Heparin-dependent Modification of the Reactive Center Arginine of Antithrombin and Consequent Increase in Heparin Binding Affinity*

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Antithrombin, the principal plasma inhibitor of coagulation proteases, circulates in a form with low inhibitory activity due to partial insertion of its reactive site loop into the A-β-sheet of the molecule. Recent crystallographic structures reveal the structural changes that occur when antithrombin is activated by the heparin pentasaccharide, with the exception of the final changes, which take place at the reactive center itself. Here we show that the side chain of the P1 Arg of α-antithrombin is only accessible to modification by the enzyme peptidyld arginine deiminase on addition of the heparin pentasaccharide, thereby inactivating the inhibitor, whereas the natural P1 His variant, antithrombin Glasgow, is unaffected, indicating that only the P1 Arg becomes accessible. Furthermore, the deimination of P1 Arg converts antithrombin to a form with 4-fold higher affinity for the heparin pentasaccharide, similar to the affinity found for the P1 His variant, due to a lowered dissociation rate constant for the antithrombin-pentasaccharide complex. The results support the proposal that antithrombin circulates in a constrained conformation, which when released, in this study by perturbation of the bonding of P1 Arg to the body of the molecule, allows the reactive site loop to take up the active inhibitory conformation with exposure of the P1 Arg.

The plasma serpin (1), antithrombin, is the major inhibitor of the serine proteinases of the coagulation network, especially thrombin and factor Xa (2). This inhibitory activity is stimulated by the complexing of antithrombin with the sulfated polysaccharide, heparin. In particular, the activity against factor Xa is mediated by a core pentasaccharide component of heparin. Antithrombin has an initial low affinity for heparin, but this immediately changes to a high affinity on initial binding, the change occurring concomitantly with the activation of inhibitory function (3).

The interaction of the heparin pentasaccharide with antithrombin and the associated mechanism of conformational activation of inhibition has recently been revealed in the crystal structure of a dimer of antithrombin complexed with the pentasaccharide (4). Linkage between the two antithrombin molecules in the dimer directly involves the reactive site loop of the inhibitory component, with a consequent constraint in the movement at its reactive site. Hence, while showing the commencement of the movement of the reactive site loop that results in activation, the crystal structure does not show the change that occurs at the reactive center itself.

There is, however, a good model of the likely unconstrained active conformation of the reactive site loop of antithrombin. This is provided by the structure of the closely related serpin, α1-antitrypsin (5), in which the loop is fixed in the optimal canonical inhibitory conformation present in all other families of serine protease inhibitors. The transition of antithrombin to this conformation would require a shift of the side chain of the P1 arginine from an internally oriented position where it is hydrogen-bonded to the body of the molecule (6), to an external and exposed orientation, as found for the P1 residue of α1-antitrypsin. Furthermore, the perturbation of the bonding of the side chain of P1 Arg in antithrombin, as occurs in the natural variant antithrombin Glasgow (P1 Arg to His), is accompanied by a shift from low to high affinity binding of heparin (7). This has led to the proposal (6–8) that antithrombin is, in part, held in a conformation with initial low heparin affinity by the constraints on reactive site loop movement imposed by the internal bonding at the side chain of P1 Arg.

To check these proposals, normal antithrombin and the Glasgow P1 Arg variant were each incubated, with and without the heparin pentasaccharide, with the enzyme peptidyld arginine deiminase (hereafter referred to as deiminase) (EC. 3.5.3.15), which acts on the side chain of arginine residues to produce the amino acid citrulline. We show that this enzyme interacts with the P1 Arg side chain, which is only vulnerable to modification in the presence of the heparin pentasaccharide. The deimination of the P1 Arg converts antithrombin to a form with higher affinity for the heparin pentasaccharide.

EXPERIMENTAL PROCEDURES

Materials

Peptidyld arginine deiminase was purchased from PanVera Corp., Madison, WI. Heparin pentasaccharide was a gift from Maurice Petiotou, Sanofi Recherche, Toulouse, France. Thrombin was a gift from Professor Stuart Stone, Monash University, Melbourne, Australia. Human neutrophil elastase was prepared as described previously (9). Factor Xa was purchased from Boehringer Mannheim (Lewes, UK). The chromogenic substrate S2238 was purchased from Quadrachem (Ep-

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som, UK). Plasma from a patient containing the variant antithrombin Glasgow was a gift from Dr. Isobel Walker, Glasgow, UK. Ampholine PAGplates for isoelectric focusing and Superdex 75 were purchased from Pharmacia Biotech Inc. (St. Albans, UK).

**Methods**

**Purification of Antithrombins**—Normal α-antithrombin was purified from time-expired, slow bleed units of human plasma as described previously (10), using a combination of heparin-Sepharose and anion exchange chromatography. Antithrombin Glasgow was purified as described (7), with the high affinity variant eluting later on the salt gradient from heparin-Sepharose, distinct from the peak for normal α-antithrombin. The forms of antithrombin were pure as judged by PAGE (data not shown).

**Treatment of Antithrombin with Deiminase**—Antithrombin (10 μM) was incubated with the deiminase at 37 °C at a molar ratio of 50:1 (inhibitor:enzyme) in 100 mM Tris-HCl, 5 mM CaCl₂, pH 7.4, for up to 16 h in the presence or absence of heparin pentasaccharide (50 μM). The reaction was stopped using 50 mM EDTA. The deaminated antithrombin was separated from both the heparin pentasaccharide and the deiminated using gel filtration chromatography on a Superdex-75 column (1.6 × 35 cm = 70 ml) in 50 mM Tris-HCl, 2 mM NaCl, 10 mM citrate, 5 mM EDTA, pH 7.4. The eluted antithrombin was concentrated using centrifugal ultrafiltration on a 10-kDa molecular mass cutoff device and buffer-exchanged into 50 mM Tris-HCl, 10 mM citrate, 5 mM EDTA, pH 7.4.

**Cleavage of Antithrombin—**Antithrombin (control and deaminated) at 4 mg/ml in 20 mM Tris-HCl, pH 8.0, was incubated with heparin pentasaccharide (50 μM) for 5 min at 37 °C, following which 120 ng of human neutrophil elastase was added. The mixture was allowed to incubate for 1 h at 37 °C, following which an equal volume of reducing SDS-PAGE sample buffer was added and the whole boiled for 5 min. SDS-PAGE was run on 10–20% Tris-Tricine gels (11), following which SDS-PAGE sample buffer was added and the whole boiled for 5 min. SDS-PAGE was run on 10–20% Tris-Tricine gels (11), following which proteins were electroblotted onto polyvinylidene difluoride membranes (12). The band corresponding to the low molecular mass subunit of cleaved antithrombin was sequenced by Alta Biosciences, Birmingham, UK.

**Activity Measurements**—Antithrombin (200 nM) was incubated with thrombin (5 nM) for 30 min at 37 °C, following which residual activity was measured against 40 μM S2238 substrate, all in 20 mM NaH₂PO₄, 250 mM NaCl, 0.1 mM EDTA, 0.1% (v/v) polyethylene glycol 8000, pH 7.4 (buffer with ionic strength of 0.3 (I₀.3 buffer)). The time of incubation and concentration of antithrombin were chosen to ensure that normally active antithrombin should inactivate thrombin to completion at the end of the incubation period. This criterion was seen to be fulfilled for control samples.

**Determination of EquilibriumBinding Constants and Rapid Kinetics for the Interaction between Antithrombin and Heparin Pentasaccharide**—The equilibrium binding constants for the interaction between the antithrombins and the heparin pentasaccharides were determined by fluorometric titrations in 10.3 buffer as described (13). The rapid kinetics of heparin pentasaccharide binding to antithrombin in 10.3 buffer were determined using stopped flow fluorometry essentially as described (3) (3).

**Electrophoresis and Isoelectric Focusing**—Polyacrylamide gel electrophoresis was carried out on 7.5% Tris/glycine gels (14). Isoelectric focusing was carried out on precast Ampholine PAGplates (Pharmacia) using ampholytes in the range from pH 4 to pH 6.5.

**RESULTS**

**Treatment of Antithrombin with Deiminase—**Incubation of antithrombin with deiminase at molar ratios of enzyme:inhibitor greater than 1:50, in the presence of heparin pentasaccharide, resulted in complete inactivation of antithrombin, as judged by its ability to inhibit thrombin under defined assay conditions, within 2 h. Lower enzyme:inhibitor ratios such as 1:100 and 1:200 resulted in less inactivation and longer times of incubation were required. No inactivation was found in the absence of heparin pentasaccharide.

The site and number of residues of arginine modified by deiminase in α-antithrombin was deduced by comparison with the effects of deiminase on antithrombin Glasgow, a natural variant of antithrombin with a mutation of P₁ Arg to His. Antithrombin, in the presence of heparin pentasaccharide, was modified such that its mobility in isoelectric focusing gels became more anodal, while antithrombin Glasgow was not changed in its mobility (Fig. 1). Since no Arg residues were therefore modified in antithrombin Glasgow and as it only differs from normal antithrombin at the P₁ position, these data strongly indicate that α-antithrombin is modified at a single site only, which is likely to be the P₁ Arg. The site of modification was further examined using cleaved deiminase-treated antithrombin (deiminated antithrombin) and control α-antithrombin. Each of these was cleaved within their reactive site loops, using human neutrophil elastase (15), thus generating the C-terminal fragment of antithrombin, which was electroblotted to polyvinylidene difluoride after separation on SDS-PAGE. This permitted N-terminal sequencing at the reactive site of antithrombin, since the sequence from the cleavage point of human neutrophil elastase should begin: AVIAGSRSLNPKR, with the R representing the reactive site P₁ Arg. N-terminal sequencing of control α-antithrombin showed the expected sequence, but sequencing of deiminated antithrombin revealed that the reactive site arginine had indeed been changed to a citrulline residue. Arg-399 was not altered, however. This confirmed the earlier hypothesis, generated by comparison with antithrombin Glasgow, that the reactive site residue had been modified and all the evidence indicates this is the only residue altered.

**Heparin Affinity of Deiminase-modified Antithrombin—**Since it was previously shown that antithrombin Glasgow, which has a substitution of its P₁ Arg residue, had increased affinity for heparin, as judged by heparin-Sepharose affinity chromatography (7), the affinity of both antithrombin Glasgow and deiminated antithrombin were measured in comparison with normal α-antithrombin. This was carried out using fluorometric titrations against the heparin pentasaccharide and revealed that both antithrombin Glasgow and deiminated antithrombin had higher affinity for the pentasaccharide than α-antithrombin, the gain being about 4.4-fold in both cases (Table 1).

The reason for the higher affinity binding of the pentasaccharide was investigated by determining the rapid kinetics of the interaction between the antithrombins and the heparin. It has been shown previously (3, 16) that antithrombin binds heparin in a two-step procedure, which may be evaluated using stopped flow fluorometry to assess the speed at which the antithrombin increases its intrinsic fluorescence upon binding heparin. When the kinetics of heparin pentasaccharide binding to antithrombin Glasgow were evaluated in this way, it was found that the increase in its affinity for pentasaccharide was due to a lower dissociation rate constant for the interaction (Fig. 2 and Table 1). This is most accurately and easily measured by stopped flow experiments at low heparin concentrations at ionic strengths of 0.3 (3). Thus, this technique was applied directly to the deiminated antithrombin which was isolated in limited quantities, and it was shown to have the...
kinetic of H5 binding to antithrombins.

- The linkage of the side chain of P1 Arg to the conformational basis for this increased affinity. The pentasaccharide induces a series of changes at its binding site on antithrombin, with a tilting of the D-helix, a shift at the commencement of the A-helix and the induction of a new 2-turn helix.

- The concept that the conformation of antithrombin was constrained with respect to inhibitory activity and that heparin released this constraint to give optimal activity as inherently presented in α1-antitrypsin arose from the finding that the mutant α1-antitrypsin Pittsburgh, with an arginine at P1, was a potent thrombin inhibitor (18). The reactive site loop of antithrombin becomes more exposed in the presence of heparin, as shown by its increased susceptibility to proteinase cleavage (15, 19), by NMR studies (20), and fluorescence studies (21). All of these studies fit with a reactive loop rearrangement and activation as modeled in Fig. 3.

The second question we have addressed relates to these concomitant changes in activation and heparin affinity, specifically whether the linkage of the side chain of P1 Arg to the body of the molecule is an inherent contributor to the structural constraints that limit the inhibitory activity and heparin affinity of circulating antithrombin. This was proposed based on the findings that antithrombins in which the P1 Arg has been replaced by a His (7, 22) or Pro (8) had an increased affinity of circulating antithrombin. This was proposed based on the findings that antithrombins in which the P1 Arg has been replaced by a His (7, 22) or Pro (8) had an increased affinity of circulating antithrombin.
helix. These changes bring a series of side chains into a stable alignment for linkage to the pentasaccharide. This conformational change at the heparin binding site is accompanied by a transition at 30 Å distance, which releases the reactive site loop from the A-β-sheet, the two changes being linked by a series of intervening conformational shifts. Reactive loop expulsion is therefore linked to the transition to the high affinity state for heparin. A recent mutation of antithrombin, which stabilized the molecule in a reactive loop expelled form, had high affinity for heparin (21), most likely because it tended to be stabilized in the high affinity state. We propose that a similar mechanism occurs for antithrombin Glasgow and deiminated antithrombin, stabilizing the molecule into a high affinity state and thus lowering the dissociation constant for heparin binding.

Taken together, the recent series of structures of antithrombin (4, 6, 23, 24), and the data presented here, allow an overall view of the mechanism of heparin activation to be formed. Tempting though it is to describe this in terms of a sequential series of changes radiating from the heparin binding site, it is better to consider the transition as the consequence of the release of the broader constraints that hold the molecule in its low activity conformation. These constraints include: the partial insertion of the reactive site loop into the A-β-sheet, the stability of the partially opened A-β-sheet, and, as shown here, the bonding of P1 Arg to the body of the molecule. Perturbation of any of these constraints, as has already been demonstrated with loop insertion (21) and A-β-sheet stability (25), will result in the reversion toward the unconstrained, high heparin affinity conformation. Thus the addition of heparin is just a special example of an induced loss of constraint, which results in reversion to a relaxed-loop inhibitory conformation. The initial low affinity of antithrombin for the pentasaccharide reflects the equilibrium between the unconstrained loop-expelled conformation, in which the pentasaccharide is tightly bound, and the constrained loop-inserted conformation, with more ready dissociation of the pentasaccharide. It follows from this that the loss of a structural constraint, as with perturbations of P1 Arg, will favor the loop-expelled, unconstrained conformation with the associated decrease in the pentasaccharide dissociation rate constant, as observed here.

All of these findings fit with the deductions, from the initial finding of a P1 Arg mutant of α1-antitrypsin (18), that α1-antitrypsin acts as a model for heparin activated antithrombin. This earlier finding, that P1 Arg-α1-antitrypsin had a thrombin inhibitory activity greater than antithrombin in the absence of the pentasaccharide, has been reinforced by a recent study of Hopkins and colleagues. They have shown that a more extensive mutation of the reactive site loop of α1-antitrypsin to give the reactive center P4-P3 conjugation with the associated decrease in the pentasaccharide dissociation rate constant, as observed here.

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