Conformational Conversion of Antithrombin to a Fully Activated Substrate of Factor Xa without Need for Heparin†

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ABSTRACT: Regulation of the inhibitory activity of antithrombin, the principal inhibitor of the blood-clotting proteinases factor Xa and thrombin, is accomplished by binding to heparin. We report here an antithrombin variant in which serine at position 380, 14 residues N-terminal from the reactive bond and at a hinge point in the structure, was replaced by cysteine to test a proposed mechanism of heparin activation of antithrombin as an inhibitor of factor Xa. By derivatization of this cysteine with a bulky group, fluorescein, the antithrombin became permanently and fully activated toward reaction with factor Xa in a manner analogous to heparin activation, albeit as a substrate. These findings establish a structural basis for the mechanism of heparin activation of antithrombin against factor Xa in agreement with that proposed from an X-ray structure of antithrombin.

Temporal and spatial regulation of the inhibitory activity of antithrombin, the principal inhibitor of the blood-clotting proteinases factor Xa and thrombin, is accomplished by binding to endogenous heparin (*2, 3*). This accounts both for the occurrence of thrombosis in patients whose antithrombin has a defect in heparin binding or activation (*4, 5*) and for the widespread clinical use of exogenous heparin as an anticoagulant (*6*). Heparin increases the rate at which human plasma antithrombin inhibits factor Xa by 200−300-fold as a result of a specific heparin-induced conformational change (*7*). The mechanism of conformational activation of antithrombin by heparin against factor Xa has, however, yet to be established. This same conformational change causes less than a 2-fold increase in the rate of inhibition of thrombin (*7*).

We have shown previously that this heparin-induced conformational change is manifested directly at the reactive center P1−P1′ [1] bond (*8*), which suggests that the activation mechanism involves adoption of a conformation for the residues at and adjacent to the P1−P1′ bond that has better substrate-like complementarity for the active site of factor Xa, but not for the active site of thrombin. Recently, we provided evidence in support of a proposal of how the conformation of the reactive center-containing loop is perturbed by heparin binding (*9*). It had been proposed, based on X-ray structures of a heterodimeric form of antithrombin (*10, 11*), that the reactive center loop of antithrombin, which stretches from residues P16 to P3′, is constrained in the native state by having residues P15 and P14 (residues 379 and 380) incorporated as part of β-sheet A and with the side chain of residue P14 consequently being buried in the protein interior (Figure 1) (*10, 12*). It was further proposed that, upon heparin binding, residues P15 and P14 are expelled from the sheet, removing constraints on the reactive center loop and permitting a more appropriate conformation of the reactive center loop for interaction with factor Xa (Figure 1). In a P14 S → W variant, we showed that heparin binding resulted in a major change in environment of the P14 residue, consistent with a change from buried to exposed environments (*9*) and thus consistent with the model.

A major prediction from this model is that, if the P14 residue could be displaced from β-sheet A, without heparin present, the rate of reaction with factor Xa would be as fast as for that of heparin-activated antithrombin. We present here a verification of this prediction and thereby establish that the proposed mechanism of heparin activation of antithrombin is correct.

MATERIALS AND METHODS

Protein Isolation and Labeling. The S380C[2] mutation was introduced on an N135Q variant background to reduce carbohydrate heterogeneity (*13*) and was accomplished by site-directed mutagenesis using the Altered sites mutagenesis kit (Amersham) and the manufacturer's directions. S380C variant antithrombin was isolated from the growth medium

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[1] The nomenclature of Schechter and Berger (*1*) is used to designate residues on a proteinase substrate close to the peptide bond that is cleaved. The residues that form the scissile bond are designated P1 and P1′. Residues N-terminal to P1 are designated P2, P3, ..., etc. and those C-terminal to P1′ are designated P2′, P3′, ..., etc.

[2] Abbreviations: CD, circular dichroism; SDS, sodium dodecyl sulfate; 5-IAF, 5-iodoacetamido-fluorescein; N135Q, variant of antithrombin in which asparagine 135 has been mutated to glutamine to eliminate one site of glycosylation; S380C, variant of antithrombin containing a serine-to-cysteine mutation at position 380 on an N135Q background.

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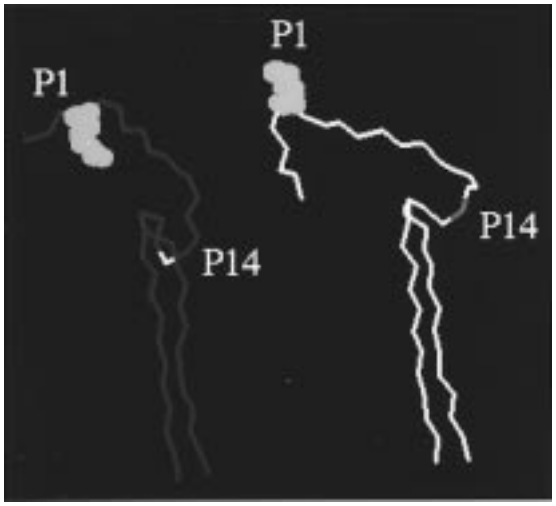

FIGURE 1: Illustration of the model proposed by van Boeckel et al. (*10*) for the heparin-induced conformational change in the reactive center of antithrombin, using published X-ray structures of native serpins. Essentially equivalent structural changes for the loop-inserted and loop-expelled conformations of the reactive center loop have also been proposed by Elliott and colleagues (*12*). Left, stucture of antithrombin, showing monomer in which the P15 and P14 residues are inserted into β-sheet A. Right, structure of α_1-proteinase inhibitor variant (*12*). The regions shown in each structure are the central strands of β-sheet A (strands 3 and 5) and the reactive center loop region extending from P16 to P3′, which is connected directly to strand 5 of β-sheet A. The residue shown in space filling is P1, the residue shown as differently colored backbone is P14, residue 380 in antithrombin. In the absence of heparin the distal end of the reactive center loop (residues P15 and P14) is integrated into β-sheet A. Upon heparin binding, the P15 and P14 residues are displaced, giving a fully exposed and possibly more flexible reactive center loop. Note the change in position of the P14 and P1 residues and the difference in stucture of the intervening residues. It was proposed that in the P14-expelled structure the reactive center bond is attacked much more rapidly by factor Xa but not by thrombin. It is possible that there is an equilibrium between inserted and expelled conformations with the heparin bound state being nearly completely loop expelled and the native conformation nearly completely loop inserted.

of confluent, stably transfected BHK cells by published procedures (*14*). The variant was isolated by chromatography on heparin-agarose and mono-Q. The P14 cysteine was labeled with fluorescein by incubation of the variant with an excess of 5-IAF in the presence of heparin-agarose to promote exposure of the P14 side chain. Labeled antithrombin was eluted with high salt, diluted to low salt, and rechromatographed on heparin-agarose. A stoichiometry of labeling of 0.7 fluorescein/antithrombin was obtained. Dansyl labeled-P14C antithrombin was prepared by a similar procedure of binding the P14C variant to heparin-agarose and then reacting with a 400-fold excess of didansyl cysteine (Molecular Probes) overnight at room temperature. Antithrombin concentrations were determined spectrophotometrically using the extinction coefficient of the plasma protein of 31 200 M^{-1} cm^{-1} (*15*).

Rates of Inhibition of Thrombin and Factor Xa. Second-order rate constants were determined at 25 °C for the reaction of control N135Q and S380C antithrombin with thrombin and factor Xa under pseudo-first-order conditions in inhibitor and 10 nM proteinase in the presence of Polybrene. The second-order rate constants were calculated from observed pseudo-first-order rate constants, obtained from the slope of the log of residual proteinase activity vs time, divided by the inhibitor concentration.

Fluorescence Measurements. Measurements of the reaction of factor Xa and thrombin with fluorescein-labeled S380C antithrombin were followed on an SLM8000 spectrofluorimeter by change in emission intensity of fluorescein, with excitation at 491 nm and observation of emission at 520 nm. Slits of 4 and 16 nm were used for excitation and emission, respectively. Data were fitted to a sequential, two step model in which the first step is spectroscopically silent and represents initial cleavage of the P1−P1′ bond (k_{cat}/K_m) and the second step represents the conformational change that results in loop insertion and change in label fluorescence. Reactions were carried out at least in duplicate for a given concentration of proteinase and antithrombin and the determined rate constants were shown to be independent of substrate (antithrombin) concentration in the concentration range studied. Data were fitted by nonlinear least-squares analysis using the program Scientist (MicroMath Inc., Salt Lake City, UT). Steady-state fluorescence emission spectra were recorded in 2 nm steps using slits of 2 nm for excitation and emission and an integration time of 5 s. For dansyl, emission excitation was at 328 nm and for fluorescein emission excitation was at 491 nm.

Gel Electrophoresis. Polyacrylamide gels (10%) were run under denaturing, but nonreducing conditions according to the procedure of Laemmli (*16*). Bands were visualized by fluorescence and photographed. Scanned image maps of the photographs were inverted, to permit quantitation by densitometry.

Rate of Reaction of Variant with 5-IAF. The rates of reaction of the S380C variant with 5-IAF in the absence or presence of heparin were determined from quantitation of fluorescein fluorescence associated with the antithrombin band on a polyacrylamide gel after reaction with 5-IAF. Antithrombin (20 μM) was reacted with a 10-fold molar excess of 5-IAF at pH 7.4 at 4 °C in the dark for different lengths of time in the presence of a 12-fold molar excess of high-affinity heparin or in its absence. The reactions were quenched by addition of L-cysteine to a concentration of 200 mM. The samples were run on 10% polyacrylamide gels under nonreducing conditions and visualized and quantitated as described above.

Heparin Affinities. Heparin-antithrombin dissociation constants for control and S380C variant antithrombins were determined by monitoring change in intrinsic fluorescence of antithrombin upon titration with stock solution of high-affinity heparin. Excitation and emission were, respectively, at 280 and 340 nm with slits of 2 and 16 nm. The dissociation constant for the fluorescein-labeled S380C variant was determined from change in fluorescence of fluorescein, with excitation at 491 nm and emission at 520 nm. All measurements were made at I 0.6 to weaken the heparin affinity sufficiently to enable accurate determination of the K_d for the high-affinity binding to the fluorescein-labeled S380C variant.

CD Measurements. CD measurements were made on a Jasco 710 spectropolarimeter in a 1 mm path-length jacketed cell. Temperature was controlled by a Neslab water bath. Antithrombin concentrations were 0.25−0.5 mg/mL in I 0.15 buffer (20 mM NaPi, 100 mM NaCl, 0.1 mM EDTA, 0.1% PEG 8000, pH 7.4). All solutions were degassed. A

bandwidth of 2 nm, step resolution of 0.5 °C, response time of 16 s, and rate of temperature change of 0.5 °C/min were used. The denaturation temperature (T_d) was determined from the minimum of the first derivative of the ellipticity at 220 nm against temperature to make more clear the midpoint of the transition. The reported values are the mean of two separate readings for each sample.

RESULTS AND DISCUSSION

We expressed a P14 S → C variant of antithrombin (S380C) to provide a means of introducing a bulky group at the P14 position by chemical modification and thus to lock the antithrombin in a conformation with the P14 residue exposed, rather than buried. By locking the reactive center loop in this conformation, we were then able to determine the rate of reaction with factor Xa and thrombin to test the hypothesis that this loop-expelled conformation represented that of antithrombin in the heparin-induced fully activated state.

Prior to modification of the P14 side chain, the variant was able to form normal 1:1 covalent complexes with blood-clotting proteinases factor Xa and thrombin, as demonstrated by the appearance of higher molecular weight SDS-stable bands of covalent complexes on polyacrylamide gels (Figure 2a). Measurements of the rates of inhibition of both factor Xa and thrombin showed no increase from that of control antithrombin (Table 1). In fact, the rate of inhibition of factor Xa was reduced 4-fold compared to control (from 4×10^3 to 1×10^3 M^{-1} s^{-1}). These properties are consistent with the serine-to-cysteine mutation not altering the fundamental properties of the antithrombin.

The reduction in rate of inhibition of factor Xa is, however, significant, since it provides further support for the existence of an equilibrium between a low activity (or even inactive) P14-inserted conformation and a high activity P14-expelled conformation for antithrombin (*8, 9*) and implies that the S → C change has further shifted the equilibrium toward the loop-inserted conformation to give an overall lower basal rate of inhibition of factor Xa. This is supported by the finding of an increase in denaturation temperature of 2.3 °C for the variant compared to control antithrombin (Table 1), as determined by the midpoint of the CD-monitored unfolding transition. This increase in stability indicated that the native, P14-inserted, state had been stabilized by the change from serine to cysteine. Also consistent with this was a lower heparin affinity for this variant compared to wild-type (K_d of 1900 compared to 78 nM at I 0.6) (Table 1), which would be expected if more of the heparin binding energy were required to expel the P14 side chain (*9*). The same type of linkage between thermal stability and heparin affinity has been seen previously with another antithrombin P14 variant (S380W) (*9*), except that for the S380W variant the mutation had the opposite effect of reducing the stability of the antithrombin native state and increasing the heparin affinity. For the S380W variant, the basal rate of factor Xa inhibition was also increased ~3-fold, consistent with a shift in equilibrium toward the loop-expelled state. From the activities of heparin-complexed and uncomplexed antithrombins, it can be estimated that such an equilibrium would be >99% in favor of the P14-inserted conformation for both wild-type and S → C variant, but may have even less of the P14-expelled conformation for the variant (<0.2%) than the

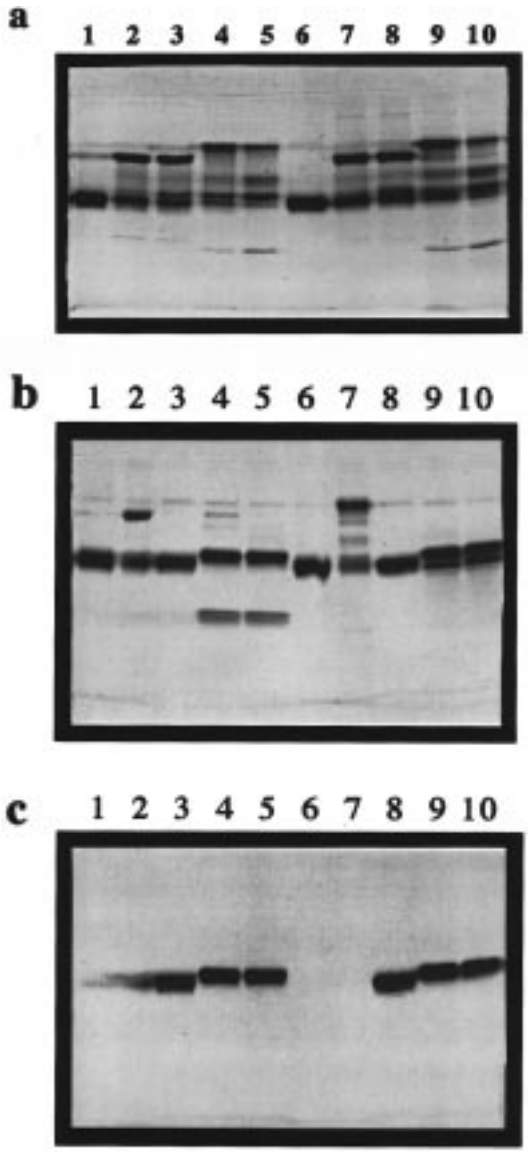

FIGURE 2: Demonstration of the ability of S380C variant antithrombin to form normal SDS-stable complex with thrombin and factor Xa and of complete conversion to substrate reaction following modification of the cysteine by the bulky fluorescein group. (Panel a) Similar ability of wild-type and P14C variant antithrombins to form SDS-stable complex with factor Xa and thrombin. Lanes 1−5, wild-type antithrombin, lanes 6−10, P14C antithrombin. Lanes 1 and 6, unreacted; lanes 2 and 7, reacted with thrombin; lanes 3 and 8, reacted with thrombin in the presence of heparin; lanes 4 and 9, reacted with factor Xa; lanes 5 and 10, reacted with factor Xa in the presence of heparin. For lanes 2−5 and 7−10, the lowest mobility band corresponds to covalent, SDS-stable complex, whereas the band with slightly lower mobility than unreacted antithrombin is antithrombin cleaved in the reactive center as a substrate. Bands were visualized by Coomassie staining. (Panel b) Loss of ability to form SDS-stable complex after labeling with fluorescein. Lanes 1 and 6, P14C antithrombin and complexes with thrombin (lane 2) and factor Xa (lane 7). Lanes 3 and 8, P14C-fluorescein antithrombin and resulting cleavage by thrombin (lanes 4 and 5) and factor Xa (lanes 9 and 10), in the presence of heparin (lanes 5 and 10) or its absence (lanes 4 and 9). Although a small amount of complex can be seen in lane 4, this is due to a small contamination with nonlabeled P14C variant, since detection by fluorescence of the same gel (panel c) shows fluorescein fluorescence only at the positions for unreacted variant (lanes 3 and 8) or cleaved labeled variant (lanes 4, 5, 9, and 10). Panel b is the Coomassie stained gel, whereas panel c is the inverted map of the same gel detected by fluorescence.

Table 1: Fluorescein Derivatization of P14C Results in Large Increase in Rate of Reaction of Variant with Factor Xa, but Not with Thrombin, Increase in Affinity for Heparin, and Decrease in Thermal Stability

antithrombin	k_{cat}/K_m (M^{-1} s^{-1}) factor Xa	k_{cat}/K_m (M^{-1} s^{-1}) thrombin	heparin K_d (nM)[a]	T_d (°C)[b]
control	4×10^3	6.0×10^3	78 ± 4	57.3 ± 0.1
control + psacc[c]	6×10^5	1.5×10^4	na[e]	
control + heparin[d]	1.3×10^6	nd		
P14C	1.02×10^3	6.1×10^3	1900 ± 80	59.6 ± 0.1
P14C + heparin[d]	4.6×10^5	nd	na	
P14C-fluorescein[f]	2×10^5	7.3×10^3	na	
P14C-fluorescein[g]	4.7×10^5	2.1×10^4	6.2 ± 0.2	51.2 ± 0.3

[a] Dissociation constant for binding of full-length high-affinity heparin at I 0.6. [b] Determined from the midpoint of the CD-monitored temperature denaturation profile. [c] From ref *7* for accelerated rate due to heparin pentasaccharide-induced conformational change. [d] Rates of inhibition in the presence of full-length high affinity heparin. [e] na, not applicable. [f] Rate constants determined from scan of SDS−PAGE gel. [g] Rate constants determined from change in fluorescein fluorescence.

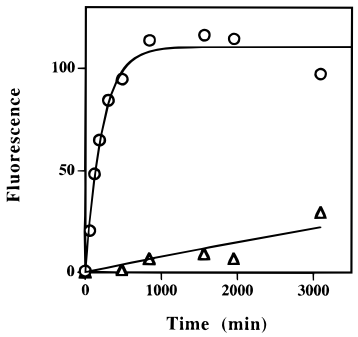

FIGURE 3: Large increase in reactivity toward 5-IAF of P14 cysteine in the presence of heparin. Time dependence of incorporation of fluorescein into S380C antithrombin variant in the absence (triangles) or presence (circles) of heparin. Reaction conditions and means of quantitation are given in the Materials and Methods.

wild-type (<0.7%). For the S380W variant, there may be as much as 1% of the loop-expelled conformation.

Such an equilibrium between loop-inserted and loop-expelled conformers is likely to be peculiar to antithrombin and to reflect the additional level of allosteric regulation of this serpin. Thus, in α_1-proteinase inhibitor, another member of the same serpin family of proteinase inhibitors as antithrombin, X-ray structures show no evidence for insertion of the P14 side chain in the native state (*12*, *17*) and consequently the stability of the protein is not affected by even drastic mutations at the P14 position, such as threonine-to-arginine (*18*).

In keeping with the dominant (>99%) conformation of the antithrombin having a buried P14 side chain in the absence of heparin, the reactivity of the P14 cysteine was very much less in the absence than in the presence of heparin. The second-order rate constant for reaction with 5-IAF in the absence of heparin was determined to be approximately 64-fold slower than in the presence of heparin (Figure 3) (30 M^{-1} s^{-1} vs 1920 M^{-1} s^{-1}, respectively), from quantitation of the intensity of fluorescein fluorescence of antithrombin on SDS gels. This very large measured difference may represent reaction only for the loop-expelled conformation, with different apparent rate constants resulting from very different concentrations of the reactive, loop-expelled species, or from reaction for both conformations, but with very

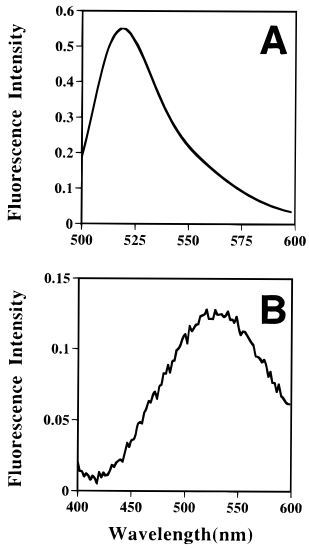

FIGURE 4: Fluorescence emission spectra of fluorescein and dansyl-labeled S380C antithrombin. Panel a, emission spectrum of P14C-fluorescein antithrombin (0.5μM). Excitation was at 491 nm. Panel b, emission spectrum of P14C-dansyl antithrombin (0.3 μM). Excitation was at 328 nm.

different rates. Given the buried location of the serine oxygen in wild-type antithrombin (*10*), it is more likely that the different rates of reaction result from reaction only with the loop-expelled conformer but with very different concentrations of the loop-expelled conformation in the absence (<1%) and presence (>99%) of heparin.

Because of the lower rate of reaction with the cysteine in the native state, the side chain could only be preparatively labeled when heparin was bound. The variant was therefore labeled at P14 with fluorescein by reaction with 5-IAF in the presence of heparin-agarose and subsequently repurified as described above. As expected from the anticipated locking of the derivatized antithrombin into a P14-expelled conformation, the introduction of the bulky fluorescein group at P14 greatly increased the affinity for heparin (from K_d of 1900 nM to 6.2 nM at I 0.6) (Table 1), while decreasing the stability of the derivative by 8.4 °C (Table 1). Evidence that the P14 side chain was exposed rather than buried in the protein interior in the P14-derivatized state was obtained from the emission spectrum of the attached fluorescein, which had the same wavelength maximum, 522 nm, as fluorescein free in solution at this pH (Figure 4a). A second fluorescent P14C derivative, containing the more environmentally sensitive dansyl fluorophore, also showed clear evidence for solvent exposure. The emission maximum of 525 nm (Figure 4b) was the same as for the free acetyl derivative (*19*), which is about 30 nm red-shifted from protein-bound dansyl in more buried locations (*19*).

The derivatization also converted the variant into a complete substrate of factor Xa and thrombin (Figure 2, panels b and c), presumably by slowing down the rate of insertion of the reactive center loop into β-sheet A during inhibition and consequently interfering with the efficiency of the inhibition mechanism. At the same time, the rate of reaction with factor Xa was greatly increased, as expected from the proposed mechanism of heparin activation. This was examined by SDS−PAGE (Figure 5), which showed very rapid cleavage of the antithrombin in the reactive center loop by factor Xa, with an estimated rate constant of 2 ×

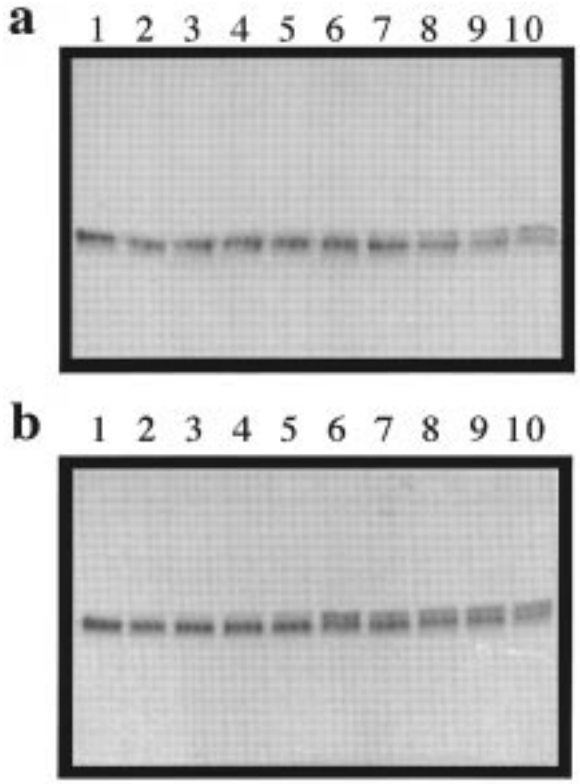

FIGURE 5: Fluorescein labeling of the P14 cysteine of S380C antithrombin resulted in a very large increase in the rate of reaction with factor Xa but not thrombin. SDS−PAGE run under nonreducing conditions of time courses of reaction of fluorescein-labeled S380C antithrombin with factor Xa (top panel) and thrombin (bottom panel), showing conversion of antithrombin from intact to P1−P1′-cleaved species (cleavage results in a reduction in mobility under nonreducing conditions). The concentrations of proteinase are very different and were chosen to give similar extents of cleavage for the same time of reaction despite the very different rate constants for reaction with the two proteinases. (Panel a) 3.45 μM P14C-fluorescein antithrombin, 2 nM factor Xa; (panel b) 3.45 μM P14C-fluorescein antithrombin, 64 nM thrombin. Samples were removed at times of 0, 30, 60, 120, 240, 360, 600, 900, 1200, and 1500 s, and the reaction with factor Xa was stopped by addition of the irreversible inhibitor FFR-chloromethyl ketone, followed after 20 s of reaction by HCl to lower the pH to 3.0, whereas the reaction with thrombin was stopped by addition of PMSF.

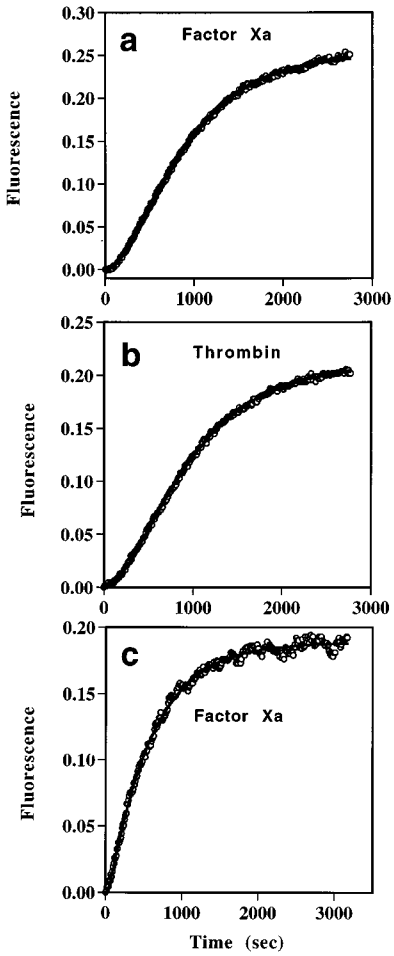

FIGURE 6: Determination of the rate constants for cleavage of the labeled variant from changes in the fluorescence intensity of the P14-fluorescein. Data are shown for reaction with both factor Xa (panel a) and thrombin (panel b) under conditions where pronounced sigmoidal behavior is evident (44 nM antithrombin and 10 nM factor Xa or 80 nM thrombin) and for factor Xa (panel c) where more hyperbolic change is seen as a result of more rapid cleavage of the P1−P1′ bond due to higher proteinase concentration (44 nM antithrombin, 50 nM factor Xa).

10^5 M^{-1} s^{-1}. In contrast, the rate of reaction with thrombin was unperturbed by the derivatization and gave an approximate rate constant of 7×10^3 M^{-1} s^{-1}. This much smaller effect on thrombin was also as expected, since the conformational change induced in antithrombin by heparin causes, at most, a 2-fold increase in the rate of inhibition of thrombin (*7*).

An independent determination of the rate constants for reaction with both factor Xa and thrombin was obtained, at lower antithrombin concentration, by using change in fluorescence of the fluorescein fluorophore upon reaction of the derivatized variant with proteinase. From the perspective of the antithrombin the reaction was a two-step process, with initial cleavage of the exposed P1−P1′ bond by proteinase causing no change in fluorescence of the exposed P14 fluorescein, followed by insertion of the cleaved reactive center loop into β-sheet A with a perturbation of the environment of the P14 side chain and thus of the fluorescein fluorescence. Consequently, the time courses were sigmoidal, except when the proteinase concentration was so high that cleavage was complete before significant loop insertion could occur (Figure 6). Fitting of these time courses (see Materials and Methods) gave rate constants both for the cleavage reaction by proteinase (Table 1) and for the subsequent rate of insertion of the cleaved reactive center loop. The second-order rate constants for reaction of factor Xa and thrombin with derivatized S → C variant antithrombin (4.7×10^5 M^{-1} s^{-1} and 2.1×10^4 M^{-1} s^{-1}, respectively) were very similar to those reported for antithrombin that has been conformationally activated by heparin pentasaccharide (6×10^5 M^{-1} s^{-1} and 1.5×10^4 M^{-1} s^{-1} respectively) and represented a 470-fold increase in rate of reaction toward factor Xa compared with the underivatized variant but only a 3-fold increase in rate of reaction toward thrombin. The similar rates of reaction of heparin pentasaccharide-activated wild-type antithrombin and the derivatized P14C variant demonstrate that the locked P14-expelled conformation of the reactive center loop of the derivatized variant must be very similar to that of heparin-complexed antithrombin. A direct determination of the heparin-accelerated rate of inhibition of factor Xa by the P14C variant gave a value of 4.6×10^5 M^{-1} s^{-1} (Table 1), which is an increase of 350-

fold over the basal rate in the absence of heparin, though it is 3-fold lower than the rate with wild-type antithrombin. However, even if the contribution to rate acceleration from heparin bridging between antithrombin and factor Xa is still 2−3-fold, this still leaves a conformationally induced acceleration of the rate of inhibition of 120−175-fold for the P14C variant. This variant is therefore capable of showing the same kind of conformationally induced large acceleration of the rate of interaction with factor Xa as is wild-type antithrombin and makes it likely that the conformations of the reactive center loop of the P14C variant in the heparin complex and in the cysteine-derivatized state are very similar, though perhaps not identical.

The rate constants for the loop insertion step were very slow (1.6 and 2.1 $\times$ 10^{-3} s^{-1} for reaction with factor Xa and thrombin, respectively), which readily accounts for the inability of the variant to act as an inhibitor with either proteinase by the serpin suicide substrate branched pathway mechanism, since the proteinase would have ample time to complete the cleavage reaction and dissociate before loop insertion could occur (*20*), so that the substrate branch of the pathway would out-compete the inhibitory branch.

Our findings thus demonstrate that the heparin-activated state of antithrombin is one in which the reactive center loop is fully exposed as far as P14 or P15 and thereby validate the proposal of van Boeckel and colleagues of how heparin activates antithrombin toward reaction with factor Xa. In addition, our findings indicate that, if expulsion of P14 could be accomplished without the need for placing a bulky group on P14, the rate of subsequent loop insertion during reaction with proteinase might not be significantly reduced. Such a variant would then have the properties of being both selectively activated toward reaction with factor Xa and of being a good inhibitor of this proteinase. Changes in the region linking the heparin binding site to β-sheet A may well be able to introduce such properties.

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