Insight into the Molecular Basis of Coagulation Proteinase Specificity by Mutagenesis of the Serpin Antithrombin†

Alireza R. Rezaie*

Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104

Received May 17, 2002; Revised Manuscript Received July 17, 2002

ABSTRACT: Specific cleavage of factor V at several P1Arg sites is critical for maintenance of hemostasis. While cleavage by procoagulant proteinases fXa and thrombin activates the cofactor, its cleavage by the anticoagulant proteinase activated protein C (APC) inactivates it. Antithrombin (AT), a specific serpin inhibitor of both thrombin and factor Xa, but not APC, was used as a model system to investigate molecular determinants of APC specificity in the inactivation reaction. Two mutants were prepared in which the P2 or the P3−P3′ residues of the reactive site loop of the serpin were replaced with the corresponding residues of the APC cleavage site in factor V spanning residues 504−509 (Asp^{504}-Arg-Arg-Gly-Ile-Gln^{509}). Kinetic analysis showed that the reactivities of mutants were impaired by ∼2−3 orders of magnitude with both factor Xa and thrombin, but improved by ∼2 orders of magnitude with APC. The saturable dependence of the observed first-order rate constants on the concentrations of AT in complex with ∼70-saccharide high-affinity heparin revealed that changes in the reactivity of the 504−509 mutant with proteinases are primarily due to an effect in the second reaction step in which a noncovalent serpin−proteinase encounter complex is converted to a stable, covalent complex. These results suggest that the P3−P3′ residues of the APC cleavage site in factor Va, particularly P2Arg, confer specificity for the anticoagulant proteinase by improving the reactivity of the catalytic pocket with the transition state of the substrate in the second step of the reaction.

Proteolytic cleavage of the single-chain polypeptide factor V at several P1Arg sites [nomenclature of Schechter and Berger (*1*)][1] by plasma serine proteinases regulates the coagulation cascade. Cleavage by the procoagulant proteinases factor Xa (fXa)[2] and thrombin upregulates the cascade by activating the cofactor (*2*, *3*). Activated cofactor (factor Va) is an essential component of the prothrombinase complex that is responsible for the conversion of prothrombin to thrombin (*2*−*4*). On the other hand, cleavage of factor Va at two P1Arg sites (positions 306 and 506) by the anticoagulant proteinase activated protein C (APC) downregulates the cascade by inactivating the cofactor (*5*−*7*). It is not completely understood how these structurally similar trypsin-like serine proteinases differentially and specifically process the cofactor in two different pathways of the coagulation cascade. Structural and mutagenesis data suggest that differences in the P3−P3′ residues of the cleavage sites may partly be responsible for determination of specificity of these catalytic reactions. The molecular basis for such specificity appears to be due to the existence of variant residues in the extended binding pocket of coagulation proteinases that specifically interact with residues surrounding the scissile bonds. In support of this, two residues at positions 99 and 192 have been demonstrated to be critical for the P2 and P3 recognition specificity of thrombin (*8*, *9*), fXa (*10*, *11*), factor VIIa (*12*), activated protein C (APC) (*11*, *13*), and other plasma serine proteinases (*14*).

APC must cleave at least two P1Arg peptide bonds at positions 306 and 506 to completely inactivate the cofactor activity of factor Va (*6*, *15*). Protein S is known to function as a cofactor in factor Va inactivation by APC (*16*). The cleavage of the Arg^{506} bond, which occurs prior to the cleavage of the Arg^{306} bond, is independent of a cofactor (*15*). In this study, antithrombin (AT) was used as a model system to evaluate the extent to which residues surrounding the Arg^{506} peptide bond contribute to the specificity of the APC reaction. AT is a specific serpin for both fXa and thrombin, but it is virtually unreactive with APC; thus, it provides a good model system for studying this question. The P3−P3′ sequence of factor Va at the Arg^{506} site is unique in that it contains an unusual Arg at the P2 position. Thus, in addition to the P3−P3′ swap mutant of AT (AT504−

† This research was supported by a grant awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (Grant R01 HL 62565 to A.R.R.).

* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104. Phone: (314) 577-8130. Fax: (314) 577-8156. E-mail: rezaiear@slu.edu.

[1] The nomenclature of Schechter and Berger (*1*) used to describe the subsites of interaction between a protease and its substrate. Amino acid residues of the substrate are termed P1, P2, etc., on the N-terminal side of the substrate scissile bond and P1′, P2′, etc., on the C-terminal side. The corresponding sites in the enzyme (subsites) where substrate residues interact are designated S1, S2, etc., and S1′, S2′, etc., respectively.

[2] Abbreviations: fXa, factor Xa; APC, activated protein C; AT, antithrombin; AT504−509, AT mutant in which the P3−P3′ residues of the reactive site loop have been substituted with the corresponding residues of the APC cleavage site of factor V spanning residues 504−509; AT P2Arg, AT mutant in which the P2Gly of the serpin (residue 392) has been replaced with an Arg; SI, stoichiometry of inhibition; serpin, serine proteinase inhibitor; PEG, polyethylene glycol; BSA, bovine serum albumin; SE, standard error.

10.1021/bi0261443 CCC: $22.00
Published on Web 09/07/2002

509), a mutant of AT was also prepared in which the P2Gly of the serpin was replaced with an Arg (AT P2Arg). Both mutants of AT were expressed in mammalian cells, and properly folded proteins were purified to homogeneity as described previously (*17*). Kinetic analysis indicated that the reactivities of both fXa and thrombin with the mutants are impaired by 2−3 orders of magnitude. On the other hand, the rates were improved by ~2 orders of magnitude with APC. Further kinetic analysis in the presence of an ~70-saccharide high-affinity heparin revealed that the impairment or improvement in the reactivity of the AT504−509 mutant with all three proteinases is primarily contained within the second reaction step in which a noncovalent serpin−proteinase encounter complex is converted to a stable, covalent complex. Analysis of these results suggests that the P3−P3′ residues of the Arg^{506} cleavage site in factor V, particularly P2Arg, play an important role in conferring specificity for the APC recognition of the scissile bond at the second step of the reaction.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins. Wild-type AT and two mutants of AT in which the P3−P3′ residues of the reactive site loop of the serpin (Ile^{391}-Gly-Arg-Ser-Thr-Ser^{396}) were replaced with the corresponding residues of the APC cleavage site on cofactor V (Asp^{504}-Arg-Arg-Gly-Ile-Gln^{509}) (AT504−509) or the P2Gly of the serpin was replaced with an Arg (AT P2Arg) were constructed by standard PCR mutagenesis methods and expressed in HEK293 cells using the RSV-PL4 expression/purification vector system as described previously (*17*). Wild-type and mutant serpins were purified from cell culture supernatants by immunoaffinity chromatography using the HPC4 antibody linked to Affi-gel 10 (Bio-Rad) followed by HiTrap-Heparin (Amersham Pharmacia Biotech, Piscataway, NJ) ion exchange chromatography with gradient elution from 0.1 to 2.0 M NaCl in 20 mM Tris-HCl (pH 7.4) as described previously (*17*). Concentrations of the AT derivatives were determined from the absorbance at 280 nm using a molar absorption coefficient of 37 700 M^{-1} cm^{-1} as described previously (*17, 18*).

The active AT-binding pentasaccharide (H_5) fragment of heparin and full-length high-affinity heparin containing the H_5 with an average molecular mass of ~21000 Da (~70 saccharides) were generous gifts from S. Olson (University of Illinois, Chicago, IL). Concentrations of heparins were based on the AT binding sites and were determined by stoichiometric titration of AT with the polysaccharides, with monitoring of the interaction by changes in protein fluorescence (*19*). Recombinant human thrombin (*8*), APC, and an APC mutant in which Glu^{192} was replaced with a Gln (APC E192Q) (*20*) were expressed and purified as described by cited methods. Human fXa and AT were purchased from Haematologic Technologies (Essex Junction, VT). The chromogenic substrates, Spectrozyme FXa (SpFXa) and Spectrozyme PCa (SpPCa), were purchased from American Diagnostica (Greenwich, CT). Polybrene was purchased from Sigma (St. Louis, MO).

Kinetic Methods. The rate of proteinase inactivation by AT in both the absence and presence of heparin was measured under pseudo-first-order conditions by a discontinuous assay method as described previously (*21*, *22*). The uncatalyzed and heparin-catalyzed rate constants for the wild-type proteins were determined as described previously (*22*). In the case of inhibition by the mutant serpins, each proteinase (1−2 nM) was incubated with 500−1000 nM AT in both the absence and presence of the high-affinity pentasaccharide fragment of heparin (2 μM) in 0.1 M NaCl and 0.02 M Tris-HCl (pH 7.4) (TBS buffer, ionic strength of 0.12) containing 1 mg/mL bovine serum albumin (BSA), 0.1% polyethylene glycol (PEG) 8000, and 2.5 mM $CaCl_2$. In the presence of full-length high-affinity heparin (~70 saccharides), the reaction conditions were the same except that each proteinase was incubated with 0.5−10 μM wild-type or mutant AT and catalytic levels of heparin (0.625−2000 nM). All reactions were carried out in 50 μL volumes in 96-well polystyrene plates at room temperature. After a period of time (10 s to 120 min, depending on the rate of reactions), 50 μL of the chromogenic substrate (500 μM SpFXa for fXa and 500 μM SpPCa for both thrombin and APC) in TBS containing 0.1% PEG 8000 and 1 mg/mL Polybrene was added to each well and the remaining enzyme activity was measured with a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The observed pseudo-first-order rate constants (k_{obs}) were determined by computer fitting of the time-dependent change of the proteinase activity to a single-exponential function, and the second-order association rate constants for uncatalyzed or catalyzed reactions were obtained from the slopes of linear plots of k_{obs} versus the concentration of AT or the AT−heparin complex, respectively, in accordance with eq 1, as described previously (*22*).

$$k_{obs} = k_{uncat}[AT]_{free} + k_H[AT{-}heparin] \qquad (1)$$

where k_{uncat} and k_H are the second-order rate constants for the uncatalyzed and heparin-catalyzed reactions, respectively, $[AT]_{free}$ and [AT−heparin] represent the free and AT−heparin complex concentrations, respectively, which were calculated from the dissociation constant for the AT−heparin interaction and total concentrations of AT ($[AT]_o$) and heparin ($[H]_o$) using the quadratic equation (*19*).

In inhibition reactions where the k_{obs} values exhibited a saturable dependence on the concentration of the AT−heparin complex, data were analyzed according to the hyperbolic equation (*19*)

$$k_{obs} = k[AT{-}heparin]/K_D + [AT{-}heparin] \qquad (2)$$

where k represents the limiting rate constant for conversion of the intermediate heparin−AT−proteinase ternary encounter complex to a stable AT−proteinase complex and K_D is the dissociation constant for binding of proteinase to the AT−heparin complex to form the ternary complex (*19*).

Fluorescence Measurements. An Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) was used for protein fluorescence measurements at 25 °C. The excitation and emission wavelengths were 280 and 340 nm, respectively. The bandwidths were set at 1 nm for excitation and 8 nm for emission. Heparin titration was performed by adding a 2−5 μL of a high-concentration stock solution of heparin into each AT sample (50 nM) in TBS containing 0.1% PEG 8000. Addition of heparin diluted

samples to less than 5% of the original volume (500 μL). Data from at least three experiments were analyzed as the ratio of change in the fluorescence intensity of the sample containing heparin to the initial intensity of the control protein lacking heparin. The affinity of each AT derivative for heparin was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation (eq 3) as described previously (*19*).

$$\Delta F = \Delta F_{max}\{[AT]_o + [H]_o + K_D - [([AT]_o + [H]_o + K_D)^2 - 4[AT]_o[H]_o]^{1/2}\}/2[AT]_o \quad (3)$$

where ΔF and ΔF_{max} represent the observed and maximum change in fluorescence intensity, respectively, $[AT]_o$ and $[H]_o$ are the total concentrations of AT and heparin, respectively, and K_D is the dissociation constant for the AT−heparin interaction.

Stoichiometry of Inhibition. Stoichiometries of inhibition of proteinases by the wild-type and mutant AT−heparin complexes were measured in TBS containing 1 mg/mL BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+} by mixing fixed levels of proteinase (100 nM) with increasing molar ratios of AT with a fixed 2-fold molar excess of heparin over the highest concentration of the serpin. After incubation at room temperature for up to 12 h, residual enzyme activity was measured by addition of the chromogenic substrate and measuring the initial rate of substrate hydrolysis as in the discontinuous kinetic assay described above. The stoichiometry was obtained from the *x*-axis intercept of the linear regression fit of a plot of enzyme activity versus the inhibitor: enzyme molar ratio.

Data Analysis. The ENZFITTER (R. J. Leatherbarrow, Elsevier, Biosoft, London) computer program was used to fit the data to appropriate equations. All values are the average of at least three independent measurements ± SE.

RESULTS

Expression and Purification of the AT Derivatives. Wild-type and mutant AT derivatives were expressed in HEK293 cells and purified to homogeneity by a combination of HPC4 immunoaffinity and HiTrap-Heparin column chromatography as described previously (*17*). SDS−PAGE analysis under nonreducing conditions suggested that the recombinant serpins have been purified to homogeneity and that they migrate with relative molecular masses identical to that of wild-type AT (Figure 1B, lanes 2−4). Moreover, like wild-type AT, fXa, thrombin (Figure 1A), and APC (Figure 1B) formed stable complexes with the mutant serpins.

Binding to Heparin. It is known that the binding of high-affinity heparin to AT is associated with an ∼30−40% intrinsic protein fluorescence enhancement (*19*). The dissociation constants (K_D) of heparin binding to AT derivatives were determined by monitoring the fluorescence emission spectra as described in Materials and Methods. As shown in Figure 2, binding of an ∼70-saccharide high-affinity heparin (H_{70}) to both wild-type and mutant serpins resulted in a similar enhancement in the intrinsic protein fluorescence. The K_D values measured for the serpin derivatives (31.1 ± 2.8 nM for wild-type AT, 28.7 ± 5.2 nM for AT504−509, and 29.8 ± 3.5 nM for AT P2R) suggested that the affinity of

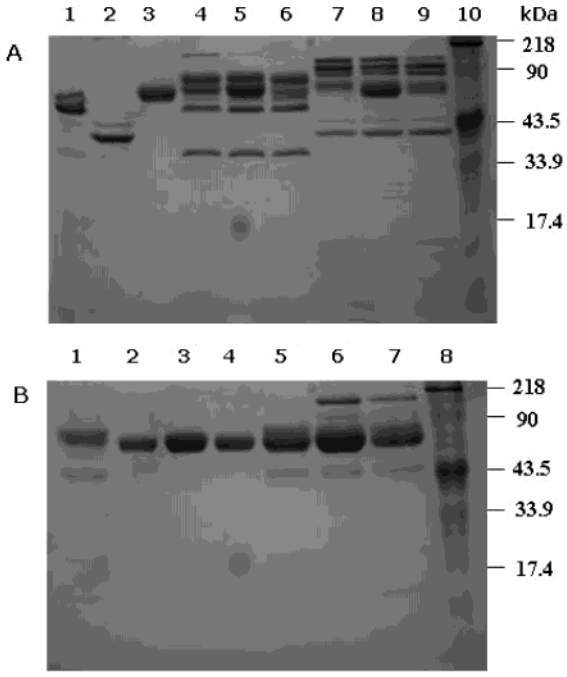

FIGURE 1: Nonreducing SDS−PAGE analysis of the stable fXa, thrombin, and APC complexes with the wild type or the AT504−509 mutant of AT. Wild-type or mutant AT (1.5 μM) was incubated with an equimolar concentration of fXa, thrombin, or APC for 5 min in the presence of ∼70-saccharide high-affinity heparin (3 μM) in 25 μL reaction mixtures in TBS buffer at room temperature. Five microliters of a 5-fold excess of nonreducing sample buffer was added to each reaction mixture, and the samples were boiled for 5 min. Twenty-five microliters of each reaction was loaded on a 10% gel. (A) Lane 1, fXa; lane 2, thrombin; lane 3, wild-type AT; lanes 4−6, fXa incubated with wild-type AT, AT504−509, and AT P2Arg, in the presence of ∼70-saccharide heparin, respectively; lanes 7−9, thrombin incubated with wild-type AT, AT504−509, and AT P2Arg, in the presence of ∼70-saccharide heparin, respectively; and lane 10, molecular mass standards in kilodaltons. The stable proteinase−AT complexes migrate as the highest-molecular mass bands, followed by degraded complexes. (B) Lane 1, APC; lane 2, wild-type AT; lane 3, AT504−509; lane 4, AT P2Arg; lanes 5−7, APC incubated with wild-type AT, AT504−509, and AT P2Arg, in the presence of ∼70-saccharide heparin, respectively; and lane 8, molecular mass standards in kilodaltons.

mutants for binding to heparin was normal. These results suggested that the mutants were properly folded.

Proteinase Inactivation. The reactivity of fXa with mutants, particularly with AT504−509, was dramatically impaired. AT504−509 and AT P2Arg inhibited fXa ∼430- and ∼45-fold slower than wild-type AT, respectively (Table 1). Consistent with previous results (*23*), H_5 promoted the wild-type AT inhibition of fXa ∼244-fold (Table 1). While the extent of the cofactor effect of H_5 was not affected in reaction with any of the mutants, the rates of fXa inhibition by AT504−509 and AT P2Arg in the presence of the cofactor were diminished 300- and ∼25-fold, respectively. The extent of impairment in the reactivity of fXa with mutants in the presence of high-affinity H_{70} was ∼340-fold for AT504−509 and ∼10-fold for the AT P2Arg mutant (Table 1). It is known that H_5 accelerates the AT inhibition of fXa by conformational activation of the serpin (*23*). However, H_{70} accelerates the inhibition reaction ∼200−300-fold through a conformational activation of the serpin and ∼200-fold through a template mechanism in the presence of physiological levels of Ca^{2+} (*22*). The data presented in Table 1

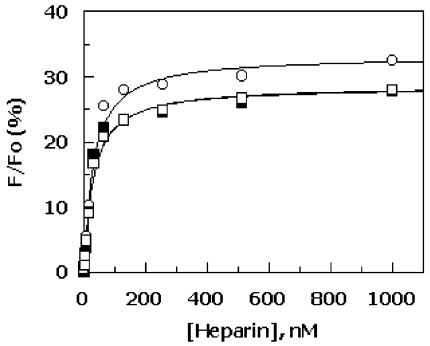

FIGURE 2: Binding of heparin to recombinant wild-type and mutant AT derivatives. Heparin-induced spectral changes were monitored at room temperature by stepwise addition of 2−5 μL of a concentrated stock solution of heparin to each AT derivative (50 nM) in 20 mM Tris-HCl, 0.1 M NaCl, and 0.1% PEG 8000 (pH 7.4). The dissociation constant of heparin for each derivative was calculated from the changes of the intrinsic protein fluorescence by nonlinear regression analysis using the quadratic equation for the tight binding inhibitors as described in Materials and Methods. The symbols are as follows: (○) rAT, (■) AT504−509, and (□) AT P2Arg.

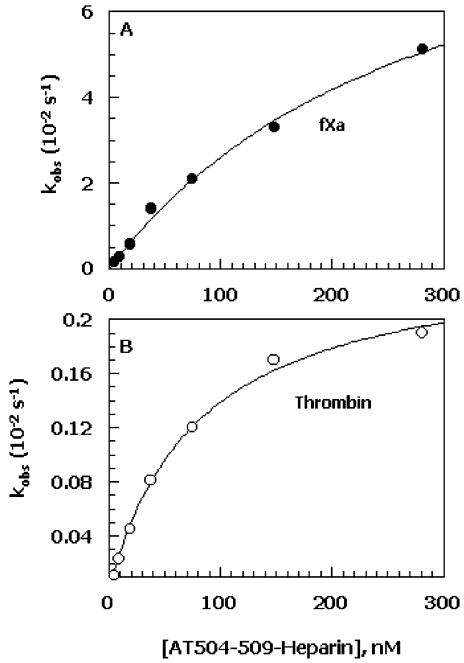

FIGURE 3: Dependence of pseudo-first-order rate constants (k_{obs}) for inactivation of fXa or thrombin on the heparin−AT504−509 complex concentration. The k_{obs} values for the heparin-catalyzed inactivation of 1 nM fXa (●, panel A) or 1 nM thrombin (○, panel B) were determined at increasing concentrations of the heparin−AT504−509 complex (shown on the *x*-axis) as described in Materials and Methods. The solid lines are least-squares computer fits of data by the hyperbolic equation (eq 2).

suggest that neither the conformational activation (the ratio of k_{H_5}/k_{uncat}) nor the template effect of heparin (the ratio of $k_{H_{70}}/k_{H_5}$) was impaired by the mutagenesis of the serpin.

Similar to that of fXa, the reactivity of thrombin with both mutants of the serpin was severely impaired in both the absence and presence of heparin (Table 1). AT504−509 and AT P2Arg inhibited thrombin ~3900- and 570-fold slower than wild-type AT, respectively. The same values were 1400- and 100-fold slower in the presence of H_5 and 4400- and 125-fold slower in the presence of H_{70}, respectively. Thus, the mutagenesis has impaired the serpin inhibition of thrombin by more than 2−3 orders of magnitude. However, as in the reaction with fXa, the rate accelerating effect of heparin was not impaired in the reaction with either mutant of the serpin (Table 1). Relative to the reaction with the wild-type serpin, the reactivity of thrombin was improved with the activated conformations of the mutant serpins. This is derived from the observation that, unlike a 2-fold enhancement in the reactivity of AT with thrombin in the presence of H_5, the reactivities of mutants were improved 5−10-fold under the same conditions (Table 1, ratio of k_{H_5}/k_{uncat}).

In contrast to the reaction with coagulant proteinases, the reactivity of APC with serpin mutants was improved in both the absence and presence of heparin. The extent of improvement in reactivities was more than 40−50-fold in the absence of heparin and ~40−110-fold in the presence of H_{70} (Table 1). H_5 promoted the reactivities of mutants with APC ~4−5-fold, suggesting that, as in the reaction with thrombin, APC reacts better with the activated conformations of mutant serpins. The observation that H_5 improved the reactivity of APC with both mutants may suggest that the cofactor-induced conformational change involves the P2 residue of the reactive site loop of the serpin.

Table 1: Second-Order Rate Constants for Uncatalyzed and Heparin-Catalyzed Reactions of Antithrombin Derivatives with fXa, Thrombin, and APC[a]

	k_{uncat} ($M^{-1}\,s^{-1}$)	k_{H_5} ($M^{-1}\,s^{-1}$)	$k_{H_{70}}$ ($M^{-1}\,s^{-1}$)
fXa			
wild-type rAT	$(2.7 \pm 0.3) \times 10^3$	$(6.6 \pm 0.2) \times 10^5$	$(8.9 \pm 0.7) \times 10^7$
AT504−509	6.3 ± 0.2	$(2.2 \pm 0.1) \times 10^3$	$(2.6 \pm 0.1) \times 10^5$
AT P2Arg	$(5.8 \pm 0.8) \times 10$	$(2.8 \pm 0.3) \times 10^4$	$(8.1 \pm 0.2) \times 10^6$
thrombin			
wild-type rAT	$(7.4 \pm 0.2) \times 10^3$	$(1.4 \pm 0.1) \times 10^4$	$(11.0 \pm 2.0) \times 10^7$
AT504−509	1.9 ± 0.1	$(1.0 \pm 0.1) \times 10$	$(2.5 \pm 0.2) \times 10^4$
AT P2Arg	$(1.3 \pm 0.5) \times 10$	$(1.4 \pm 0.1) \times 10^2$	$(8.8 \pm 0.3) \times 10^5$
APC			
wild-type rAT	0.14[b]	ND[c]	$(2.2 \pm 0.2) \times 10^2$
AT504−509	6.7 ± 0.5	$(3.0 \pm 0.1) \times 10$	$(2.5 \pm 0.1) \times 10^4$
AT P2Arg	5.7 ± 0.2	$(2.0 \pm 0.1) \times 10$	$(9.5 \pm 0.2) \times 10^3$

[a] The uncatalyzed (k_{uncat}) and pentasaccharide-catalyzed (k_{H_5}) second-order rate constants were determined from the residual chromogenic activities of fXa, thrombin, or APC (1−2 nM) after incubation at room temperature with recombinant wild-type (rAT) or mutant AT (10−1000 nM) in the absence or presence of H_5 (2 μM) for 0.5−60 min in TBS containing 1 mg/mL BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+}. The full-length heparin-catalyzed values ($k_{H_{70}}$) were determined by the same procedures except that 0.2−2 nM enzymes were incubated with 0.6−100 nM H_{70} and 500 nM AT. [b] Incubation of APC with AT (2 μM) for 15 h did not result in a decline in the chromogenic activity; thus, this value was taken from ref *30*. [c] Not determined.

Table 2: Kinetic Constants for the High-Affinity H_{70}-Catalyzed Reactions of Wild-Type and Mutant Antithrombins with fXa, Thrombin, APC, and APC E192Q[a]

	K_D ($\times 10^{-9}$ M)	k (s^{-1})	k_2 ($\times 10^6$ M^{-1} s^{-1})
wild-type AT			
fXa	90 ± 10	18 ± 1	$(200 \pm 20) \times 10^6$
thrombin	20 ± 1	3.2 ± 0.1	$(160 \pm 10) \times 10^6$
APC	632 ± 125	$(1.9 \pm 0.1) \times 10^{-4}$	$(3.0 \pm 0.7) \times 10^2$
APC E192Q	1900 ± 200	$(6.9 \pm 0.3) \times 10^{-3}$	$(3.6 \pm 0.5) \times 10^3$
AT504−509			
fXa	350 ± 30	$(1.1 \pm 0.1) \times 10^{-1}$	$(3.1 \pm 0.5) \times 10^5$
thrombin	80 ± 6	$(2.5 \pm 0.1) \times 10^{-3}$	$(3.6 \pm 0.4) \times 10^3$
APC	322 ± 53	$(1.2 \pm 0.1) \times 10^{-2}$	$(3.7 \pm 1.0) \times 10^4$
APC E192Q	1100 ± 100	$(2.6 \pm 0.1) \times 10^{-1}$	$(2.4 \pm 0.3) \times 10^5$

[a] The kinetic constants were determined from computer fits of the saturable dependence of the observed pseudo-first-order rate constant (k_{obs}) on the AT−heparin complex concentration according to eq 2. All reactions were carried out in TBS buffer containing 1 mg/mL BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+}. In all cases, a molar excess of AT was used to saturate the high-affinity heparin (~70 saccharides) as described in Materials and Methods. The overall second-order rate constants (k_2) were calculated from the ratio of k/K_D.

AT inhibits both fXa and thrombin by a two-step reaction mechanism in which an enzyme−inhibitor encounter complex, formed in the initial reaction step, is converted to a stable, covalent complex in a second reaction step (*22*, *24*). High-molecular mass heparins are known to lower the dissociation constant (K_D) for the formation of the initial enzyme−inhibitor encounter complex with a minimum effect on the rate constant (k) for formation of the stable, covalent complex (*24*). Rapid kinetic methods have previously been employed to resolve the two-step reaction of AT with both fXa and thrombin (*22*, *24*). In the case of the heparin-catalyzed reaction of AT504−509 with both fXa and thrombin, however, because the k values were dramatically impaired, high concentrations of the mutant serpin−heparin complexes could be used in the reactions to measure kinetic values for both steps in a discontinuous assay method. As shown in Figure 3, in the presence of the full-length heparin, H_{70}, the k_{obs} values for the reaction with both fXa and thrombin showed a saturable dependence on the concentration of the heparin−AT504−509 complex, indicating the saturation of an intermediate heparin−AT504−509−proteinase encounter complex prior to formation of a stable, covalent complex. Nonlinear regression analysis of data by the hyperbolic equation (eq 2) yielded K_D values for the ternary complex dissociation constants and k values for the rate constants of formation of the stable complex according to the following scheme:

$$\text{fXa} + \text{AT504}-509 + \text{H}_{70} \xleftrightarrow{K_D=300\text{ nM}}$$
$$\text{fXa}\cdot\text{AT504}-509\cdot\text{H}_{70} \xrightarrow{k=0.1\text{ s}^{-1}} \text{fXa}-\text{AT504}-509 + \text{H}_{70}$$

$$\text{Th} + \text{AT504}-509 + \text{H}_{70} \xleftrightarrow{K_D=80\text{ nM}}$$
$$\text{Th}\cdot\text{AT504}-509\cdot\text{H}_{70} \xrightarrow{k=0.0025\text{ s}^{-1}} \text{Th}-\text{AT504}-509 + \text{H}_{70}$$

The second-order rate constants (k_2), calculated from the ratio of the rate constants, k, to K_D values for both fXa and thrombin (Table 2), are in agreement with the values in Table 1, determined at subsaturating concentrations of the serpin using the linear equation (eq 1). Comparisons of these values with the corresponding values for the ternary wild-type serpin−heparin−proteinase complexes [$K_D = 20$ nM and $k = 3.2$ s^{-1} for thrombin; $K_D = 90$ nM and $k = 18$ s^{-1} for fXa (*22*)], as determined previously by rapid kinetic methods, suggest that the defect of mutation in both cases is primarily caused by a dramatic impairment in the rate constant of the second reaction step (Table 2).

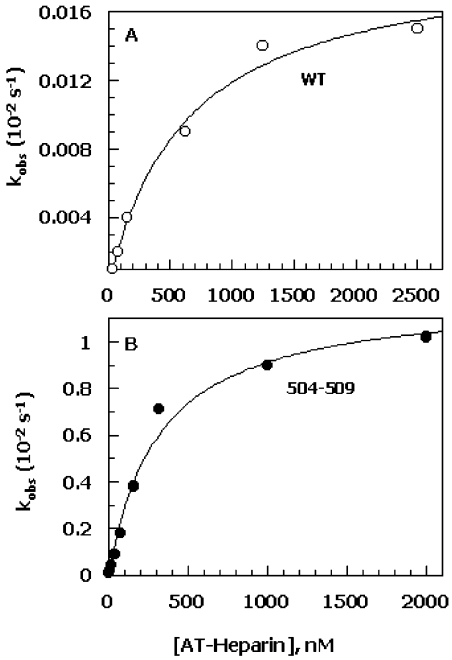

FIGURE 4: Dependence of pseudo-first-order rate constants (k_{obs}) for inactivation of APC on the heparin−AT or heparin−AT504−509 complex concentration. The k_{obs} values for the heparin-catalyzed inactivation of 2 nM APC by either the wild-type AT (○, panel A) or AT504−509 (●, panel B) were determined and analyzed as described in the legend of Figure 3.

To evaluate which kinetic step is affected in the AT−APC interaction, k_{obs} values for the H_{70} heparin-catalyzed reactions were determined for both the wild-type and mutant AT as described above. In both cases, k_{obs} values showed a saturable dependence on the concentration of the heparin−AT complex (Figure 4). Nonlinear regression analysis of the data by the hyperbolic equation (eq 2) yielded K_D and k values according to the following scheme:

$$\text{APC} + \text{AT} + \text{H}_{70} \xleftrightarrow{K_D=630\text{ nM}}$$
$$\text{APC}\cdot\text{AT}\cdot\text{H}_{70} \xrightarrow{k=0.00019\text{ s}^{-1}} \text{APC}-\text{AT} + \text{H}_{70}$$

$$\text{APC} + \text{AT504}-509 + \text{H}_{70} \xleftrightarrow{K_D=320\text{ nM}}$$
$$\text{APC}\cdot\text{AT504}-509\cdot\text{H}_{70} \xrightarrow{k=0.012\text{ s}^{-1}} \text{APC}-\text{AT504}-509 + \text{H}_{70}$$

Similarly, the second-order rate constants, calculated from the ratio of the rate constants, k, to K_D values (Table 2), are in agreement with the values in Table 1. Comparison of the k values listed in Table 2 clearly suggests that the 2 order of magnitude higher second-order rate for the APC−AT504−509 reaction is primarily due to an improvement in the rate constant of the second reaction step.

We previously demonstrated that substitution of Glu^{192} of APC with a Gln (E192Q) improves the catalytic efficiency

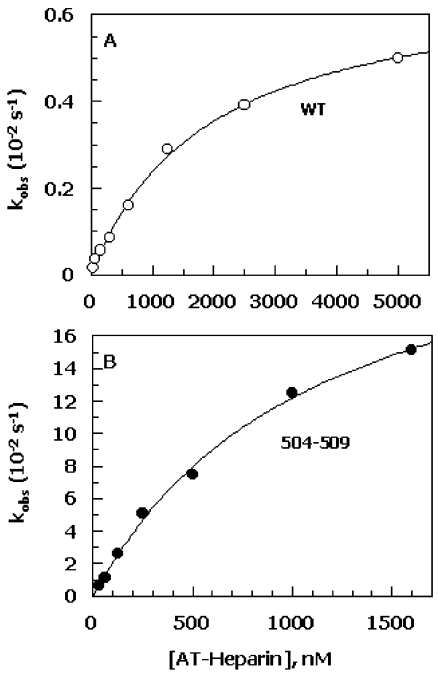

FIGURE 5: Dependence of the heparin-catalyzed pseudo-first-order rate constants (k_{obs}) for inactivation of APC E192Q on the heparin−AT or heparin−AT504−509 complex concentration. The k_{obs} values for the heparin-catalyzed inactivation of 2 nM APC by either wild-type AT (○, panel A) or AT504−509 (●, panel B) were determined and analyzed as described in the legend of Figure 3.

of the proteinase to inactivate factor Va (*13*). To determine whether this was due to better recognition of the Arg^{506} cleavage site by the mutant enzyme, k_{obs} values for the APC E192Q inhibition by the H_{70} heparin−AT504−509 complex were also determined. A saturable dependence on the AT−heparin complex concentration was also observed in the reaction with both wild-type and mutant serpin (Figure 5). Kinetic constants for the APC E192Q inhibition by the serpins are listed in Table 2. The K_D values for inhibition of the APC mutant by either the wild-type or mutant serpin were minimally affected (1.1−1.9 μM); however, relative to that of wild-type AT, the k value with the mutant serpin was improved ~38-fold (Table 2). The second-order rate constant for the AT504−509 inhibition of APC E192Q, determined at subsaturating concentrations of the mutant serpin−heparin complex [$(2.0 \pm 0.1) \times 10^5$ M^{-1} s^{-1}], was in agreement with the k/K_D value [$(2.4 \pm 0.3) \times 10^5$ M^{-1} s^{-1}] presented in Table 2. These results strongly suggest that the improvement in the catalytic function of APC E192Q toward degradation of factor Va is due to the better recognition of the Arg^{506} cleavage site by the mutant enzyme. It should, however, be noted that this previous mutagenesis approach did not improve the anticoagulant function of the APC E192Q mutant, since it became highly susceptible to inactivation by plasma serpins, including AT and α_1-antitrypsin (*9*).

To ensure that mutagenesis did not convert the mutant serpins into substrates of proteinases under study, or increase the substrate pathways of the reactions, the stoichiometries of inhibition (SI) for all proteinases were determined with mutant serpins. Consistent with previous results (*22*), an SI value of 1.2−1.5 for the wild-type AT inhibition of fXa was observed in the presence of heparin. Corresponding values for the heparin-catalyzed AT inhibition of fXa were elevated to 2.0−2.2 for AT504−509 and 2.5−2.7 for AT P2Arg mutants. The SI values for the mutant serpin inhibition of APC were ~1.2−1.5 with both serpins in the presence of heparin. Similar SI values of ~1.5 were observed for the heparin-catalyzed inhibition of thrombin by the wild-type and AT504−509 serpins; however, this value was elevated to ~2 for the AT P2Arg mutant. SDS−PAGE analysis of the proteinase−serpin complexes confirmed the kinetic results and further suggested that all three proteinases form stable complexes with both mutants of the serpin (Figure 1).

DISCUSSION

AT binds to the active site pocket of coagulation proteinases through an exposed reactive site loop with the P1Arg of the serpin fitting into the primary specificity pocket, as occurs in the reaction of serine proteinases with true substrates (*25*). Unlike the reaction with true substrates, however, attack of the P1Arg by the catalytic residue, Ser^{195}, is accompanied by a large-scale conformational change in the reactive site loop of the serpin that leads to disruption of the catalytic machinery, thereby trapping the proteinase in an inactive and kinetically stable complex (*26*, *27*). Since the initial phases of the interaction of serine proteinases with both serpins and true substrates are similar until the acylation step, the reactive site loop mutants of AT provide a good model system for investigating the extent to which the P3−P3′ residues of substrates contribute to determination of specificity in coagulation proteinases.

The results suggest that substitution of the P3−P3′ residues of AT with the corresponding residues of the APC-specific cleavage site in factor V can dramatically change the recognition specificity of the inhibitor in favor of the reaction with APC. This is derived from the observation that the several orders of magnitude difference in the reactivity of AT with target proteinases thrombin and fXa and the nontarget proteinase APC was entirely eliminated in the reaction with the AT504−509 mutant (Table 1). This was also true in the presence of heparin. Resolution of the two-step reaction of wild-type or mutant serpin with all proteinases that were studied suggested that the improvement or impairment in reactivities was primarily contained within the second reaction step in which a noncovalent serpin−proteinase encounter complex is converted to a stable, covalent complex (Table 2). It is worth noting that, relative to the heparin-catalyzed rate constant for the APC−AT reaction ($k = 0.00019$ s^{-1}), the rate of the APC E192Q−AT504−509 reaction ($k = 0.26$ s^{-1}) was improved by ~1368-fold (Table 2). These results clearly suggest that the nature of the P3−P3′ residues of the Arg^{506} cleavage site can dramatically influence the specificity of cofactor recognition by coagulation proteinases. The results further suggest that the role of these residues in proteinase specificity is to stabilize the transition state for cleavage of the scissile bond in the second step of the reaction. The observations that the reactivity of AT P2Arg with APC was improved by more than 40-fold and impaired to a nearly similar extent with both fXa and thrombin suggest that the P2Arg in factor V and P2Gly in AT are key residues at the cleavage sites that restrict the recognition specificity of both the cofactor and the serpin in the reaction with nontarget coagulation proteinases.

The implication of these results for determination of substrate specificity of APC is that following interaction of factor Va with an exosite of APC, the P3−P3′ residues of the position 506 cleavage site can dock optimally into the catalytic pocket of APC, leading to efficient cleavage of the Arg^{506} peptide bond by the anticoagulant enzyme. In support of this, recently a positively charged exosite on APC has been identified, the interaction of which with factor Va is required for efficient recognition and subsequent cleavage of the Arg^{506} bond by the proteinase (*28*, *29*). In this mutagenesis model system, the bridging function of heparin partly mimics the cofactor effect of the putative exosite interaction that facilitates the APC inactivation of the cofactor.

In summary, the results of this study suggest that productive interactions between S3−S3′ subsites of APC and P3−P3′ sites of factor Va markedly stabilize the transition state for the cleavage of the Arg^{506} bond in the second step of the reaction. Thus, the specificity of the coagulation protease reaction is determined at the level of both subsite and exosite interactions.

ACKNOWLEDGMENT

I thank Dr. Steven Olson for useful discussions and Audrey Rezaie for proofreading of the manuscript.

REFERENCES

1. Schechter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162.
2. Foster, W. B., Nesheim, M. E., and Mann, K. G. (1983) *J. Biol. Chem. 258*, 13970−13977.
3. Monkovic, D. D., and Tracy, P. B. (1990) *J. Biol. Chem. 265*, 17132−17140.
4. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A. Z., and Hemker, H. C. (1980) *J. Biol. Chem. 255*, 274−283.
5. Walker, F. J., and Fay, P. J. (1992) *FASEB J. 6*, 2561−2567.
6. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) *J. Biol. Chem. 269*, 31869−31880.
7. Esmon, C. T. (1993) *Thromb. Haemostasis 70*, 1−5.
8. Rezaie, A. R. (1997) *Biochemistry 36*, 7437−7446.
9. Rezaie, A. R., and Esmon, C. T. (1996) *Eur. J. Biochem. 242*, 477−484.
10. Rezaie, A. R., and Esmon, C. T. (1995) *J. Biol. Chem. 270*, 16176−16181.
11. Rezaie, A. R. (1996) *J. Biol. Chem. 271*, 23807−23814.
12. Neuenschwander, P. F., and Morrissey, J. H. (1995) *Biochemistry 34*, 8701−8707.
13. Rezaie, A. R., and Esmon, C. T. (1993) *J. Biol. Chem. 268*, 19943−19948.
14. Ahang, Y., Hervio, L., Strandberg, L., and Madison, E. L. (1999) *J. Biol. Chem. 274*, 7153−7156.
15. Rosing, J., Hoekema, L., Nicolaes, G. A. F., Thomassen, M. C. L. G. D., Hemker, H. C., Varadi, K., Schwarz, H. P., and Tans, G. (1995) *J. Biol. Chem. 270*, 27852−27858.
16. Walker, F. J. (1980) *J. Biol. Chem. 255*, 5521−5524.
17. Rezaie, A. R., and Yang, L. (2001) *Biochim. Biophys. Acta 1528*, 167−176.
18. Nordenman, B., Nystrom, C., and Björk, I. (1977) *Eur. J. Biochem. 78*, 195−203.
19. Olson, S. T., Bjork, I., and Shore, J. D. (1993) *Methods Enzymol. 222*, 525−560.
20. Rezaie, A. R. (2001) *J. Biol. Chem. 276*, 15567−15570.
21. Rezaie, A. R. (1998) *J. Biol. Chem. 273*, 16824−16827.
22. Rezaie, A. R., and Olson, S. T. (2000) *Biochemistry 39*, 12083−12090.
23. Olson, S. T., Björk, I., Sheffer, R., Craig, P. A., Shore, J. D., and Choay, J. (1992) *J. Biol. Chem. 267*, 12528−12538.
24. Olson, S. T., and Shore, J. D. (1982) *J. Biol. Chem. 257*, 14891−14895.
25. Gettins, P. G. W., Patston, P. A., and Olson, S. T. (1996) in *Serpins: Structure, Function and Biology*, pp 33−63, R. G. Landis Co., Austin, TX.
26. Lawrence, D. A. (1997) *Nat. Struct. Biol. 4*, 339−341.
27. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) *Nature 407*, 923−926.
28. Friedrich, U., Nicolaes, G. A. F., Villoutreix, B. O., and Dahlbäck, B. (2001) *J. Biol. Chem. 276*, 23105−23108.
29. Yang, L., Manithody, C., and Rezaie, A. R. (2002) *Biochemistry 41*, 6149−6157.
30. Hopkins, P. C. R., Crowther, D. C., Carrell, R. W., and Stone, S. R. (1995) *J. Biol. Chem. 270*, 11866−11871.

BI0261443