lys133Pro variant is partially activated by pentasaccharide binding, implying that the conformational changes induced in the heparin binding site of the variant, and presumably also the intermediate, involve activating changes in the proteinase binding site that do not require helix D extension and RCL expulsion from sheet A as discussed earlier.

Together, these observations suggest that the activating conformational changes induced by the heparin pentasaccharide in native antithrombin occur in two stages in an overall 3-step binding and activation mechanism (Fig. 9) [40]. The first stage conformational changes are induced following the binding of pentasaccharide in the initial two steps of the 3-step mechanism and involve the heparin binding site as well as partially activating changes in the proteinase binding site. These changes resemble those induced in the antithrombin intermediate structure and those induced in the heparin binding site of latent antithrombin. The second stage conformational changes involve additional changes in the heparin binding site and the proteinase binding region that are unique to native antithrombin. Simulations of such a three-step mechanism in which the second conformational change is assumed to be much faster than the first (to account for the similar rates of conformational change caused by heparin binding to native and latent antithrombins) and the parameters are constrained so that the overall binding affinities agree with measured values indicate binding kinetics that are indistinguishable from a two-step mechanism [57].* This supports the 3-step mechanism for heparin binding to native antithrombin and implies that the two successive conformational changes are driven by progressive induced-fits that increase heparin affinity and lock the serpin in the activated state and thus account for the reduced heparin off-rate.

Model for allosteric activation of antithrombin by heparin

Collectively, the accumulated evidence suggests the following detailed molecular model for allosteric activation of antithrombin by the heparin pentasaccharide (Fig. 9). The nonreducing end trisaccharide unit of the pentasaccharide initially recognizes and binds antithrombin through electrostatic interactions involving Lys125 and Lys11 of the serpin and the 3-O-sulfate and other critical sulfates and carboxylates of the trisaccharide. This binding causes a first set of induced-fit conformational changes in the heparin binding site that involve rotation of helix D to better position Lys125 and Arg129 in the helix and Lys11 in the N-terminus for interactions with the trisaccharide. They also include the formation of the P helix at the N-terminal end of helix D that positions Lys114 for interaction with the 3-O-sulfate and allows the reducing end disaccharide to bind through interactions with Lys114 as well as with Arg46, Arg47 and Arg13. The repositioning of Lys125 and Arg129 results in improved but still sub-optimal interactions with the nonreducing end trisaccharide in this site. This first set of conformational changes partially activates antithrombin reactivity with factors Xa and IXa by causing a rearrangement of the allosteric core similar to that produced either by mutating buried Tyr131 to Leu or by pentasaccharide activation of a Lys133Pro variant that blocks the second

*Because the second set of conformational changes occur so much faster than the first, the two conformational changes can be thought of as concerted events. However, it is clear that these changes can be uncoupled in native antithrombin either through crystal packing forces in the intermediate structure or through mutations such as the Lys133Pro variant.

set of activating changes (helix D extension and RCL expulsion). The changes in the allosteric core alter the electrostatics of the surface around the RCL so as to partly alleviate unfavorable interactions with bound factors Xa or IXa and strengthen favorable exosite interactions between Tyr253 of antithrombin and Arg150 of these proteinases. A second set of induced-fit conformational changes then results in the extension of helix D in the heparin binding site and shifts in the positions of Lys125 and Arg129 to further enhance the affinity of these interactions and increase the stability of the activated state. Helix D extension augments the activation of antithrombin reactivity with factor Xa and factor IXa by exposing the buried Tyr131 sidechain and compacting the hydrophobic core, causing sheet A to close and the RCL to be expelled from the sheet. This causes further changes in the serpin surface electrostatics around the RCL and allows the bound proteinase to extend away from sheet A so as to fully alleviate negative interactions with the serpin body and further enhance positive exosite interactions.

Concluding remarks

Antithrombin circulates in the bloodstream in a repressed reactivity state as a result of the serpin RCL providing only the minimal specificity determinants for recognizing the three main target proteinases, thrombin, factor Xa and factor IXa, and because favorable exosite interactions with the latter two proteinases are downregulated by unfavorable interactions. Binding to heparin or heparan sulfate molecules of the luminal and subluminal blood vessel walls activate antithrombin reactivity with its three target proteinases through allosteric changes that alleviate the unfavorable interactions with factor Xa and IXa and strengthen exosite interactions with these proteinases. The glycosaminoglycans also act as bridging cofactors to provide additional exosites for binding proteinases next to bound antithrombin so as to augment antithrombin-proteinase interactions. Antithrombin has thus evolved to regulate the activity of multiple blood clotting cascade proteinases with only a minimal P1 Arg RCL determinant as bait by acquiring proteinase binding exosite determinants on the serpin and by exploiting proteinase binding exosite determinants on heparin.

The reason for what seems to be an overly complex design appears two-fold. First, it serves to repress antithrombin reactivity until it is needed either at the blood vessel wall to prevent surface-mediated activation of blood clotting proteinases or at an injury site to control and localize blood clotting proteinase activity. Second, it acts to prevent antithrombin reactivity with another anticoagulant serine proteinase, activated protein C [59]. This critical anticoagulant proteinase is activated by thrombin in a feedback loop for the purpose of shutting down thrombin formation. This is accomplished by activated protein C proteolytically inactivating the nonenzymatic cofactor proteins, factors Va and VIIIa, that are essential for thrombin formation. Since activated protein C cooperates with antithrombin to regulate hemostasis by preventing unwanted activation of blood coagulation, it would be counterproductive for antithrombin to inhibit activated protein C. Although activated protein C recognizes the P1 Arg bait of antithrombin, it is in fact inactivated by heparin-activated antithrombin at a nonphysiologic rate that is seven orders of magnitude slower than the rate at which target proteinases are inactivated [60]. This greatly compromised reactivity appears to arise from an unfavorable RCL sequence since it can be closely replicated when the antithrombin RCL is put into an α_1-proteinase inhibitor scaffold with a P1 Arg [60]. Antithrombin has thus evolved to selectively regulate procoagulant blood clotting proteinases in a manner that provides low reactivity until high reactivity is needed at vascular and extravascular sites where blood clotting proteinases may be activated and heparan sulfate or heparin are present. The need for such a complex mode of proteinase regulation may explain nature's exploitation of the serpin fold and its multiple conformational states for regulating proteinase cascades in numerous physiologic processes. Indeed, serpins such as heparin cofactor II and ZPI control their target proteinases at specific sites of action through cofactors

that act to relieve a repressed reactivity state of the serpin much like the paradigm of heparin regulation of antithrombin reactivity [8].

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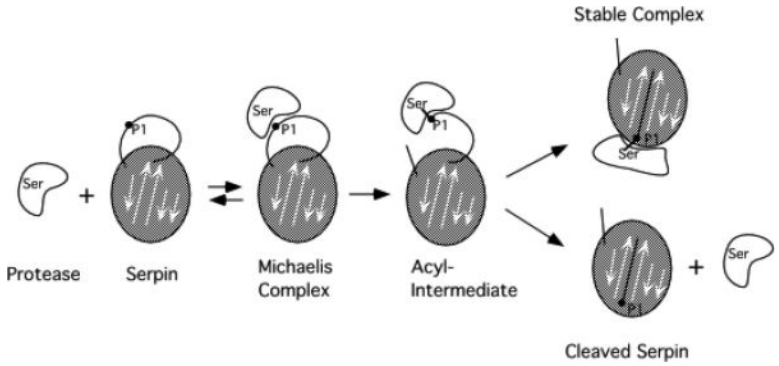

Fig. 1. Branched pathway suicide substrate mechanism by which serpins inhibit serine and cysteine proteinases

The pathway begins with the proteinase active-site recognizing and binding to a substrate sequence contained within an exposed reactive center loop (RCL) of the serpin (gray-shaded) to form a Michaelis complex. The active-site serine or cysteine of the proteinase proceeds to attack a reactive P1-P1' bond in the serpin RCL sequence to form an acyl-intermediate complex in which the bond is cleaved and the P1 residue has become covalently linked to the attacking serine or cysteine. RCL cleavage triggers the suicide mechanism by inducing the metastable serpin to undergo a large conformational change in which the RCL inserts into the center of β-sheet A (shown as arrows), causing the tethered proteinase to be dragged to the opposite end of the protein and inactivated by conformational deformation. The trapped acyl-intermediate complex is kinetically stable and deacylates extremely slowly. A normal deacylation of the acyl-intermediate competes with conformational trapping to release active proteinase and cleaved serpin.

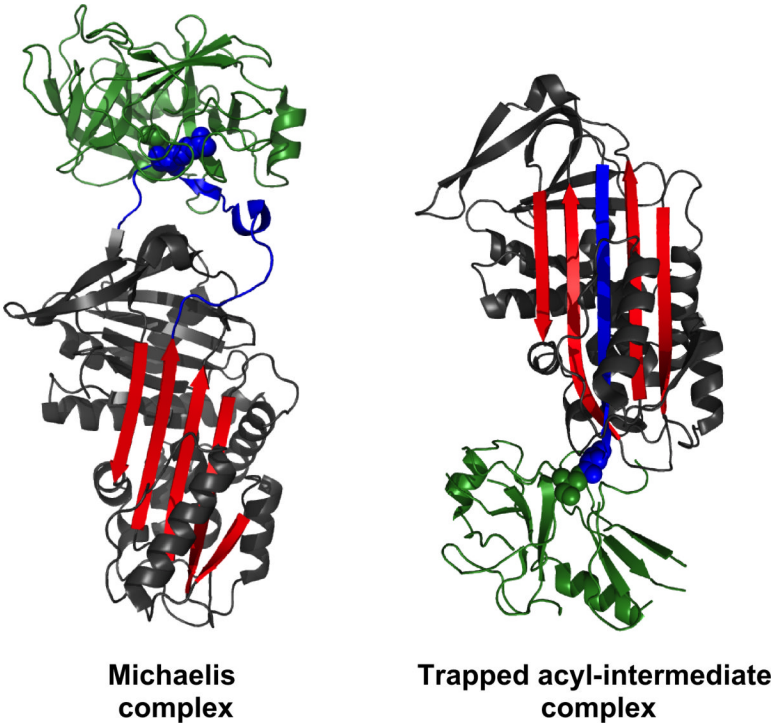

Fig. 2. X-ray structures of the intermediate serpin-proteinase Michaelis complex and the final trapped acyl-intermediate complex

Ribbon representations of the Michaelis complex of a P1Arg variant of the serpin, α_1-proteinase inhibitor, with catalytically inactivated S195A trypsin on the left (pdb 1OPH) and the trapped acyl-intermediate complex of wild-type α_1-proteinase inhibitor (P1Met) with porcine pancreatic elastase on the right (pdb 1EZX). The proteinase is depicted in green and the serpin in gray with the A-sheet highlighted in red, the RCL in blue and the serpin P1 residue and proteinase catalytic serine shown in space-filling representation.

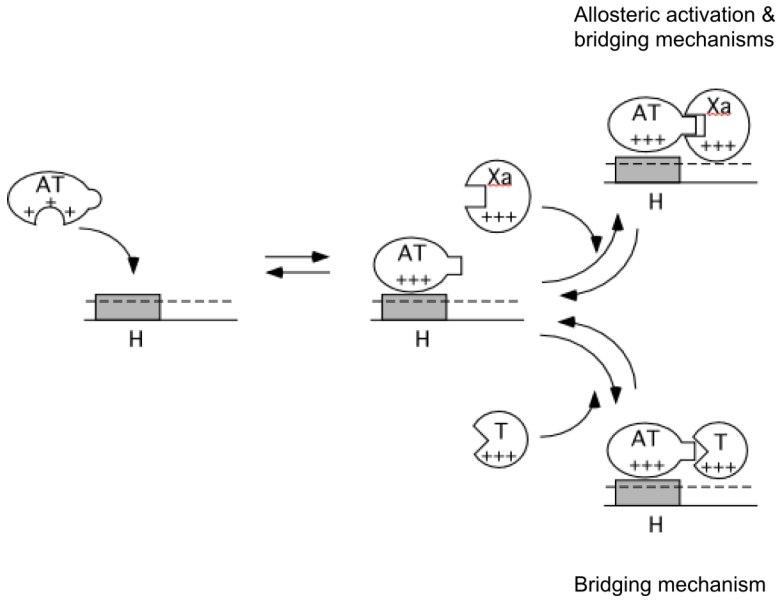

Fig. 3. Mechanisms of heparin activation of antithrombin

The scheme depicts two mechanisms by which heparin or heparan sulfate glycosaminoglycans activate antithrombin to rapidly inhibit blood clotting proteinases. In both mechanisms a positively charged site on antithrombin (AT) binds to a sequence-specific negatively charged pentasaccharide (shaded) on the heparin or heparan sulfate polysaccharide (H). This binding induces an activating conformational change in the heparin binding site of the serpin that is allosterically transmitted to the proteinase binding region. The allosteric changes specifically activate antithrombin reactivity with factors Xa and IXa by relieving unfavorable interactions and strengthening favorable interactions of these proteinases with the serpin RCL and exosites. The allosteric mechanism minimally affects antithrombin reactivity with thrombin since thrombin does not interact with the factor Xa and IXa-specific exosites. Longer chain heparin or heparan sulfate glycosaminoglycans activate antithrombin reactivity with thrombin and further augment antithrombin reactivity with factors Xa and IXa through a second bridging mechanism in which positively charged sites on the proteinase bind to the negatively charged polysaccharide at an exosite adjacent to bound antithrombin so as to position the proteinase for engagement of the serpin RCL.

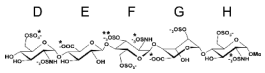

Fig. 4. Structure of the antithrombin binding pentasaccharide sequence of heparin and heparan sulfate

The O-methyl glycoside of the pentasaccharide sequence is shown from the nonreducing end to the reducing end with sulfates and carboxylates that are important determinants of pentasaccharide binding to antithrombin labeled with an asterisk. The unique 3-O-sulfate marker of the pentasaccharide sequence is indicated by a double asterisk. The saccharides are designated DEFGH based on the historical record of discovery of the sequence [15]. The dominant structure of heparin and of certain highly sulfated domains of heparan sulfate is a polymer of repeating GH disaccharide units.

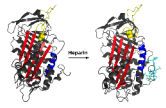

Fig. 5. X-ray structures of free and heparin pentasaccharide-complexed antithrombin

Ribbon representations of free antithrombin on the left (pdb 1E05) and heparin pentasaccharide complexed antithrombin on the right (1E03) reveal the activating conformational changes induced in antithrombin by the binding of the pentasaccharide (a mimetic shown in cyan stick representation). These include an extension of helix D and formation of a new P helix in the heparin binding site and an expulsion of the P14 serine residue (space-filling) of the serpin RCL (yellow), initially buried in β-sheet A (red) in free antithrombin, from the A sheet and closing of the gap in the A sheet.

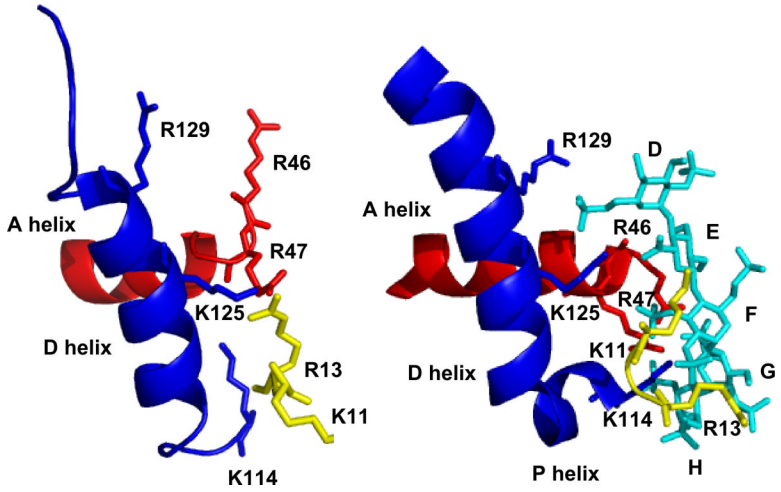

Fig. 6. Closeup of the heparin binding site of free and heparin pentasaccharide-complexed antithrombin structures

Highlighted in ribbon representation are the three regions of antithrombin that comprise the heparin binding site: helix D and the loops that extend from its C- and N-terminal ends (blue), helix A (red) and the N-terminal region (yellow). Residues involved in binding the pentasaccharide are shown in stick representation and include Lys11, Arg13, Arg46, Arg47, Lys114, Lys125 and Arg129. The pentasaccharide is represented in stick (cyan). Conformational changes induced in antithrombin by pentasaccharide binding include an extension of helix D at the C-terminal end, formation of a new P helix in the loop preceding the N-terminal end of helix D and rotations of the three heparin binding regions to position basic residues for binding the critical sulfates and carboxylates of the pentasaccharide.

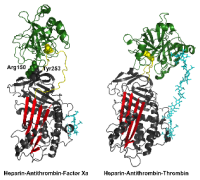

Fig. 7. X-ray structures of heparin-antithrombin-proteinase Michaelis complexes

Shown in ribbon representation are the structures of ternary Michaelis complexes of heparin pentasaccharide-antithrombin with S195A factor Xa on the left (pdb 2GD4) and heparin hexadecasaccharide-antithrombin with S195A thrombin on the right (1TB6). The hexadecasaccharide contains a pentasaccharide mimetic, an uncharged saccharide linker and five terminal sulfated glucose saccharides. Heparin molecules are depicted in cyan (stick), proteinases in green and antithrombin in gray. The A-sheet is highlighted in red and the RCL in yellow. The critical Tyr253 exosite residue in strand 3C of antithrombin and the complementary Arg150 exosite residue of factor Xa as well as the P1 Arg are shown in space-filling representation. The structures reveal distinct orientations of the proteinase bound to the serpin RCL, with factor Xa bending downward toward the serpin body to form the critical exosite-exosite interaction and thrombin extending away from the serpin surface and bending in the opposite direction to interact with a heparin exosite on the extended polysaccharide chain.

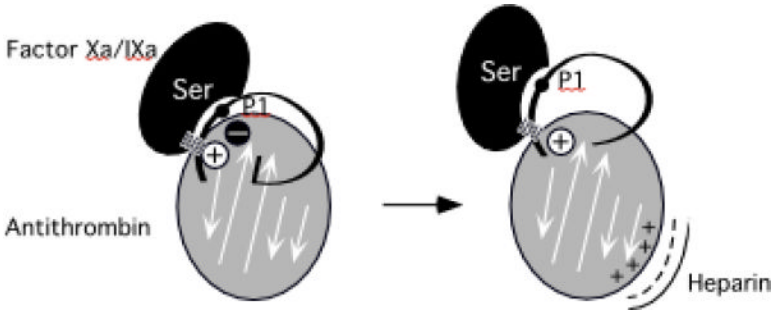

Fig. 8. Model for allosteric activation of antithrombin reactivity with factor Xa and IXa

Antithrombin is proposed to exist in a repressed reactivity state as a result of unfavorable interactions (symbolized by the black circled minus sign) that mitigate favorable interactions of an exosite (symbolized by the white circled plus sign) and the RCL with factors Xa and IXa (hatched rectangle denotes the favorable exosite interaction). This mitigation is exacerbated by the burial of the RCL hinge in sheet A (shown as arrows) which causes the bound proteinase to closely approach the serpin surface. Heparin releases antithrombin from its repressed reactivity state by inducing allosteric activating changes that diminish the negative interactions but retain the positive exosite interactions. The unfavorable interactions with proteinase are reduced both by changes in the serpin surface electrostatics and by extension of the RCL away from the serpin surface.

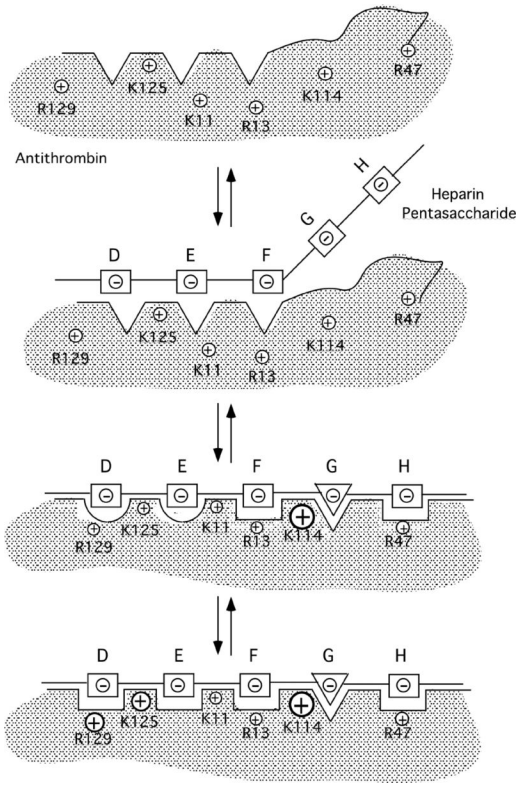

Fig. 9. Induced-fit model for heparin pentasaccharide binding to and allosteric activation of antithrombin

The pentasaccharide is proposed to bind and allosterically activate antithrombin in 3-steps. ln step one, the conformationally rigid nonreducing end DEF trisaccharide recognizes and binds to the D helix and N-terminal segments of the heparin binding site of antithrombin primarily through electrostatic interactions involving Lys125 and Lys11. The conformationally flexible GH disaccharide, the consequence of an equilibrium between chair and skew boat forms of iduronate residue G, minimally interacts in this step [16]. DEF binding triggers a first set of induced-fit conformational changes in the heparin binding site in step two that include formation of the P helix, rotation of the D helix and bending of the A helix. These changes allow the critical Lys114 interaction with the pentasaccharide 3-O-sulfate to be made and serve to position Lys114 together with Arg13, Arg46, and Arg47 for binding the reducing end GH disaccharide in the skew boat form (triangle). The changes additionally improve the DEF trisaccharide interactions with Lys11, Lys125 and Arg129. A second set of induced-fit conformational changes follows in step three in which the D helix extends at its C-terminal end, causing the buried Tyr131 to become exposed and the hydrophobic core to compact. Such changes increase pentasaccharide complementarity with the heparin binding site by repositioning Lys125 and Arg129 for optimal interactions with the DEF trisaccharide. The relative importance of each basic residue in binding the pentasaccharide in each step is reflected by the size of the positive charge symbols for each residue.

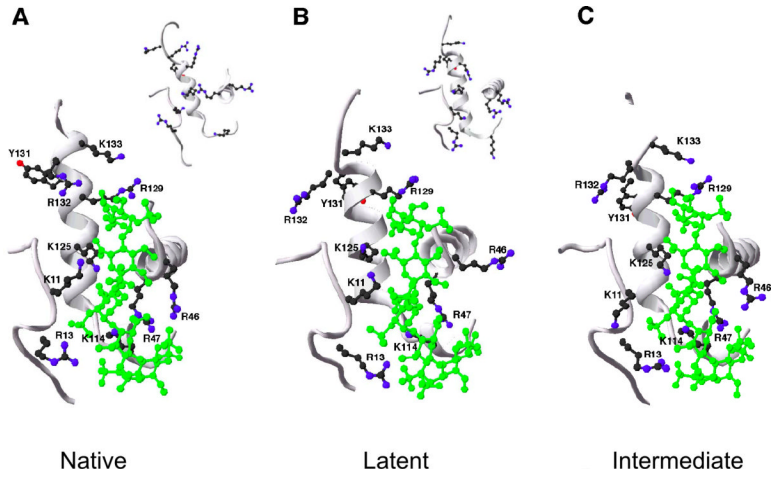

Fig. 10. Comparison of the structures of the antithrombin-heparin pentasaccharide complex in native, latent and intermediate forms

A closeup of the D- and P-helices and parts of the N-terminal region and the A-helix of the heparin binding site of native antithrombin (A), latent antithrombin (B) and an intermediate native form (C) in complex with a heparin pentasaccharide mimetic (large images, pdb codes 1E03 and 1NQ9) and in the unbound state (inset, pdb code 1E05) are shown in ribbon representation (gray). Antithrombin basic residues that participate in binding the heparin pentasaccharide as well as Tyr131, Arg132 and Lys133 in the C-terminal extension of helix D are shown in ball-and-stick representation. Carbon atoms are in black, nitrogen atoms are blue and oxygen atoms are red. The pentasaccharide atoms are shown in green ball-and-stick. The latent and intermediate antithrombin-pentasaccharide complex structures show similar induced-fit conformational changes including P helix formation, D helix rotation and A helix bending but have not undergone helix D extension and the flipping of Tyr131 that is evident in the native antithrombin structure.