Topology of the Stable Serpin-Protease Complexes Revealed by an Autoantibody That Fails to React with the Monomeric Conformers of Antithrombin

Véronique Picard, Pierre-Emmanuel Marque, Francis Paolucci, Martine Aiach, and Bernard F. Le Bonniec

From INSERM, Unité 428, Université Paris V, 75270 Paris Cedex 06 and Sanofi Research, 34000 Montpellier, France

Solving the structure of the stable complex between a serine protease inhibitor (serpin) and its target has been a long standing goal. We describe herein the characterization of a monoclonal antibody that selectively recognizes antithrombin in complex with either thrombin, factor Xa, or a synthetic peptide corresponding to residues P14 to P9 of the serpin’s reactive center loop (RCL, ultimately cleaved between the P1 and P′1 residues). Accordingly, this antibody reacts with none of the monomeric conformers of antithrombin (native, latent, and RCL-cleaved) and does not recognize heparin-activated antithrombin or antithrombin bound to a non-catalytic somatic inhibitor (12A5) that provides definite insight into the structure of the stable complex, ultimately split into regenerated enzyme and RCL-cleaved (consumed) serpin (9–12). The RCL sustains a variety of conformations (13–16). In antithrombin (AT) that is heparin-activated (17, 18) and other inhibitory serpins such as α1-antitrypsin (also called α1-proteinase inhibitor; 19–21) or α1-antichymotrypsin (22), the RCL is wholly exposed, whereas in the AT monomer, residue P14 of the RCL disrupts β-sheet A (23–25). In latent AT, an intact but non-inhibitory conformer (25), and in latent type-1 plasminogen activator inhibitor (16), residues P14 to P3 of the RCL are completely inserted into β-sheet A, constituting an additional, sixth, strand. The same conversion from a five- to six-stranded β-sheet A occurs in the inhibitory serpins, following cleavage of the RCL (14, 26).

To date, no x-ray analysis of a serpin-protease complex has been reported; thus, its structure remains largely hypothetical. Based on functional studies of serpin variants and immunological investigations, a number of reports nevertheless suggest that, in the stable complex, the RCL is inserted into β-sheet A. Antibodies have been characterized that fail to react with native serpin, but recognize binary complexes with a synthetic tetradecapeptide corresponding to residues P14 to P1 of the RCL, as well as consumed inhibitors (27–33). Thus, antibodies revealed that insertion of the RCL in β-sheet A exposes neoepitopes that are not present in the intact inhibitor. The same neoepitopes being exposed in stable serpin-protease complexes led to the conclusion that, during trapping of the protease, the RCL inserts at least in part into β-sheet A. Convincing evidence also suggests that the protease translocates away from the site at which initial attack occurs. Wright and Scardale (34) even proposed that the enzyme ends in a location almost opposite to that of the RCL in intact serpins, i.e. that, in the stable serpin-protease complexes, β-sheet A is a six-stranded sheet. Stratikos and Gettins (35) demonstrated that, at least 21 Å separates position of the protease in the initial (reversible) Michaelis complex from that in the (virtually irreversible) final complex. Modeling considerations suggest a range of plausible stable structures: from full insertion of the RCL with upside-down translocation of the defeated protease, to limited RCL insertion (up to the P9 residue) with concomitant stacking of the target on the F-helix. Following cross linking experiments with stable complexes of type-1 plasminogen activator inhibitor, Wilczynska et al. (36) favored stacking on the F-helix, and partial rather than complete insertion of the cleaved RCL.

We report herein characterization of a monoclonal antibody (12A5) that provides definite insight into the structure of the stable AT-protease complex; unless AT was unfolded, the antibody failed to react with any AT monomers, while endorsing stable complexes with thrombin or factor Xa and binary complex with a short peptide derived from the RCL. The unexpected location of the neoepitope, on the distal part of β-sheet...
A, has several fundamental implications. The conformations of β-sheet A and/or of the F-helix in the stable complex must simultaneously differ from the five- and six-stranded structures of native and RCL-cleaved AT, respectively. Steric hindrance considerations also limit drastically the possible topologies of the complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human thrombin and its S195A variant were prepared as described previously (8). Factor Xα was purchased from ERL (South Bend, IL). Porcine pancreatic elastase (type IV) and bovine serum albumin were purchased from Sigma (St-Louis, MO, France), as well as mouse AT and bovine, sheep, porcine, rabbit, and chicken plasma. Standard, unfractionated heparin (Heparin Choay) and pentasaccharide with high affinity for AT (M, 1714) were from Sanofi-Winthrop (Gentilly, France). Peptide Pα-Pβ derived from the RCL of AT (Ac-SEAAAS) was synthesized by Althergen (Schiltigheim, France).

**Preparation and Characterization of the Various AT Conformers**—Human AT was purified from citrated frozen plasma essentially according to McKay (37) by affinity chromatography on heparin-Sepharose (Pharmacia, St-Quentin-en-Yvelines, France), followed by anion-exchange chromatography on a Mono-Q column (Pharmacia). AT cleaved by porcine pancreatic elastase was prepared by incubating AT (5 μM) with 50 nM enzyme for 4 h at 37 °C in 50 mM Tris–HCl, pH 8.5, containing 0.15 M NaCl and 0.1% polyethylene glycol (M, 8000; w/v). The resulting C-terminal fragment was isolated by transfer onto polyvinylidene difluoride membrane after denaturing polyacrylamide gel electrophoresis (15% acrylamide), and analyzed by N-terminal sequencing (Biotechnology Department, Institut Pasteur, Paris, France). Two sites of cleavage were identified: Val392–Ile393 and Ile399–Ala400, corresponding to the Pα-Pβ and Pβ-Pγ residues of the RCL, respectively. Identical cleavages are obtained following incubation of AT with human neutrophil elastase (38). Thrombin-cleaved AT was prepared by incubating AT (7 μM) with thrombin (0.1 μM) for 3 h at 37 °C in 50 mM Tris- HCl, pH 7.5, containing 0.15 M NaCl and trace amounts of SDS (0.02%, w/v; Refs. 39 and 40). Thrombin was neutralized by the addition of 1 mM phenylmethylsulfonyl fluoride (Sigma). Traces of SDS and excess phenylmethylsulfonyl fluoride were removed by extensive dialysis against 50 mM Tris- HCl, pH 7.5, containing 0.15 M NaCl. Denaturing polyacrylamide gel electrophoresis (Fig. 1) suggested that in these conditions AT was fully cleaved by thrombin, at a single site. In addition to the N terminus of AT, N-terminal sequencing of the reaction mixture revealed a single sequence starting with the Pα residue of the RCL, indicating that thrombin cleaved a single covalent bond (39). Reversible complexes were assumed to be that of the added AT (5 μM). Reversible complex of AT with S195A thrombin was prepared by mixing AT (2 μM) with S195A thrombin (2 μM) in the presence of heparin (1 unit/ml); association was presumed to be almost instantaneous (8), and concentration of the reversible complex was assumed to be that of the added AT.

**Preparation of the Monoclonal Antibodies**—Marine invertebr- otoxonin antibodies directed against human AT were selected, isolated and characterized by standard procedures (43). Four 6-week-old BALB/c females were immunized by subcutaneous injection of 20 μg of AT in complete Freund’s adjuvant followed 3 weeks later by a further subcutaneous injection of 20 μg of AT in incomplete Freund’s adjuvant. One hundred days later, mice received 5 μg of AT subcutaneously and 12 μg of AT intravenously. One day prior to the AT injection, and for 5 days, mice also received a daily intraperitoneal injection of 20 μg of pentasaccharide. Three days after the final injection, spleen cells were fused with the mouse myeloma cell line P3-X63-Ag.8.653 by using 50% polyethylene glycol 1540, and cultured in hypoxanthine/aminopterin/thymidine media. Positive clones were detected by enzyme-linked immunosorbent assay (ELISA); Maxisorp microplates from Nunc (Plytolabo, Strasbourg, France) were coated overnight at 4 °C with 5 μg/ml AT in 0.1 M NaHCO3/Na2CO3, pH 9.5, and washed three times in 20 mM NaHPO4/Na2PO4, pH 7.4, containing 0.15 M NaCl (PBS) and 0.5% Tween 20 (v/v). Residual sites were blocked for 1 h with 1 mg/ml BSA in PBS, and microplates were washed as above. Each hybridoma supernatant to be tested was added to one of the wells of the microplate and allowed to bind for 1 h at room temperature.

**Determination of the AT Conformers Recognized**—To identify the conformers of AT recognized by the monoclonal antibodies, a sandwich ELISA was designed in which microplates were coated with purified IgG (5 μg/ml). Each AT conformer, diluted in PBS containing 0.5% Tween 20 (v/v) and 1 mg/ml BSA, was added to one of the wells of the microplate and allowed to bind for 2 h at room temperature. Sandwich ELISA was developed with a peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Ivry-sur-Seine, France; monoclonal 12A5 described in this study was typed as IgG2a. Reduction conformational antibodies, a sandwich ELISA was developed with a peroxidase-conjugated goat anti-mouse antibody (Bio-Rad, Ivry-sur-Seine, France) was added and allowed to bind for 1 h at room temperature. Following a final wash, ELISA was developed by adding 150 μl of orthophenylenediamine (0.5 mg/ml; Bio-Rad) in 0.1 M sodium citrate, pH 5.5, containing 0.3% H2O2 (v/v). The reaction was stopped after 30 min by the addition of 50 μl of H2SO4 (12.5%, v/v), and the absorbance at 490 nm was recorded. Selected clones were grown in pristane-primed BALB/c mice, and antibodies purified from the ascitic fluid by affinity chromatography on protein A-Sepharose (Pharmacia). IgG appeared pure by denaturing polyacrylamide gel electrophoresis analysis and were stored at −80 °C after dialysis against PBS. The subclass was determined with a commercial kit (Amersham, Les Ulis, France; monoclonal 12A5 described in this study was typed as IgG2a.

**Topography of the Serpin-Protease Complexes**

**FIG. 1.** Polyacrylamide gel electrophoresis of the various AT conformers used. Left panel, electrophoresis in denaturing conditions of native AT (lane 1), AT cleaved by porcine pancreatic elastase (lane 2), AT cleaved by thrombin (lane 3), thrombin (lane 4), complex of AT with thrombin (lane 5), factor Xα (lane 6), and complex of AT with factor Xα (lane 7). Right panel, electrophoresis in non-denaturing conditions of native AT (lane 1), latent AT (lane 2), and AT partially in complex with the Pα-Pβ peptide derived from the RCL (lane 3).
bionylated peptides linked to a cleavable polyethylene pin (44). A four-amino acid spacer (SGSG) separated the N terminus of each peptide from the pin, except for the peptide encompassing the first 14 amino acids of AT where the spacer was coupled to the C terminus. Binding assays were performed according to the supplier's instructions. Briefly, 100 μl of streptavidin (5 μg/ml in water) was added to each well of a microplate and evaporated to dryness at 37 °C. Bionylated peptides (about 1 μM in PBS containing 1 mg/ml BSA) were added and incubated for 1 h at room temperature. Monoclonal antibody (0.1 μg/ml) in PBS containing 0.5% Tween 20 (v/v) and 1 mg/ml BSA was incubated for 2 h at room temperature. Washing and development were otherwise performed as described above for the ELISA, using the peroxidase-conjugated goat anti-mouse IgG.

**Immunoblotting**—Immunoblotting of the various AT conformers (after polyacrylamide gel electrophoresis in denaturing conditions) was performed on nitrocellulose membrane (Bio-Rad) essentially as described previously (45). Incubation and washing were all completed at room temperature in 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.5% Tween 20 (v/v). The membrane was saturated with nonfat dry milk (3% w/v), washed, and incubated for 1 h with monoclonal antibody 12A5 (0.5 μg/ml). After washing, the membrane was incubated for 1 h with an alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad). The immunoblot was further washed, and developed by adding a phosphatase substrate solution consisting of 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg/ml nitro blue tetrazolium in 0.1 M Tris, pH 9.5, containing 0.5 mM MgCl2.

**Inhibition Kinetics**—The influence of monoclonal antibody 12A5 on thrombin and factor Xa inhibition by AT was studied in the absence and in the presence of heparin, essentially as described previously (46, 47). Assays were performed at 37 °C in 50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl and 0.1% polyethylene glycol (M, 6000; w/v); 1 ng/ml BSA was included in the assay of factor Xa inhibition. Without heparin, estimates of the association rate constant (k association) in the presence of 1 μM 12A5 (or control IgG), were obtained from kinetic experiments performed in microplates, under pseudo-first-order conditions (20–100 nM AT with 2 nM thrombin, and 150–300 nM AT with 15 nM factor Xa). First-order rate constants were estimated by non-linear regression analysis of the residual activities versus time (up to 90 min), and k association values deduced from the linear plot of the first order rate constants as a function of AT. The k association values in presence of heparin (1.4 units/ml), were estimated by analysis of data from progress-curve kinetics also completed in pseudo-first-order conditions (0.2–0.6 μM AT with 10 pM thrombin, and 1–2 nM AT with 100 pM factor Xa). Inhibition of thrombin was initiated by its addition to a microtiter well containing 0.2 μM 12A5 (or control IgG); AT at various concentrations, and 100 μM H-D-Phe-Arg-p-nitroanilide (S-2238, Biogenic, Montpellier, France). Before initiating factor Xa inhibition, the release of p-nitroaniline from 400 μM benzyl-CO-Ile-Glu-γ-(OR)-Gly-Arg-p-nitroanilide (S-2222, Biogenic) by factor Xa was monitored for 10–15 min, until a steady state velocity of about 0.2 μM substrate/min was reached. The inhibition reaction was initiated by the addition of a mixture of 0.2 μM 12A5 (or control IgG) and AT at various concentrations. The release of p-nitroaniline was monitored for up to 3 h using a Lambda 14 Perkin Elmer spectrophotometer, but only data corresponding to less than 10% substrate hydrolysis were used in the analysis. Estimates of the k association values were obtained by fitting data to the equation for slow-binding inhibition and corrected for the competition introduced by the substrate.

## RESULTS

12A5, a Monoclonal Antibody That Distinguishes AT-Protease Complexes from Native, Latent, and RCL-cleaved AT—AT may adopt at least four conformations: native, heparin-activated, latent, and RCL-cleaved. A fifth conformation is likely to occur when AT is trapped in a complex with one of its targets (thrombin or factor Xa), but the precise structure of AT within this complex remains largely unknown. In an attempt to probe the elusive conformation of trapped AT, we prepared a panel of monoclonal antibodies; 12 were selected, because they recognized AT that had been coated on a microplate. To determine which conformer of AT was recognized, the monoclonal antibodies were coated onto a microplate, then AT (native, RCL-cleaved, or in complex with thrombin) was added, and sandwich ELISA developed with a polyclonal antibody directed against AT. Two monoclonal antibodies retained our attention, because they did not react with native or RCL-cleaved AT, while giving a strong signal with complexes; the other monoclonal antibodies reacted with all three forms of AT, indicating that selectivity of the former was not due to an experimental artifact. Thus, even though AT-protease complexes were not included in the immunization mixture, all the antibodies reacted with AT in complex. Although surprising at first, we reasoned that complexes might have formed with endogenous mouse proteases, and that a neoepitope inaccessible in the native protein might be exposed in partially denatured AT. To delineate more precisely the specificity of one of the monoclonal antibodies (12A5), we evaluated its affinities for native, latent, and heparin-activated AT, for AT with the RCL cleaved by thrombin or porcine pancreatic elastase, for AT in stable complex with thrombin or factor Xa, and for AT in complex with a peptide derived from the P14-P9 residues of its RCL. AT in complex with either thrombin or factor Xa bound to 12A5 in a dose-dependent fashion, as did the binary complex of AT with the P14-P9 peptide derived from the RCL (Fig. 2) and allowed to coag with AT. 2 h. Sandwich ELISA was developed with a peroxidase-conjugated polyclonal IgG directed against AT. **FIG. 2. Antibody 12A5 recognizes only complexes of AT with thrombin, factor Xa, or with the P14-P9 peptide derived from the RCL.**

Antibody 12A5 was coated onto microtiter wells, and AT in complex with thrombin (●), AT in complex with factor Xa (○), native AT (△), latent AT (■), heparin-activated AT (▲), AT cleaved by porcine pancreatic elastase (□), AT cleaved by thrombin (□), or AT in complex with the P14-P9 peptide derived from the RCL (▼) was added and allowed to bind to AT for 2 h. Sandwich ELISA was developed with a peroxidase-conjugated goat anti-mouse IgG. The immunoblot was further washed, and developed by adding a phosphatase substrate solution consisting of 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.3 mg/ml nitro blue tetrazolium in 0.1 M Tris, pH 9.5, containing 0.5 mM MgCl2.

12A5 Recognizes Pentapeptide DAFHK on the Penultimate Strand of β-Sheet A—As 12A5 recognized unfolded AT after reduction of the disulfide bridges with β-mercaptoethanol. Thus, a motif recognized by 12A5, inaccessible in native, heparin-activated, latent, and RCL-cleaved AT, was exposed in denatured and target-complexed AT. Taken together, these data suggested that steric hindrance, rather than a need for a specific conformation, limited the ability of 12A5 to interact with native or RCL-cleaved AT. 12A5 Recognizes Pentapeptide DAFHK on the Penultimate Strand of β-Sheet A—As 12A5 recognized unfolded AT after reduction of the disulfide bridges, it was reasonable to expect that the neoepitope was a contiguous sequence of amino acids. We scanned two sets of bionylated peptides, covering the entire sequence of AT. The first set simply divided AT sequence into 31 peptides that each were 14 amino acids in length, starting with His1 of AT; the second set divided AT sequence...
Table shows binding of 12A5 to peptides derived from sequence Val364 to Phe368 of AT. Biotinylated peptides (linked to a microplate) were considered as able to grasp 12A5 (+) when signal was in excess of 8-fold that of the background. The minimum sequence recognized was DAFHK; within this sequence, only D, F, and K were found to be critical.

| Sequence     | 12A5 binding |
|--------------|--------------|
| FHKAF        | –            |
| AFHKAF       | –            |
| DAHKF        | +            |
| SDAHKF       | +            |
| VSDAFHKAF    | +            |
| VSDAFHK      | +            |
| VSDAFH       | –            |
| VSDAF        | –            |
| SAAHKA       | –            |
| SDVHKKA      | +            |
| SDAHKRA      | –            |
| SDAHKRA      | +            |
| SDVHKA       | –            |

Our data establish that AT in complex with thrombin or factor Xa differs from all conformers of known structure (native, heparin-activated, latent, or RCL-cleaved); they also imply that protease permits access to neoepitope DAFHK within the complex.

DISCUSSION

The Conformational Change Unveiled by 12A5 Is Subsequent to the Step Controlled by Ser195 of the Protease—To achieve formation of a stable complex, serpin-protease interactions involve multiple steps, but in the path of AT inhibition, only the very last concerns the catalytic serine of the protease. S195A thrombin is an inactive variant, in which the catalytic serine has been replaced by alanine (8). S195A binds to AT with a $k_{on}$ value similar to that of thrombin, indicating that Ser195 of thrombin is not involved in the rate-limiting step of complex formation. The main difference between thrombin and S195A actually resides in the rate of complex dissociation; in contrast to the thrombin-AT complex, the S195A-AT complex is reversible ($K_d = 3$ nM with heparin, 3 $\mu$M otherwise). Unless AT was denatured, the motif DAFHK appeared accessible to 12A5 only when AT was in complex with a peptide or a target. Thus, the combination of S195A and 12A5 constituted a remarkable tool to probe the concluding step of thrombin-AT complex formation; if the particular folding of AT unveiled by 12A5 takes place after formation of the initial complex, but before the irreversible step, AT bound to S195A must react with 12A5. On the contrary, if neoepitope exposure is concomitant with final stabilization of the complex, then motif DAFHK must remain invisible to 12A5, whether or not S195A adheres to AT. The high $K_d$ value of AT for S195A in the absence of heparin precluded study of 12A5 binding to the reversible complex, but saturation experiments were feasible in the presence of heparin. Hence, saturating amounts of S195A-AT complexes were not retained on a microplate coated with 12A5 (Fig. 5). This observation implied that neoepitope display was independent from initial binding of AT to thrombin; it was instead concomitant (or subsequent) to the catalytic step involving Ser195. Consistent with this hypothesis, 12A5 had no detectable influence on thrombin or factor Xa inhibition by AT; $k_{on}$ values were virtually identical, whether or not the target had been preincubated with saturating concentration of 12A5, and that reaction proceeded in the presence or not of heparin (Table II and Fig. 6). In fact, mice producing 12A5 were not abnormally ill, suggesting that autoantibody did not interfere with normal hemostasis, at least until neoepitope DAFHK was exposed (i.e., after formation of the stable complex). In this model, the autoantibody would not be harmful; the only consequence might have been a faster clearance of the protease-AT complexes.
tion that 12A5 also recognized unfolded AT (i.e. blotted on nitrocellulose membrane or adsorbed on plastic wells). However, such a model would be difficult to reconcile with the dynamics of protease trapping by a serpin; in general, bonds are cleaved more efficiently in denatured substrates than in folded proteins (48). Thus, if formation of an initial Michaelis complex triggers gross disruption of AT folding, it would be expected that AT is a good substrate rather than an inhibitor. More satisfactory is a model in which the overall folding of AT is preserved: failure of 12A5 to react would originate from a steric hindrance that complex formation releases. Epitopes have been characterized that are recognized in the latent, RCL-cleaved, and complexed conformers, not in the native serpin; hence, RCL-cleaved and native conformers share a common overall folding, implying that limited conformational change indeed can trigger exposure of a neoepitope (27, 33, 49). Two limited alterations in the structure of AT would permit motif DAFHK to access solvent: partial disruption of β-sheet A and/or motion of the loop connecting the F-helix to strand 3 of β-sheet A (Fig. 3).

A number of studies suggest that several events, including formation of the stable complex, dramatically modify the structure of β-sheet A in serpins. In type-1 plasminogen activator inhibitor, for instance, a temperature-dependent transition exposes the penultimate strand of β-sheet A, as attested by its susceptibility to non-target proteases (50); at 0 °C, the native (inhibitory) conformer is cleaved within motif QALQK (i.e. within the region homologous to neoepitope DAFHK of AT); at 37 °C, the motif resists hydrolysis. The main structural rearrangement that seems to accompany complex formation consists of an insertion of the N-terminal (P) side of the RCL into β-sheet A, but it is unclear which of the P₁ to P₉ residues are concerned (49, 51–54). It is likely that insertion is subsequent to the release by the protease of the C-terminal (P') side of the cleaved RCL (11, 55), whereas the P₁ residue remains attached to the charge stabilizing system of the protease. Insertion of peptides with the same sequence as the RCL also causes conformational changes in AT; there, the extent of the insertion...
triggers various consequences, suggesting that many conformations are achievable (29, 42, 56, 57). Structural data obtained by x-ray diffraction (18, 25), fluorescence spectra data on the S380W variant of AT (17), immunochemical data (58), and susceptibility of the P₁ arginine to chemical modification (59) all suggest that at least the P₁₄ residue of the RCL is inserted in native (inhibitory) AT; this conformer was not recognized by 12A5 antibody. Insertion of a 7-amino acid peptide, homologous to residues P₁₄–P₈ of the RCL, induces a slow polymerization of 12A5 antibody. Insertion of a 7-amino acid peptide, homologous in native (inhibitory) AT; this conformer was not recognized by directed against AT. Saturation suggests that washing does not disso-

cmations are achievable (29, 42, 56, 57). Structural data ob-

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its RCL. On the other hand, the neoepitope was no longer recognized by 12A5 in the conformer of AT having the RCL cleaved at the P₅-P₄ bond (i.e. with the RCL inserted up to the P₅ residue), nor in AT cleaved at the P₁-P₁ bond by thrombin (i.e. with the RCL inserted up to the P₁ residue; Ref. 26). Finally, when a synthetic tetradecapeptide homologous to residues P₁₄ to P₁₅ of the RCL is locked in β-sheet A, AT is a substrate rather than an inhibitor (30, 31). Thus, insertion of not less than two, but no more than nine, residues in β-sheet A induced a particular conformation in AT that allowed 12A5 to bind the motif DAFHK. This neoepitope was also accessible to 12A5 in the stable complexes with thrombin or factor Xa. Therefore, it is tempting to conclude that AT in complex with either a target or the P₁₄–P₉ peptide share similar structural rearrangements: absent from the five- as well as from the six-stranded β-sheet A conformers. Combined with the assumption that part of the RCL occupies β-sheet A within the stable complex, the inference is that insertion concerns at least the P₁₄–P₁₅ residues of the RCL, at most the P₁₄ to P₉ segment.

An alternative to partial disruption of β-sheet A would be that failure of 12A5 to react originates from a steric hindrance that is unrelated to the conformation of the penultimate strand of β-sheet A. Unless it is masked, the motif DAFHK would be recognized by 12A5 in the five- as well as in the six-stranded conformers: structure of β-sheet A would not be the limiting factor per se. Displacement of the loop connecting the F-helix to strand 3 of β-sheet A would authorize neoepitope recognition by 12A5. Somehow, insertion of the RCL into β-sheet A (as observed in latent and RCL-cleaved serpins) requires that the connection to the F-helix moves: otherwise, the RCL would have to sneak into a very narrow passage underneath the connecting loop. Fairly limited motion of this surface loop would suffice to clear access to motif DAFHK. Once the RCL is inserted, the surface loop could return to a location similar to that which it occupied initially, with the result that the neoepitope is masked again in the conformer with fully inserted RCL. It is unlikely that surface loop moves alone; comparison of RCL-cleaved and native serpins suggests that strands 1–3 of β-sheet A, the F-helix, and the connecting loop constitute a mobile block that shifts relative to the remainder of the structure (60). Considering that the neoepitope was masked in both the native and RCL-cleaved conformers of AT, the inference is that the motif DAFHK is accessible to 12A5 only in the utmost distorted position of the mobile block, and that this distorted conformation is similar in the stable complex with a target. Consistent with this hypothesis, studies of a functional epitope in type-1 plasminogen activator inhibitor and of natural vari-

aments are achievable (29, 42, 56, 57). Structural data ob-

tained by x-ray diffraction (18, 25), fluorescence spectra data on the S380W variant of AT (17), immunochemical data (58), and susceptibility of the P₁ arginine to chemical modification (59) all suggest that at least the P₁₄ residue of the RCL is inserted in native (inhibitory) AT; this conformer was not recognized by 12A5 antibody. Insertion of a 7-amino acid peptide, homologous to residues P₁₄–P₈ of the RCL, induces a slow polymerization of AT (42), but insertion beyond the P₈ residue, prevents polymer-

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The observation that motif DAFHK remained accessible within the complex AT-protease restricts the possible topologies of the complex (34–36). One of the proposals is that the enzyme ends in a location almost opposite to the RCL in intact serpins. This model is compatible with our data, as the protease would not cover motif DAFHK. However, it is difficult to rec-

ocincle the hypothesis that β-sheet A is a six-stranded structure in the stable complex with our observation that neoepitope is not exposed following full insertion of the cleaved RCL. Thus, our data imply that if the protease rotates fully around the serpin, then either β-sheet A is grossly unfolded within the complex (i.e. it is neither a five nor a six-stranded structure), or the F-helix has moved away to release motif DAFHK. Assuming on the contrary that the overall folding of AT is preserved, and that the cleaved RCL inserts partially into β-sheet A, the complexed protease cannot rotate further than the F-helix around AT (i.e. about half-way), as it would otherwise cover the

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Fig. 4. Antibody 12A5 is an autoantibody. Immunoblotting of sheep (lane 1), rabbit (lane 2), porcine (lane 3), and bovine plasma (lane 4); AT from all species share the motif DAFHK and were recognized by 12A5. In chicken AT (lane 5), residue 366 is a glutamate (instead of an aspartate); chicken plasma was not recognized by 12A5. Surprisingly, purified mouse AT (lane 6) was recognized by 12A5. Mouse AT contains a DAFHK sequence, implying that immunized mice synthesized an autoantibody against a cryptic region of their own protein. The last lane was obtained with purified human AT; the small lower band corre-
sponds to β-AT (45).

Fig. 5. Antibody 12A5 fails to recognize the reversible complex of AT with S195A thrombin. Binding of the stable thrombin-AT complex (□) and of the reversible S195A-AT complex (▲) to microtiter wells coated with 12A5. Sandwich ELISA was developed with a perox-
idase-conjugated goat IgG directed against human AT. Lack of recog-
nition does not originate from S195A-AT complex dissociation during the washing steps; middle curve (▼) represents binding of the S195A-AT complex to microtiter wells coated with a rabbit IgG directed against thrombin and revealed by a peroxidase-conjugated rabbit IgG directed against AT. Saturation suggests that washing does not disso-

ciate the S195A-AT complex.
motif DAFHK. In this respect, it is interesting to note that 12A5 was incapable of even slowing down the inhibition reaction, and did not recognize the complex of AT with the S195A motif DAFHK. In this respect, it is interesting to note that 12A5 antibody (○) or in the presence of a monoclonal antibody that does not recognize AT, thrombin, or factor Xa (□). Solid lines were obtained by non-linear regression analysis of the data according to the equation for slow binding inhibition.

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References—The Serpin-Protease Complexes

Table II

|           | Thrombin | Factor Xa |
|-----------|----------|-----------|
| Heparin   | + Heparin|           |
| ~12A5     | 1.0 × 10^4 (± 6%) | 6.6 × 10^4 (± 6%) |
| + 12A5    | 9.5 × 10^5 (± 5%) | 6.7 × 10^6 (± 5%) |

FIG. 6. Antibody 12A5 does not influence thrombin and factor Xa inhibition by AT. Progress curves of thrombin inhibition (upper panel) and factor Xa inhibition (lower panel) in the presence of heparin. Reactions were performed in the presence of 12A5 antibody (○) or in the presence of a monoclonal antibody that does not recognize AT, thrombin, or factor Xa (□). Solid lines were obtained by non-linear regression analysis of the data according to the equation for slow binding inhibition.

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