Conformational Change in Antithrombin Induced by Heparin, Probed with a Monoclonal Antibody against the 1C/4B Region†

Joan Dawes,*,‡ Keith James,§ and David A. Lane‖

Heart Research Institute, 145 Missenden Road, Camperdown, NSW 2050, Australia, Department of Surgery, University of Edinburgh, Edinburgh, U.K., and Department of Haematology, Charing Cross and Westminster Medical School, Hammersmith, London, U.K.

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ABSTRACT: A murine monoclonal antibody (MAb) raised against a covalent antithrombin–heparin complex was used to probe the conformational change resulting when the serpin antithrombin binds to heparin. This MAb completely inhibited the progressive activity of antithrombin against thrombin. However, although the MAb remained bound to antithrombin in the presence of heparin, it did not significantly inhibit heparin cofactor activity against thrombin, and increasing concentrations of the antithrombin-binding pentasaccharide progressively unblocked the inhibitory action of the MAb. The MAb bound to antithrombin without affecting either heparin-binding affinity or heparin-induced fluorescence enhancement, and it did not convert antithrombin from inhibitor to substrate. The MAb failed to interact with reduced and *S*-carboxymethylated antithrombin, indicating the conformational nature of its epitope. Antithrombin variants with N-terminal substitutions (Arg47→Cys or His, Leu99→Phe, Arg129→Gln) modifying heparin binding, and C-terminal substitutions affecting the reactive site (Arg393→Cys) or resulting in substrate-variant antithrombin (Ala384→Pro), were all recognized normally, as were normal reactive site cleaved antithrombin and the thrombin–antithrombin complex. However, interaction of the MAb with antithrombin was reduced by several substitution mutations (Phe402→Cys, Phe402→Ser, Phe402→Leu, Ala404→Thr, Pro407→Thr) in the 402–407 sequence which codes for amino acid residues of strand 1C and the polypeptide leading to strand 4B. Pro429→Leu also blocks recognition [Olds et al. (1992) *Blood* 79, 1206–1212], and this residue is believed to be spatially approximated to strand 1C. These observations support the concept of a mobile reactive loop which is conformationally linked to the heparin-binding site, but is independently mobile of the adjacent strand 1C/4B region. When heparin binds to antithrombin, spatial separation of a thrombin-binding site and the 1C/4B region appears to increase.

Antithrombin, also known as antithrombin III and heparin cofactor, is the most important physiological inhibitor of thrombin and the other proteinases generated during the activation of blood coagulation (Abildgaard, 1969; Rosenberg & Damus, 1973; Bjork et al., 1989), and a deficiency of functional antithrombin results in an increased risk of thromboembolism. It is a member of the serine protease inhibitor (serpin) superfamily, a single-chain glycoprotein the primary structure of which has been elucidated by protein and cDNA sequencing (Petersen et al., 1979; Chandra et al., 1983). The structures of the serpins can be related to a model derived from the crystallographic studies of Lobermann et al. (1984) of α_1-proteinase inhibitor (α_1-antitrypsin), with nine α-helices denoted A–I constituting 30% of the molecule and three β-sheets (A–C) making up a further 40%. This model has been validated for cleaved bovine antithrombin (Mourey et al., 1990) and extended to intact serpins using ovalbumin, the superfamily prototype (Stein et al., 1991). The crystallographic structure of intact human antithrombin has not yet been described, though preliminary data have been reported (Schreuder et al., 1993).

Unlike most serpins, however, antithrombin has two distinct functional domains: the reactive site and the heparin-binding site. The reactive site at the Arg393–Ser394 bond acts as a pseudosubstrate for the target proteinase, and a conformational change in the inhibitor then traps the proteinase in a highly stable inactive complex. The rate of this reaction is low in the absence of heparin, but is accelerated over 2000-fold in its presence, with heparin acting as a catalyst [reviewed by Bjork et al. (1989)]. Interaction of heparin molecules containing a unique pentasaccharide sequence (Choay et al., 1983) with the heparin-binding site of antithrombin induces a conformational change in the inhibitor, accompanied by spectroscopic changes indicative of alterations in the environments of tryptophan (Einarsson & Andersson, 1977; Olson & Shore, 1981), histidine, and basic amino acid residues (Gettins, 1987). It is clear from extensive studies involving limited proteinase digestion, chemical modification of specific amino acid residues, and investigation of congenital variants of antithrombin which do not bind heparin [reviewed by Lane et al. (1992b)] that the heparin-binding site is not a linear sequence of amino acids but includes residues from widely separated regions of the molecule. Borg et al. (1988) have proposed that seven basic residues are aligned to form a primary contact positive site stretching across the molecule from the A helix (Arg47) to the D helix (Lys125, Arg129, Arg132), and the additional residues Lys136 (Chang, 1989), Lys257, and Lys275 (Peterson et al., 1987) have also been implicated in heparin binding. However, congenital variants in strand 1C near the C-terminus also have reduced affinity for heparin which has yet to be explained, though it may be due to

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‡ Heart Research Institute.
§ University of Edinburgh.
‖ Charing Cross and Westminster Medical School.

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alterations in the secondary structure of the molecule (Lane et al., 1992a).

The conformational change induced in antithrombin by heparin remains to be fully described at a molecular level. In this study, a monoclonal antibody (MAb)[1] raised against a covalent heparin–antithrombin complex has been used to examine this conformational change in relation to the ability of antithrombin to inhibit thrombin. The results provide further evidence of the importance of strand 1C, the sequence leading into strand 4B and the hydrophobic region at the C-terminus, and suggest that the relationship of the active-site region itself to these contiguous parts of the molecule is modified when heparin binds to antithrombin.

EXPERIMENTAL PROCEDURES

Materials. Human antithrombin was either purified from fresh citrated plasma by affinity chromatography on heparin–agarose (Miller-Andersson et al., 1974) or purchased from Stago (purified protein) or Kabi Diagnostica (containing human serum albumin). The chromogenic substrate S-2236 was from Kabi Diagnostica and heparin–agarose from Kemen-Tec, Copenhagen, Denmark. Porcine mucosal heparin, human thrombin, and $NaCNBH_3$ were purchased from Sigma. Rabbit polyclonal antibody against antithrombin and alkaline phosphatase-conjugated affinity-purified swine and rabbit immunoglobulins to rabbit and mouse immunoglobulins were from Dakopatts A/S (Denmark). Streptavidin–alkaline phosphatase conjugate, nitrocellulose membranes, and $Na^{125}I$ were from Amersham and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (sodium salt) from Boehringer-Mannheim. The antithrombin-binding pentasaccharide was a generous gift of Dr. J. Choay. Plasma samples containing the antithrombin congenital variants Alger (Arg47→Cys), Paris 1 (Arg47→Cys), Padua 1 (Arg47→His), Budapest 3 (Leu99→Phe), Geneva (Arg129→Gln), Charleville (Ala384→Pro), Northwick Park (Arg393→Cys), Rosny (Phe402→Cys), Torino (Phe402→Ser), Maisons-Laffitte (Phe402→Leu), Paris 3 (Ala404→Thr), La Rochelle (Asn405→Lys), and Budapest 5 (Pro407→Thr) were kind gifts of Drs. A.-M. Fischer, M. Aiach, A. Girolami, D. Howarth, G. Sas, J. Conard, M.-H. Horellou, and G. Tamponi.

Preparation of Heparin–Antithrombin Complex. Heparin oligosaccharides were prepared by partial deaminative cleavage with nitrous acid essentially as described by Thunberg et al. (1982), and without further fractionation were covalently linked to the heparin-binding site of antithrombin by reduction of the unstable Schiff base formed between the terminal anhydromannose residue on the oligosaccharide and the amino group of a neighboring lysine (Bjork et al., 1982). Briefly, antithrombin (40 mg) was incubated overnight at room temperature with 40 mg of depolymerized heparin (140 units/mg against factor Xa, but no detectable activity against thrombin) and 30 mg of $NaCNBH_3$ in 20 mL of 0.1 M phosphate buffer, pH 7.5. The reaction mixture was then extensively dialyzed against 0.1 M Tris-HCl buffer, pH 7.4, containing 2 M NaCl, concentrated by ultrafiltration, and chromatographed on a 3 $cm^2 \times 95$ cm column of Sephadex G75 to remove unbound heparin. Protein-containing fractions were pooled, diluted to a concentration of 0.15 M NaCl, concentrated by ultrafiltration, and fractionated by fast protein liquid chromatography (Pharmacia) on a Mono Q ion-exchange column using a 0–1 M NaCl exponential gradient in Tris-HCl buffer, pH 7.0. The peak which eluted at >0.5 M NaCl comprised 65% of the total protein and was shown to be the covalent antithrombin–heparin complex by two-dimensional electrophoresis. It had an activity measured by amidolytic assay (see below) of 5.2 heparin units/mg against factor Xa, but no additional detectable activity against thrombin.

Modification of Antithrombin. Purified normal antithrombin was reduced and *S*-carboxymethylated as previously described (Erdjument et al., 1987). It was then fragmented with CNBr, and the resulting peptides were separated on a 15-μm C18 Pep RPC column using the FPLC delivery system (Erdjument et al., 1988). Antithrombin cleaved at the reactive site was prepared as described (Caso et al., 1991).

Purification of Antithrombin Congenital Variants. Antithrombins Alger (Brunel et al., 1987) and Budapest 3 (Olds et al., 1992a) occurred in the homozygous state, and the plasma was used directly. In all other cases, the propositi were heterozygous for antithrombin. The Northwick Park variant was purified as previously described (Erdjument et al., 1987, 1988), completely cleaved antithrombin Charleville was purified from the variant (Aiach et al., 1985) by additional affinity chromatography on thrombin–Sepharose (Caso et al., 1991), and the remaining variants were separated from the normal antithrombin component by affinity chromatography on heparin–agarose, from which they eluted at 0.4 M NaCl (Lane et al., 1992a).

Preparation of Thrombin–Antithrombin Complex. Thrombin–antithrombin complex was prepared by the method of Fish and Bjork (1979), using thrombin labeled with ^{125}I by the chloramine T method (Dawes, 1988).

Production of Monoclonal Antibodies. Murine monoclonal antibodies were raised in 12-week-old Balb/c female mice as described previously (Dawes et al., 1984), except that the spleen cells were fused with NS-0 myeloma cells. Mice were immunized by three consecutive injections of 50 μg of covalent antithrombin–heparin complex, and two fusions were performed. Media of growing hybridomas were screened for antibodies against heparin, antithrombin, and the antithrombin–heparin complex using liquid-phase radioimmunoassays. Heparin, antithrombin, and antithrombin–heparin complex were radiolabeled with ^{125}I using previously described methods (Dawes & Pepper, 1979; Dawes, 1988) designed to retain relevant biological activities. Samples of culture supernatant (50 μL) were incubated overnight at room temperature with 50 μL of ^{125}I-labeled antigen (10 ng/mL) and 100 μL of 0.05 M phosphate buffer, pH 7.5, containing 0.15 M NaCl, 0.25% gelatin, and 1% Tween 20. The buffer used minimized nonspecific binding, which was measured in tubes containing only radiolabeled antigen and buffer. Antigen–antibody complexes were separated from unbound antigen using Sepharose 4B-coupled sheep anti-mouse immunoglobulin antiserum (Dawes et al., 1984).

A selected clone was grown in pristane-primed mice and the antibody, ESAH 1, purified from ascitic fluid by affinity chromatography on protein A–Sepharose (Dawes et al., 1984) and stored at a concentration of 4.5 mg/mL at −80 °C. It was shown to be an IgG1 by immunodiffusion in agarose against specific sera.

A control IgG1 murine monoclonal antibody against human serum albumin, ESA 3, was raised similarly.

Amidolytic Assays. Inhibition of thrombin by antithrombin in the absence of heparin was measured using the chromogenic substrate S-2238. Antithrombin (10 μg/mL) was incubated with human thrombin (2.5 units/mL) at 37 °C in 0.05 M Tris

[1] Abbreviations: MAb, monoclonal antibody; FPLC, fast protein liquid chromatography.

buffer, pH 7.4, containing 0.15 M NaCl, 0.25% gelatin, and 0.1% Tween 20 (assay buffer). At 5, 10, 15, 20, and 30 min, samples (15 μL) were removed and added to 135 μL of S-2238 (0.2 mM) in a microtiter plate at pH 8.4 to assay residual thrombin activity. Substrate hydrolysis at 20 °C was linear for 15 min, when it was stopped by the addition of 90 μL of 50% acetic acid before reading the absorbance at 405 nm. Assay in the absence of antithrombin gave the uninhibited rate of substrate cleavage by thrombin (V_0).

Inhibition of thrombin by antithrombin in the presence of the heparin-binding pentasaccharide was measured by including a range of concentrations (0–10 μg/mL pentasaccharide) in the incubation of antithrombin with thrombin, and 50 μg/mL Polybrene in the amidolytic assay of residual thrombin.

For measurement of the activity of antithrombin against thrombin in the presence of heparin, antithrombin (2–10 μg/mL) was incubated with thrombin (1.7 units/mL) at 4 °C in assay buffer containing 25 μg/mL heparin. At 10-s intervals, 30 μL was removed and added to 135 μL of S2238 (0.2 mM) in assay buffer at pH 8.4 containing 50 μg/mL Polybrene. Samples were incubated at 20 °C for 15 min, and the reaction was stopped with 90 μL of 50% acetic acid before reading the absorbance at 405 nm.

Effects of ESAH 1 on Antithrombin Activities. To assess the effect of binding to ESAH 1 on the ability of antithrombin to inhibit thrombin, the antibody (100 μg/mL) was incubated with antithrombin (20 μg/mL) overnight at room temperature before being tested. Antithrombin was similarly incubated alone, and with a monoclonal antibody of the same isotype (ESA 3) directed against human serum albumin as controls.

Quantitation of Antithrombin Binding to ESAH 1. To measure the amount of antithrombin complexed to ESAH 1, the antibody was incubated with antithrombin, and aliquots were then shaken with excess Sepharose-coupled sheep anti-mouse immunoglobulin and with bland Sepharose for 45 min before centrifugation. After centrifugation, both progressive and heparin cofactor activities of the supernatants were measured as described above. Controls included both antithrombin incubated with ESA 3 and antithrombin which had not been preincubated with monoclonal antibody.

Effect of ESAH 1 on Antithrombin Binding to Heparin. ESAH 1 and the control MAb ESA 3 (10 μg) were radiolabeled with ^{125}I (Dawes, 1988) and incubated at a 3-fold molar excess with antithrombin overnight as described above. Iodination did not affect the ability of the MAb to bind to antithrombin. The incubation mixture was then fractionated by heparin–Sepharose affinity chromatography (Miller-Andersson et al., 1974) using a 0.15–1.5 M NaCl gradient in 0.1 M Tris-HCl buffer, pH 7.4. The elution positions of radiolabeled material were compared with that of antithrombin alone, which was quantitated by radioimmunoassay (Olds et al., 1992b) using a rabbit polyclonal antibody against antithrombin.

Effect of ESAH 1 on Heparin-Enhanced Antithrombin Fluorescence. ESAH 1 (100 μg/mL) was incubated overnight at room temperature with antithrombin (20 μg/mL) in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. After dilution to a final antithrombin concentration of 4.5 μg/mL with the same buffer, fluorescence emission was monitored at 340 nm using a 10-nm band-pass following excitation at 280 nm with a 5-nm band-pass (Einarsson & Andersson, 1977). Heparin was then added to a final concentration of 8 μg/mL and a second spectrum recorded. Antithrombin was similarly incubated with a monoclonal antibody of the same isotype (ESA 3) directed against human serum albumin as a control. Other controls, which were also incubated overnight, included ESAH 1 alone and antithrombin alone.

Binding of ESAH 1 to Different Forms of Antithrombin. To assess the binding of ESAH 1 to different forms of antithrombin for epitope characterization, serial dilutions of normal antithrombin and congenital antithrombin variants [25 μL in 225 μL of 10 mM Tris-HCl (pH 7.4) containing 0.9% NaCl (TBS)] were applied to a nitrocellulose membrane (0.45 μm) using a slot-blotting apparatus (Bio-Rad), and washed with 500 μL of TBS. The membrane was blocked in 3% skim milk powder in TBS for 1 h at room temperature, followed by 3 washes for 5 min in TBS containing 0.05% Tween-20 (TTBS). It was then incubated for 2 h at room temperature with ESAH 1 (10 μg/mL) in 1% (w/v) skim milk powder in TBS. Control membranes were incubated with a 1:1000 dilution of rabbit polyclonal antibody against antithrombin. After unbound antibody was removed by washing 3 times for 5 min in TTBS, the blot was incubated with the appropriate alkaline phosphatase-conjugated secondary antibody (1:1000 dilution) in 1% skim milk powder in TBS for 1 h at room temperature, washed 3 times for 5 min in TTBS, and developed with 0.3 mg/mL 4-nitroblue tetrazolium chloride and 0.15 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 5 mM $MgCl_2$. The staining was stopped by washing in 20 mM sodium phosphate (pH 7.4) and 20 mM EDTA for 5 min and subsequently air-dried for storage.

Densitometric analysis of nitrocellulose membranes was carried out using an XRS 6cx OmniMedia scanner in conjunction with an Apple Macintosh Image 1.44 program. Standard curves were constructed and the concentration of each variant calculated from the data obtained using polyclonal antibody, and the ability of ESAH 1 to detect each variant was then compared with its interaction with normal antithrombin and expressed quantitatively as the ratio of the apparent concentration of the variant measured with ESAH 1 to that measured with polyclonal antibody.

The ^{125}I-thrombin–antithrombin complex, prepared as described above, was similarly quantitated by slot-blotting with both ESAH 1 and polyclonal antibody. In addition, serial dilutions were analyzed by SDS–PAGE in 10–15% reducing gels (Phast-Gel, Pharmacia). After Western blotting, they were quantitated as described for the slot-blots using polyclonal antibody, and duplicate gels were autoradiographed to confirm the position of the thrombin–antithrombin complex.

RESULTS

Selection of Antibody-Producing Clones. The monoclonal antibody ESAH 1, which was of IgG1 isotype, was selected from 43 antibodies which bound antithrombin or the antithrombin–heparin covalent complex. No antibodies directed against heparin alone were produced.

Effect of ESAH 1 on Inhibition of Thrombin by Antithrombin. After incubation of ESAH 1 (100 μg/mL) with antithrombin (20 μg/mL) overnight, the progressive activity of antithrombin against thrombin was inhibited by >95% (Figure 1), whereas preincubation with a monoclonal antibody against human serum albumin was without effect. However, antithrombin still expressed full heparin cofactor activity after complexing to ESAH 1 (Figure 2a). The reciprocals of the observed pseudo-first-order rate constants measured after antithrombin was incubated with ESAH 1 and the control antibody are plotted against reciprocal antithrombin concentrations in Figure 2b. Three experiments yielded a zero ordinate intercept and a second-order rate constant of

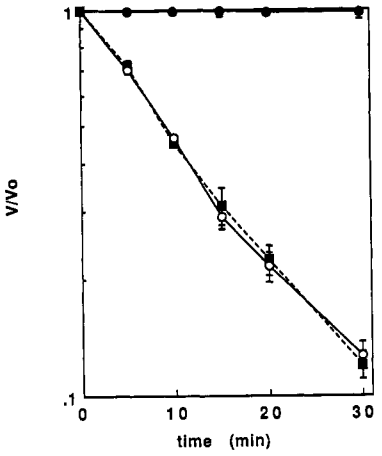

FIGURE 1: Effect of ESAH 1 on the progressive activity of antithrombin against thrombin. ESAH 1 and the control monoclonal antibody ESA 3 against human serum albumin were incubated at room temperature with antithrombin overnight, and the progressive activities of antithrombin against human thrombin were then measured for antithrombin incubated alone (■), antithrombin + ESA 3 (○), and antithrombin + ESAH 1 (●) as described under Experimental Procedures. V/V_0 indicates the relative velocity of thrombin inactivation in the presence and absence of antithrombin. Points represent the mean ± SD of data from three experiments.

inhibition of $(1.79 \pm 0.08) \times 10^7$ M^{-1} min^{-1}, compared with $(2.04 \pm 0.22) \times 10^7$ M^{-1} min^{-1} after preincubation with the MAb against human serum albumin; these values are not significantly different, though lower than other published values because the experiments were conducted at 4 °C to permit manual measurement. The same results were obtained whether the antithrombin was incubated with ESAH 1 in the presence or absence of heparin before assay. Moreover, when the antithrombin-binding pentasaccharide was substituted for unfractionated heparin, the blocking action of ESAH 1 that prevented thrombin inhibition by antithrombin was similarly ameliorated. As the rate of thrombin inhibition by antithrombin is not greatly stimulated by the pentasaccharide, it was possible to demonstrate unblocking of the inhibitory action of ESAH 1 by increasing concentrations of pentasaccharide without altering the experimental conditions (Figure 3). Progressive activity was completely restored at a pentasaccharide concentration of 1.1 μg/mL, approximately equimolar with the concentration of antithrombin in the test system. Results with the control MAb were identical with those obtained with antithrombin alone (not shown), and indicate low but reproducible stimulation of the rate of thrombin inhibition by pentasaccharide; this was also observed in the presence of ESAH 1 at a higher pentasaccharide concentration.

To investigate whether ESAH 1 converted antithrombin from inhibitor to substrate, the antithrombin–ESAH 1 complex was preincubated with thrombin at 37 °C for 40 min before addition of the pentasaccharide (2.5 μg/mL). The rate of thrombin inhibition was identical with that measured before preincubation (data not shown); evidently the MAb does not convert antithrombin to a substrate conformation.

Effect of Heparin on the Interaction between Antithrombin and ESAH 1. When ESAH 1 was incubated with antithrombin under the conditions described above and then precipitated with Sepharose-coupled sheep anti-mouse immunoglobulin, no progressive or heparin cofactor activity could be detected in the supernatant, indicating that 100% of the antithrombin bound to ESAH 1 and had been removed from solution. By contrast, after incubation with an antibody against human serum albumin, 92–97% of the original

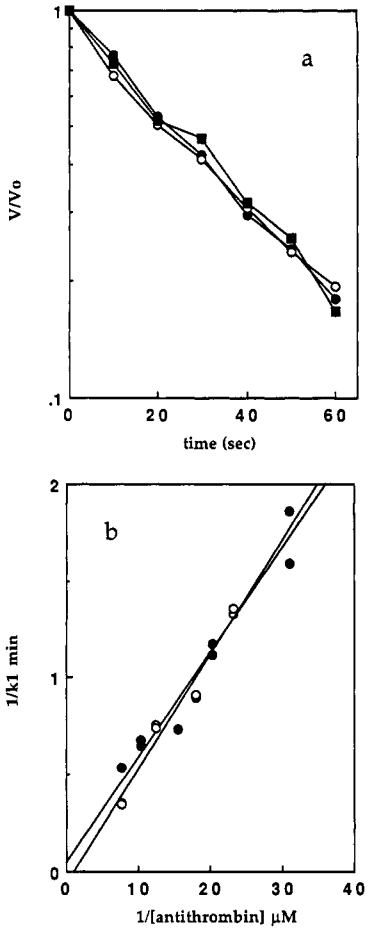

FIGURE 2: Effect of ESAH 1 on the heparin cofactor activity of antithrombin against thrombin. ESAH 1 and the control monoclonal antibody ESA 3 against human serum albumin were incubated at room temperature with antithrombin overnight, and (a) the heparin cofactor activities of antithrombin against human thrombin were then measured for antithrombin incubated alone (■), antithrombin+ ESA 3 (○), and antithrombin + ESAH 1 (●) over the antithrombin concentration range 2–10 μg/mL as described under Experimental Procedures. (b) The reciprocals of the observed pseudo-first-order rate constants are plotted against reciprocal antithrombin concentrations. These data are from one of three experiments.

activities of the antithrombin were recovered. When heparin (10 μg/mL) was included at all stages of this experiment and heparin cofactor activity measured, identical results were obtained ($n = 3$ throughout). Clearly heparin did not displace ESAH 1 from its complex with antithrombin.

Effect of ESAH 1 on the Interaction of Antithrombin with Heparin. ESAH 1 was radiolabeled and incubated with antithrombin before affinity chromatography on heparin–agarose. Labeled ESAH 1 was present in a 3-fold molar excess over antithrombin; the uncomplexed MAb (68% of the radiolabel) did not bind during the subsequent heparin–Sepharose chromatography step, but the fraction complexed to antithrombin bound to the column and eluted at the same ionic strength as a control sample of antithrombin alone (Figure 4). After incubation of labeled ESA 3 with antithrombin, the MAb did not bind to heparin–agarose (data not shown). In a further control experiment, ESAH 1 alone did not bind to heparin–Sepharose.

The effect of ESAH 1 on heparin-enhanced antithrombin fluorescence was examined after overnight incubation of a 2-fold molar excess of the antibody with antithrombin. Higher ratios of antibody to antithrombin decreased the sensitivity of the measurements because of the high background con-

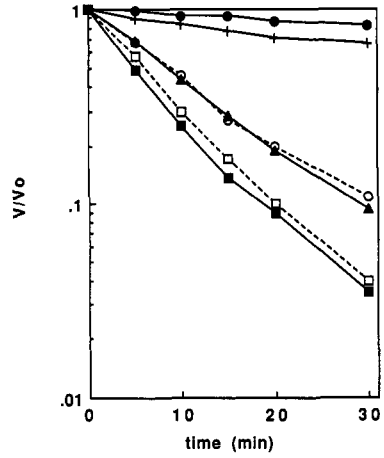

FIGURE 3: Unblocking of antithrombin activity by increasing concentrations of antithrombin-binding pentasaccharide. ESAH 1 and control monoclonal antibody ESA 3 directed against human serum albumin were incubated at room temperature with antithrombin overnight, and the activity of antithrombin against human thrombin was then measured in the presence of increasing concentrations of the antithrombin-binding pentasaccharide. Results are shown for ESAH 1 without pentasaccharide (●) and with 110 ng/mL (+), 1.1 μg/mL (▲), and 10 μg/mL (■) pentasaccharide, and for ESA 3 without pentasaccharide (○) and with 10 μg/mL (□) pentasaccharide.

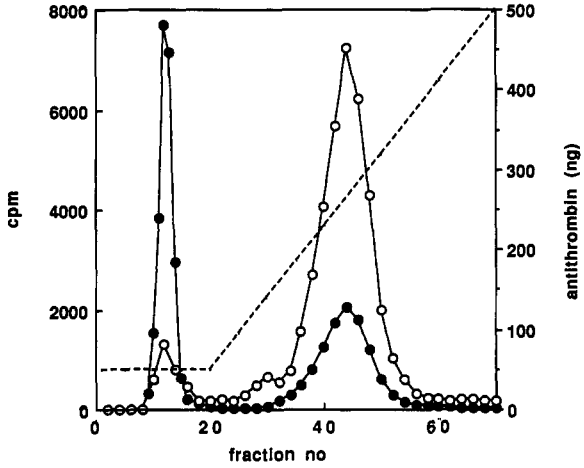

FIGURE 4: Effect of ESAH 1 on the affinity of antithrombin for heparin. ESAH 1 was radiolabeled with ^{125}I, and a 3-fold molar excess was incubated overnight with antithrombin. The products were then fractionated by heparin–agarose affinity chromatography, using a 0.15–1.5 M NaCl gradient in 0.1 M Tris-HCl buffer, pH 7.4 [(- - -); axis range 0–1.5 M NaCl]. The elution profile of radiolabeled material (●) was compared with that of antithrombin alone, which was quantitated by radioimmunoassay (○).

tributed by fluorescence of the immunoglobulin. Heparin enhanced the fluorescence emission at 340 nm of antithrombin alone by 28 ± 2% (n = 3). After incubation with ESAH 1 and the control MAb ESA 3, the fluorescence enhancement was 91 ± 4% and 89 ± 3%, respectively, of that measured for antithrombin alone. In control experiments, there was no effect of heparin on the emission spectra of the antibodies.

Epitope Analysis of ESAH 1. Normal antithrombin was reduced, *S*-carboxymethylated, and converted to CNBr fragments. The ability of a range of concentrations of these fragments and of the intact polypeptide chain to interact with ESAH 1 was compared with that of intact antithrombin by slot-blot analysis. No activity was detected against ESAH 1 with any of the fragments or with the complete reduced and carboxymethylated chain.

ESAH 1 detected antithrombin cleaved at the reactive site as well as congenital variants resulting from mutations within

Table 1: Detection of Antithrombin Congenital Variants by ESAH 1[a]

variant	mutation	concn measured by ESAH 1 (% concn measured by polyclonal)
Alger	Arg47→Cys	106 ± 4
Paris 1	Arg47→Cys	94 ± 7
Padua 1	Arg47→His	97 ± 4
Budapest 3	Leu99→Phe	96 ± 6
Geneva	Arg129→Gln	103 ± 6
Charleville	Ala384→Pro	101 ± 5
Northwick Park	Arg393→Cys	96 ± 7
Rosny	Phe402→Cys	<1
Torino	Phe402→Ser	7 ± 1
Maisons-Laffitte	Phe402→Leu	17 ± 3
Paris 3	Ala404→Thr	38 ± 3
La Rochelle	Asn405→Lys	97 ± 4
Budapest 5	Pro407←Thr	32 ± 2

[a] Experiments were performed as described for Figure 5. Values represent the mean ± SD of data from three experiments.

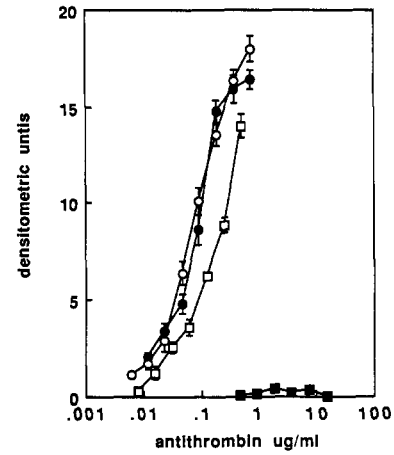

FIGURE 5: Comparison of the interaction of normal antithrombin (●) and antithrombins Rosny (■), La Rochelle (○), and Paris 3 (□) with ESAH 1. Serial dilutions of antithrombins were bound to a nitrocellulose membrane using a slot-blotting apparatus. Duplicate membranes were incubated with ESAH 1 (10 μg/mL) or rabbit polyclonal antibody against antithrombin followed by alkaline phosphatase-conjugated secondary antibody, and developed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as described under Experimental Procedures. Densitometric analysis of nitrocellulose membranes was carried out using an XRS 6cx OmniMedia scanner in conjunction with an Apple Macintosh Image 1.44 program. The concentrations of antithrombin in each sample were calculated from the results with polyclonal antibody. Points represent the mean ± SD of data from three experiments.

codons 47, 99, 129, 384, 393, and 405 with the same sensitivity as normal antithrombin (see Table 1). However, other variants located in the 402–407 region of the antithrombin molecule were detected poorly. The interactions of antithrombins Paris 3 (Ala404→Thr), La Rochelle (Asn405→Lys), and Rosny (Phe402→Cys) with ESAH 1 are compared with that of normal antithrombin in Figure 5. Antithrombin Rosny at concentrations up to 10 μg/mL did not react with ESAH 1, the concentration of antithrombin Paris 3 measured by binding to ESAH 1 was about 40% of that measured using the polyclonal antibody, and antithrombin La Rochelle was indistinguishable from normal antithrombin. The apparent concentrations of all the variants tested, measured by binding to ESAH 1, are expressed as a percentage of their concentration detected by polyclonal antibody in Table 1.

Antithrombin present in the thrombin–antithrombin complex was also recognized by ESAH 1. A preparation of the thrombin–antithrombin complex labeled with ^{125}I in the

thrombin moiety was analyzed by SDS–PAGE and autoradiography, and quantitated by Western blotting with polyclonal antibody against antithrombin. It comprised a major radiolabeled component which was identified as the thrombin–antithrombin complex and contained 88% of the antithrombin in the sample, and a minor band of uncomplexed antithrombin. On slot-blot analysis, ESAH 1 detected the antithrombin in this preparation with the same sensitivity as unmodified antithrombin.

DISCUSSION

There are several reports of monoclonal antibodies against antithrombin which affect its activity. Some prevent inhibition of thrombin by antithrombin in the presence and absence of heparin, while another moderated thrombin inhibition in the presence of heparin, but had little effect on the progressive activity of antithrombin (Herion et al., 1985). Others are directed against the heparin-binding site (Watton et al., 1993). One MAb blocked the heparin-induced conformational change (Knoller & Savion, 1989), while another, the epitope for which was localized to residues 382–386 of antithrombin (i.e., P8–P12 of the reactive bond loop), facilitated hydrolysis of the inhibitor by thrombin (Asakura et al., 1990). However, an antibody with the properties of that detailed here has not been previously described. We used a covalent antithrombin–heparin complex as immunogen in the present study, and obtained a MAb which, when its effects on the biological activities of antithrombin were tested, yielded data pertinent to the conformational changes induced in antithrombin when it interacts with heparin.

Binding of ESAH 1 to its epitope on antithrombin almost fully inhibited the intrinsic activity of the inhibitor against thrombin, suggesting that the epitope is located at or near either the reactive site Arg393–Ser394 bond of antithrombin or a secondary proteinase-binding site. By contrast, the same antithrombin–MAb complex displayed full heparin cofactor activity against thrombin, indicating that the conformational change consequent on interaction with heparin unblocked the inhibitory action of ESAH 1 on the activity of antithrombin against thrombin. Moreover, the same unblocking effect was achieved by increasing concentrations of the antithrombin-binding pentasaccharide, and unblocking was complete at a pentasaccharide concentration which was approximately equimolar with that of antithrombin.

It is thought that as a result of the conformational change resulting from the interaction of antithrombin with heparin molecules, the reactive bond of antithrombin is presented to certain of its target proteinases in a more accessible form (Rosenberg & Damus, 1973; Huber & Carrell, 1989). The nature of this conformational change still has to be clarified, but recent evidence obtained from a study of recombinant antithrombin with an Arg393 to Cys substitution fully supports an induced change in the environment of the P1 reactive center residue (Gettins et al., 1993). The pentasaccharide is reported to induce a very similar (Gettins & Choay, 1989) or identical conformational change (Olson et al., 1987; Gettins et al., 1993). The ability of both heparin and the pentasaccharide to enhance cleavage of the active center by elastases (Jordan et al., 1987) is in accordance with this view. Nevertheless, unlike heparin the pentasaccharide does not greatly affect the rate of reaction between antithrombin and thrombin. A major component of the chain-length dependence of heparin rate enhancement of thrombin inhibition is believed to arise from a requirement for a single heparin molecule to bind both antithrombin and thrombin and act as a surface to approximate the two molecules. A considerable body of evidence supports this hypothesis [reviewed by Bjork et al. (1989)]. Our data are in accord with the view that the primary conformational change induced by the pentasaccharide, though insufficient to contribute greatly to enhancement of the thrombin–antithrombin reaction, closely resembles that resulting from interaction with heparin and is sufficiently major to restore activity to the antithrombin–ESAH 1 complex in which the reactive center is blocked.

Despite the ability of ESAH 1 to completely inhibit the intrinsic activity of antithrombin against thrombin, binding of the antibody to its epitope did not interfere with the heparin-induced thrombin–antithrombin reaction in any detectable way. As interaction of thrombin with the Arg393–Ser394 reactive site bond is required for both progressive and heparin cofactor activity, these residues are clearly not themselves components of the ESAH 1 epitope, and the same is true for any secondary thrombin-binding sites on antithrombin which are functional in the presence of heparin. The antibody did not affect the heparin-enhanced fluroescence of antithrombin. Moreover, it was not displaced from antithrombin by heparin and failed to modify the affinity of antithrombin for heparin, and so did not interact with the primary heparin-binding site. Importantly, unlike the MAb reported by Asakura et al. (1990), ESAH 1 did not convert antithrombin from an inhibitor to the substrate conformation. These results are consistent with the epitope being closely approximated to a thrombin-binding site on antithrombin in the absence of heparin but topographically removed from this thrombin-binding site following the conformational change consequent on heparin binding. Thus, formation of the antithrombin–ESAH 1 complex would prevent interaction with thrombin by steric hindrance, which would not occur following the heparin-induced conformational change.

Full reduction of the disulfide bonds in antithrombin destroyed the ESAH 1 epitope, which are clearly conformational rather than being composed of consecutive residues in the peptide chain. This restricted analytical options to relatively nondenaturing conditions such as the slot-blotting technique described. Using this method, the ability of ESAH 1 to recognize a range of naturally occurring antithrombin variants [reviewed in Lane et al. (1992b, 1993)] was tested. The antibody failed to distinguish naturally occurring antithrombin variants at the reactive site bond and at residues 47, 99, and 129, which solely affect the primary heparin-binding site, from the normal protein. These results support the conclusions drawn from the effects of ESAH 1 on antithrombin activity, that the ESAH 1 epitope is neither at the reactive bond nor at the primary contact site for heparin binding.

ESAH 1 also bound normally to antithrombin Charleville, in which the mutation Ala384→Pro converts antithrombin from a thrombin inhibitor to a substrate. This variant was studied in a form cleaved at the reactive site but otherwise undenatured. In addition, normal reactive site cleaved antithrombin and the stable thrombin–antithrombin complex were recognized by the antibody. The three-dimensional structures of the cleaved forms of antithrombin (Mourey et al., 1990) and α_1-proteinase inhibitor, another inhibitory serpin (Loebermann et al., 1984), have been elucidated by X-ray crystallography. Cleavage of the reactive bond was found to separate the P1 and P1′ residues by as much as 70 Å, with the P1–P16 region directly amino-terminal to the reactive site bond comprising the central strand (s4A) of a six-stranded β-sheet in the core of the cleaved protein. The crystal structure of uncleaved ovalbumin, a noninhibitory serpin, has also been

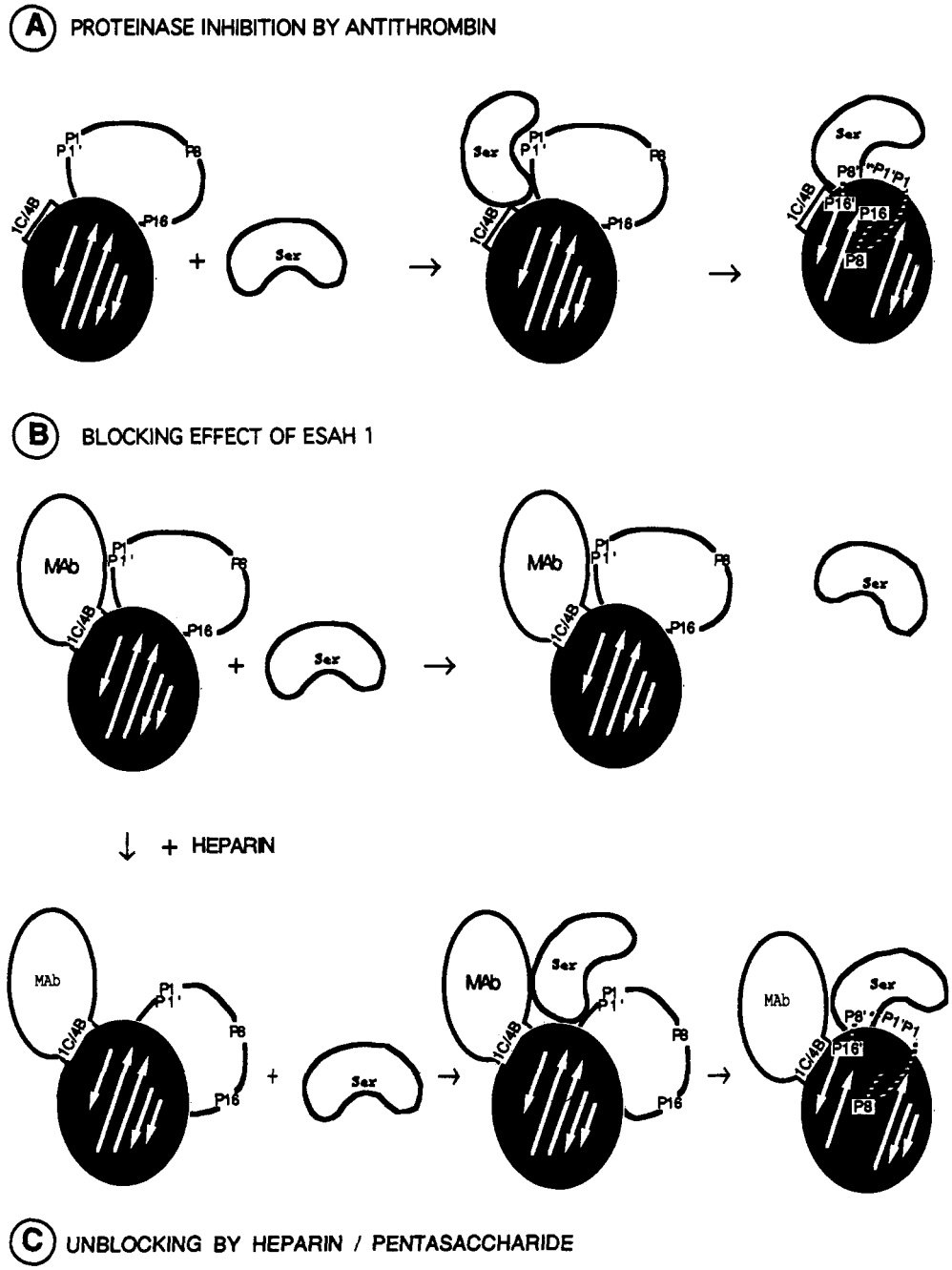

FIGURE 6: Model of the conformational change induced in the reactive loop of antithrombin by interaction with heparin. According to the model of Bjork and colleagues (Olson & Bjork, 1992; Bjork et al., 1992), formation of a stable proteinase–serpin complex involves partial insertion of the P1–P16 sequence in the β-sheet (A). The monoclonal antibody ESAH 1 binds to antithrombin in the region of the 402–407 and 429 residues (shown by box), preventing interaction of the reactive site with thrombin by steric hindrance (B). The conformational change resulting from heparin binding to antithrombin increases the spatial separation between the reactive site and/or an associated secondary thrombin-binding region and the 402–407 region, so that thrombin is no longer sterically hindered from binding to the ESAH 1–antithrombin complex (C). Abbreviations: Ser, serine proteinase (thrombin); MAb, monoclonal antibody ESAH 1.

described (Stein et al., 1991), and together with molecular dynamic modeling of α_1-proteinase inhibitor (Engh et al., 1990), these structural determinations have suggested that in the native form of antithrombin the P1–P16 region forms an exposed peptide loop extending away from the protein core. Conformational changes also accompany formation of the serpin–proteinase complexes, and there is increasing evidence to suggest that induced partial insertion of the exposed peptide loop into the β-sheet may be involved in trapping the proteinase in a stable complex (Schulze et al., 1990; Skriver et al., 1991; Bjork et al., 1992). Although heparin binding may not involve loop insertion (Bjork et al., 1993), ESAH 1 reacted equally well with antithrombin, its complex with thrombin, and cleaved normal and variant molecules; it seems highly unlikely, therefore, that the P1–P16 region of antithrombin forms part of the ESAH 1 epitope.

However, of six antithrombin variants with mutations C-terminal to the reactive bond in the 402–407 region, four were detected only poorly by ESAH 1, and the protein containing the substitution Phe402→Cys was not recognized at all; only the substitution Asn405→Lys failed to affect the interaction with antibody. These mutations have pleiotropic effects, with reduced antithrombin activities, heparin-binding abnormalities, and low plasma concentrations (Lane et al., 1992a). We have previously reported that another variant (Pro429→Leu) with both reactive site and heparin-binding

defects also fails to bind to ESAH 1 (Olds et al., 1992b), and in a model derived from the crystal structure of native ovalbumin, residue 429 lies close to the 402–407 region in the tertiary structure of antithrombin (Lane et al., 1992a). The available evidence indicates that the conformational epitope of ESAH 1 is located near the reactive loop in the C-terminal region of antithrombin, and involves residues 402–407 and 429. The alternative interpretation is that a major structural perturbation in the variant molecules results in their pleiotropic defects and also disrupts an epitope at some distance from the substituted residues. This appears unlikely because the Asn405→Lys variant has the same phenotype as others in the group, but was fully recognized by the antibody. This is a conservative substitution which could conceivably retain the appropriate characteristics of an antibody recognition site. Alternatively, residue 405 may not constitute part of the epitope, despite its proximity to residue 402. The 402–407 region maps to strand 1C and the polypeptide leading into strand 1B and thus connects two of the three β-sheets in the molecule. In the intact molecule, it lies in close proximity to the reactive loop, and Lane et al. (1992) have suggested that it may constitute part of an extended enzyme–inhibitor contact surface. It is spatially distinct from the primary contact heparin-binding region, but could be conformationally linked to it through the internal β-sheet which connects at its distal end to the α-helices forming the primary heparin-binding site.

It appears, therefore, that the epitope of ESAH 1 is located within or close to a conformationally significant region of antithrombin. It will be recalled that the substitution mutations in strands 1C, 4B, and 5B alter both reactive site and heparin-binding site functions. On the other hand, while the antithrombin–ESAH 1 complex was inactive toward thrombin in the absence of heparin, heparin interaction and induction of the associated conformational change were unaffected by the binding of the MAb. The results therefore favor the concept of a mobile reactive loop, in which a conformational change is induced by heparin or the pentasaccharide. Our data suggest that a feature of the mobility of the loop is the potential independence of thrombin-binding sites from the strand 1C, 4B region, indicated by the ability of the pentasaccharide to unblock the action of ESAH 1. The ESAH 1 epitope, located near residue 402, may sterically block thrombin access either to the reactive site bond directly or to a secondary binding or contact region of thrombin (Lane et al., 1992a; Nishioka & Suzuki, 1992). In either case, the blocked site is exposed following the heparin-induced conformational change, which increases the spatial separation of the reactive site bond from the strand 1C, 4B region. It seems unlikely that in the presence of heparin the epitope itself constitutes part of any secondary binding region for thrombin.

These experiments support the concept of a mobile reactive loop which is conformationally linked to the heparin-binding site. In addition, they provide new insight into the involvement of the C-terminal part of the antithrombin molecule in the heparin-induced conformational change, indicating that the reactive loop is independently mobile of the adjacent strands 1C and 4B as illustrated in the model (Figure 6), based on that proposed by Skriver et al. (1991) and Bjork and colleagues (Olson & Bjork, 1992; Bjork et al., 1992). In the model, the proteinase approaches the reactive P1–P1′ bond and induces a partial insertion of the reactive loop region around the P12 residue into the A β-sheet (Figure 6A). Binding of the ESAH 1 MAb to antithrombin sterically impedes the interaction of thrombin with the reactive site either directly or by blocking a putative thrombin contact site close to the ESAH 1 epitope (Figure 6B). In the presence of pentasaccharide or heparin, a conformational change is induced (Figure 6C) which involves increased separation between the thrombin binding site and the 1C/4B region, exposing the reactive site and allowing thrombin to interact to form a stable complex.

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