Heparin Binding Affinity of Normal and Genetically Modified Antithrombin III Measured Using a Monoclonal Antibody to the Heparin Binding Site of Antithrombin III

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ABSTRACT: The inhibitory activity of the plasma serine proteinase inhibitor antithrombin III (AT III) is enhanced about 1000-fold upon binding to heparin. We have determined the dissociation constants, K_d, of 48.8 nM for the heparin–AT III interaction, of 175 nM for the specific pentasaccharide–AT III interaction, and of 13 μM for the low-affinity heparin–AT III interaction, using a binding assay based on a monoclonal antibody (MAb) that recognizes an epitope at or close to the heparin binding site of AT III. The heparin binding affinities and proportions of normal and variant AT III in plasma from patients with mutations of AT III have been quantitated for the first time using the binding assay. Substitution mutations in three regions of AT III have been investigated: (i) mutations in the reactive site loop affecting Ala382, Arg393, and Ser394 have no discernible effect on heparin binding; (ii) mutations in the previously identified N-terminal heparin binding region, affecting Arg47, Leu99, and Arg129, produce variant AT III molecules with heparin affinities reduced 11–924-fold, the largest reduction being observed for the substitution mutation Arg47–Cys in Padua 2, which has an affinity of 65.6 μM; (iii) mutations in the hydrophobic regions around strand 1C of the C terminus, affecting Phe402, Ala404, Asn405, Pro407, and Pro429, have pleiotropic effects that include the production of reduced amounts of low-affinity AT III with dissociation constants from 6 to 43 μM. The MAb-based method described is a simple means of determining the heparin binding affinity of variant AT III in complex solutions, and it will be useful in the study of novel natural mutations or genetically engineered variants.

Antithrombin III (AT III),[1] the main inhibitor of blood coagulation, is a member of the serpin family of proteinase inhibitors (Hunt & Dayhoff, 1980; Carrell & Travis, 1985). It acts by forming a stable equimolar complex with serine proteases—mainly factors IIa (thrombin) and Xa but also factors IXa, XIa, and XIIa (Damus et al., 1973)—blocking the enzyme active site with a peptide loop. This loop is located in the C-terminal region with the P1–P1′ (393Arg–394Ser) peptide bond as its crucial elements (Schecter & Berger, 1967; Björk et al., 1982). The rate of proteinase inactivation is enhanced 3–4 orders of magnitude by catalytic amounts of heparin (Rosenberg & Damus, 1973) which binds specifically to a second important functional domain in AT III located near the N terminus.

Inheritance of AT III deficiency follows an autosomal dominant pattern and occurs in about 1 in 2000–5000 of the population (Thaler & Lechner, 1981). It is a risk factor for the development of venous thrombotic disease (Brandt, 1984). Mutations in the AT III gene have two main consequences—either reduced levels of AT III due to the absence of a gene product from one allele, type I deficiency, or normal immunoreactive but reduced functional levels due to the presence of variant AT III, type II deficiency. The lack of homozygotes with type I deficiencies is indicative of the importance of the role of AT III in the regulation of hemostasis. Information about all of the known AT III mutations has recently been compiled into a database (Lane et al., 1991).

AT III type II deficiencies and variant AT III production arise from point substitution mutations that affect one or both of the important functional domains of AT III. Mutations at or N-terminal to the reactive site loop (amino acids 382–394, P12–P1′) have attracted interest because of their (sometimes complex) effects upon proteinase inhibition and their association with an increased risk of development of venous thrombosis [for a comprehensive list of references see the database (Lane et al., 1991)]. Until recently, there have been fewer identifications of mutations C-terminal to the reactive site loop in hydrophobic regions around strand 1C, strand 4B (amino acids 407–415, P14′–P22′), and strand 5B (amino acids 416–427, P23′–P34′). The tertiary structures of these C-terminal strands of AT III are described in a computer model of active site cleaved AT III, based on the X-ray crystal structure of α_1-antitrypsin (Huber & Carrell, 1989). These hydrophobic areas are emerging as being important for maintenance of the structural integrity of AT III (Bock et al., 1988; Hultin et al., 1988; Nakagawa et al., 1991), and mutations disrupting this region have been proposed to cause or impair conformational changes, thus having pleiotropic or multiple effects upon the AT III molecule (Lane et al., 1992). There is less evidence of an association between mutations solely affecting the heparin binding domain of AT III and the development of thrombosis, but information on the extent to which various mutations affect heparin binding is extremely useful in elucidating the nature of the primary interaction of this glycosaminoglycan and AT III. To date, studies of the interaction of heparin with these heparin binding variants of AT III have been limited by use of semiquantitative

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[1] Abbreviations: AT III, antithrombin III; MAb, monoclonal antibody; UFH, unfractionated heparin; HAH, high-affinity heparin; LAH, low-affinity heparin; PBS, phosphate-buffered saline; IRMA, immunoradiometric assay; K_d, dissociation constant; PEG, poly(ethylene glycol).

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methods such as heparin–Sepharose chromatography or crossed immunoelectrophoresis. These methods can only distinguish large changes in heparin affinity and have given incomplete and often conflicting information.

We have previously described production and use of a monoclonal antibody (MAb) directed against the heparin binding site of AT III (Watton et al., 1990). Addition of AT III to the MAb in the presence of increasing concentrations of heparin quantitatively reduces MAb binding of AT III. The MAb has been used in IRMA format to characterize both AT III and heparin preparations and has the particular advantage that the interaction of heparin with AT III in plasma can be studied without the need for prior purification. In the present study we have used this binding assay to measure dissociation constants for the heparin–AT III interaction of normal AT III and of a number of variant AT IIIs in their plasma milieu. In this way, the role of particular amino acids in the heparin–AT III interaction has been quantitated for the first time, without use of any potentially denaturing purification procedures. We provide evidence that substitution mutations in the hydrophobic regions of strands 1C and 4B in the C terminus cause or prevent conformational changes which affect heparin binding as severely as substitutions in the N-terminal region.

MATERIALS AND METHODS

Determination of Dissociation Constants. Unfractionated heparin (UFH) (Leo Laboratories, Princes Risborough, U.K.) has a high-affinity heparin (HAH) content of 30–40% (Lam et al., 1976; Andersson et al., 1976). The concentration of HAH was calculated from the weight of UFH added, assuming a MW of 13 000 (Johnson & Mulloy, 1976; Linhart et al., 1985) and a HAH content of 30%; 1 μg/mL UFH = 0.023 μM HAH. Low-affinity heparin (LAH) of MW 9500, prepared by affinity chromatography from porcine mucosal heparin, was kindly provided by Dr. J. C. Lormeau (Sanofi Research, Paris, France); 1 μg/mL = 0.105 μM.

Pentasaccharide (MW 1714) with high affinity for AT III was kindly provided by Dr. J. Choay (Sanofi Research); 1 μg/mL = 0.583 μM.

A range of concentrations of each heparin (0.001–5 μM HAH, 0.05–400 μM LAH, and 0.001–60 μM pentasaccharide), chosen to cover the expected affinities, was prepared in blocking buffer [5% skimmed milk powder in phosphate-buffered saline (pH 7.4) (PBS)].

The binding method used is a modification of the previously described IRMA (Watton et al., 1990) which allows studies of the effect of heparin on AT III. A MAb known to recognize an epitope at or close to the heparin binding site of AT III was adjusted to 10 μg/mL in PBS. Aliquots of 100 μL were used to coat each well of a microtiter plate overnight at 4 °C. The plate was washed three times with 200 μL of PBS before being incubated with 200 μL of blocking buffer for 30 min at 25 °C. To 300 μL of each heparin concentration was added 10 μL of 310 ng/mL purified or plasma AT III [the 1st International Reference Preparation for human plasma AT III (NIBSC code 72/1) is used, assuming a normal plasma AT III concentration of 125 μg/mL], giving a final concentration of 10 ng/mL AT III. Aliquots of 100 μL of each heparin–AT III mixture were added in duplicate to the microtiter plate and incubated for 4 h at 37 °C. The AT III binding capacity of the MAb in the wells was determined by adding 100 μL of 10 ng/mL AT III in blocking buffer without heparin to six to eight wells. After three washings with blocking buffer, approximately 100 000 cpm of ^{125}I-affinity-purified rabbit polyclonal antibody directed against AT III was added and incubated at 37 °C for 2 h. As described previously (Watton et al., 1990), the antibody was iodinated by the chloramine T method, following its purification from rabbit serum by affinity chromatography.

The plates were washed three times in blocking buffer before the counts bound to the wells were detected by gamma counting.

Analysis of binding curves for a single population of AT III and heparin provides an estimate of K_d for the reaction

$$\text{AT III} + \text{heparin} \rightleftharpoons \text{AT III–heparin}$$

$$K_d = \frac{[\text{AT III}][\text{heparin}]}{[\text{AT III–heparin}]}$$

Values for K_d were generated by curve fitting experimental data using nonlinear regression analysis (Fig P, Biosoft, Cambridge, U.K.). In normal plasma samples, where a single population of wild-type AT III is present, curve fitting was to the equation for a simple adsorption isotherm (eq 1). However, because the assay actually measures the amount of AT III bound to the immobilized MAb at equilibrium, the amount of heparin–AT III complex must be calculated by subtracting the observed amount of AT III–MAb complex at each heparin concentration from the maximum observed level AT III–MAb complex with no heparin. As the heparin concentration increases, the binding to immobilized MAb reaches a minimum. In experiments with wild-type AT III, the highest heparin concentrations were routinely 4.46 μM, approximately 90K_d, corresponding to 98.9% of maximum possible heparin–AT III complex formation. Because both maximum and minimum levels of binding and detection of AT III–MAb complex were subject to error and scatter, both were treated as independent variables (i.e., not set at 100% or 0%) for curve fitting

$$\text{heparin complexed} = \frac{\text{max}}{1 + K_d/[\text{heparin}]} + \text{min} \quad (1)$$

where max – min → 100%. For data presentation plots of log[heparin] vs percent complex are used since these display the data more clearly over a large concentration range

Where plasma samples contained a heterozygous mixture of wild-type and mutant inhibitor (AT III and AT III′), experimental data could be fitted to eq 2. Here the inhibitor pool is composed of two populations of equal size of AT III and AT III′ having dissociation constants for heparin of K_d and K_d', respectively

$$\text{heparin complexed} = \frac{\text{max}}{1 + K_d/[\text{heparin}]} + \text{min} + \frac{\text{max}}{1 + K_d'/[\text{heparin}]} + \text{min} \quad (2)$$

where max – min → 50% for AT III and AT III′.

A third set of plasma samples fitted poorly to eq 2, but fitting could be improved by allowing variable proportions of AT III and AT III′ in the inhibitor pool. In this way, using eq 3, relative amounts of each inhibitor and their dissociation constants could be determined. Initial estimates for K_d and K_d' in eq 3 were first generated from eq 2, but all K_d values

Table I: Patient Plasmas Used in This Study—Their Amino Acid Mutations

name	mutation	P	ref
	A.Reactive Site Mutations		
Glasgow 2	Ala382 to Thr	P12	Ireland et al. (1991)
Chicago	Arg393 to His	P1	Erdjument et al. (1989)
Pescara	Arg393 to Pro	P1	Lane et al. (1989a)
Northwick Park	Arg393 to Cys	P1	Erdjument et al. (1988)
Milano 2	Ser394 to Leu	P1′	Olds et al. (1989)
	B.Heparin Binding Site Mutations		
Padua 1	Arg47 to His		Caso et al. (1990)
Padua 2	Arg47 to Cys		Olds et al. (1990)
Budapest 3	Leu99 to Phe[a]		Olds et al. (1992a)
Geneva	Arg129 to Gln		Gandrille et al. (1990)
	C.Pleiotropic Effect Mutations		
Torino	Phe402 to Ser		Lane et al. (1992)
Maisons Laffitte	Phe402 to Leu		Lane et al. (1992)
Paris 3	Ala404 to Thr		Lane et al. (1992)
La Rochelle	Asn405 to Lys		Lane et al. (1992)
Budapest 5	Pro407 to Thr		Lane et al. (1992)
Budapest 1	Pro429 to Leu[a]		Olds et al. (1992b)

[a] Homozygous mutation.

and proportions were treated as independent variables

$$\text{heparin complexed} = \frac{\max_1}{1 + K_d/[\text{heparin}]} + \min_1 + \frac{\max_2}{1 + K_d'/[\text{heparin}]} + \min_2 \quad (3)$$

where $\max_1 - \min_1$ is the proportion of AT III and $\max_2 - \min_2$ is the proportion of AT III′.

K_d values determined in these assays are apparent K_d values since heparin affinity for AT III is reduced by the presence of MAb. True K_d values may be calculated from the relationship

$$K_d = K_d(\text{apparent})/(1 + [L]/K_L) \quad (4)$$

where [L] is the concentration of competing ligand and K_L is the dissociation constant.

Crossed Immunoelectrophoresis. Crossed immunoelectrophoresis was carried out as described (Barrowcliffe, 1979) using 15 IU/mL heparin in the first dimension. The polyclonal antiserum described above was used in the second dimension without any purification.

Heparin–Sepharose Chromatography of AT III Concentrates and Patient Plasmas. The heparin binding of clinical AT III concentrates (BPL, Elstree, U.K.) was studied before and after affinity chromatography on heparin–Sepharose (Pharmacia AB, Uppsala, Sweden). These concentrates are known, due to the presence of a slow moving peak upon crossed immunoelectrophoresis (Figure 3), to contain some heat-denatured material with a reduced affinity for heparin. AT III binding to a heparin–Sepharose column in 0.15 M NaCl and 0.05 M Tris-HCl (pH 7.4) was eluted with a salt gradient from 0.15 to 1.5 M NaCl in 0.05 M Tris-HCl (pH 7.4) to produce high- and low-affinity AT III fractions for analysis.

Heparin–Sepharose chromatography, as described previously, was also used to separate variants AT III Geneva (Gandrille et al., 1990), Budapest 1 (Olds et al., 1992b), and Budapest 5 (Lane et al., 1992) from patient plasmas.

Source of Genetically Abnormal AT III. Table I lists the plasma taken from patients with type II AT III deficiencies used in this study together with their amino acid substitution mutations. The plasmas have been divided into three groups based on the location of the mutations: (Ia) those amino acids known to be in the reactive site loop (residues 382–394, P12–P1′); (Ib) those in the N-terminal heparin binding region; and (Ic) those in hydrophobic regions of the C terminus in or adjacent to strands 1C and 4B.

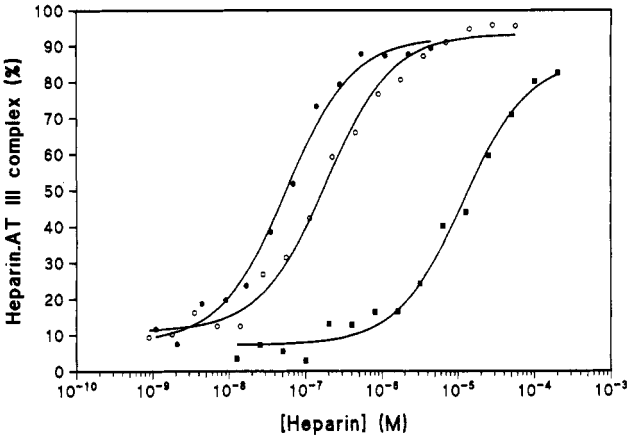

FIGURE 1: Binding curves produced by nonlinear regression analysis of experimental data for (●) unfractionated heparin, (■) low-affinity heparin, and (○) pentasaccharide. AT III (10 ng/mL) was incubated with various heparin concentrations in a microtiter plate previously coated with a MAb which recognizes the heparin binding site of AT III. AT III not bound to heparin at equilibrium forms a AT III–MAb complex which, after washing, is detected using ^{125}I-affinity-purified polyclonal antibody to AT III. The percentage of heparin–AT III complex is determined by subtracting the percentage of AT III–MAb complex at each heparin concentration from the AT III–MAb complex observed in the absence of heparin, taken as 100%.

Fluorescence Measurements. Fluorescence emission spectra of AT III [1st International Concentrate Standard for AT III (NIBSC code 88/548)] and heparin–AT III complex were produced on a Spex FluoroMax spectrofluorometer in the ratio mode at 1-nm intervals with excitation at 280 nm (4.25-nm bandpass) and an emission bandpass of 8.5 nm, with 1-s integrations of the signal at each wavelength. The buffer spectrum was subtracted automatically to correct for Raman bands and background signals. AT III (10 nM) in PBS (pH 7.4) and 1% PEG 8000 (Sigma Chemical Co., Poole, U.K.) was added to a series of dilutions of UFH (Leo Laboratories) in PBS (pH 7.4) containing from 5 to 320 nM HAH, and the fluorescence spectra from 300 to 400 nm were recorded at 25 or 37 °C. The heparin-induced fluorescence enhancement was measured as the increase in peak height or area under the spectrum over that of 10 nM AT III in the absence of heparin. Bound and free heparin concentrations were calculated from the change in fluorescence at each point, and then the K_d value was determined using a normal binding isotherm for a plot of percentage change in fluorescence vs free [heparin], as in eq 1 (Materials and Methods) (Fig P, Biosoft).

RESULTS

Determination of Dissociation Constant for Normal AT III–Heparin Binding. The mean K_d from 14 measurements of the normal plasma AT III–UFH interaction in the binding assay was 48.8 ± 3.2 nM (mean ± SEM) with a range from 21 to 68 nM.

Similar measurements of the interaction of normal AT III with LAH and a synthetic pentasaccharide gave K_d values of 13 ± 2.7 μM and 175 ± 15 nM, respectively.

Typical binding curves for these different heparins are shown in Figure 1.

The interaction of AT III with the same UFH was also measured by analysis of the enhancement of AT III intrinsic fluorescence by heparin. The K_d values determined according to this method were 15.0 ± 1.5 nM (n = 3) at 25 °C and 34.5

Table II: Dissociation Constants for AT III in Plasma from Patients with AT III Reactive Site Defects

name	dissociation constant (μM), K_d ● SEM
Glasgow 2 (n = 1)	0.027
Chicago (n = 3)	0.055 ± 0.003
Pescara (n = 2)	0.052 ± 0.016
Northwick Park (n = 1)	0.038
Milano 2 (n = 1)	0.055

Table III: Dissociation Constants for AT III in Plasma from Patients with AT III Heparin Binding Site Defects

name	dissociation constants (μM) K_{d1}	K_{d2}	reduction in affinity (x-fold)
Padua 1 (n = 2)	0.061 ± 0.001	0.654 ● 0.302	11
Padua 2 (n = 1)	0.071	65.6	924
Budapest 3 (n = 3)	0.328 ± 0.052	4.43 ± 0.767	14
Geneva (n = 6)	0.059 ± 0.013	8.02 ± 2.23	136

± 9.5 nM (n = 2) at 37 °C; thus, the value at 37 °C is slightly lower than the value determined in the binding assay (48.8 nM, also at 37 °C). This was expected since fluorescence gives an estimate of the true K_d value, whereas the binding assay gives an apparent K_d value (see eq 4, Materials and Methods); however, these results suggest that the correction factor is small. Henceforth, only apparent K_d values will be expressed to demonstrate the differences between normal and variant AT III molecules.

Determination of the Heparin Affinities of AT III in Plasma from Functionally AT III Deficient Patients. (*a*) *Reactive Site Mutants.* Table II shows the K_d values determined with the plasmas listed in Table IA from five patients heterozygous for different substitution mutations of amino acids in the reactive site loop of AT III. The binding curves produced (using eq 1, Materials and Methods) for all plasmas were essentially unchanged from that of the normal plasma control AT III examined concurrently. Clearly, mutations of amino acids around the reactive site of AT III had no detectable effect on the interaction with heparin.

(*b*) *Heparin Binding Mutants.* Table III shows the K_d values determined with plasmas listed in Table IB from four patients (three heterozygous and one homozygous) for different substitution mutations of amino acids in the N-terminal region of AT III, known to affect heparin binding. For maximal AT III binding to UFH, higher concentrations were required than in earlier experiments, up to 600 μM HAH. This indicated the presence of a second population of AT III with reduced heparin affinity. Poor fits were obtained for the experimental data with the equation used for normal AT III. To calculate K_d and K_d', eq 2 was used (Materials and Methods), where K_d is the dissociation constant for the binding of the normal AT III and K_d' the variant AT III'. Good fits were obtained to experimental data assuming equal populations of normal and variant AT III. If K_d and K_d' are sufficiently different, the resultant binding curves clearly show the two phases of heparin interaction. The binding affinities for AT III in plasmas from this group of patients vary considerably. Plasma from the Padua 1, Padua 2, and Geneva kindreds all have 50% AT III with a K_d essentially unchanged from that of the control; this is the normal moiety. The remaining 50% AT III in each plasma has reduced affinity for heparin—the variant moieties: their binding affinities vary from 0.65 to 66 μM, which is a reduction of 11–924-fold.

Unlike the other cases in this group, Budapest 3 has no AT III with normal heparin affinity, the propositus being homozygous for the abnormality. Yet in the binding assay there are two populations of AT III with abnormal heparin binding, the determined affinities differing 14-fold.

Table IV: Dissociation Constants for AT III in Plasma from Patients with AT III Pleiotropic Effect Defects

name	dissociation constants (μM) K_{d1}	K_{d2}	reduction in affinity (x-fold)	proportion
Torino (n = 3)	0.048 ± 0.007	10.1 ± 4.80	210	76:24
Maisons Laffitte (n = 1)	0.050	9.71	194	81:19
Paris 3 (n = 1)	0.041	43.4	1056	72:28
La Rochelle (n = 1)	0.032	39.7	1241	64:36
Budapest 5 (n = 1)	0.019	6.09	321	77:23
Budapest 1 (n = 5)	0.051 ● 0.013	29.5 ± 12.9	578	38:62

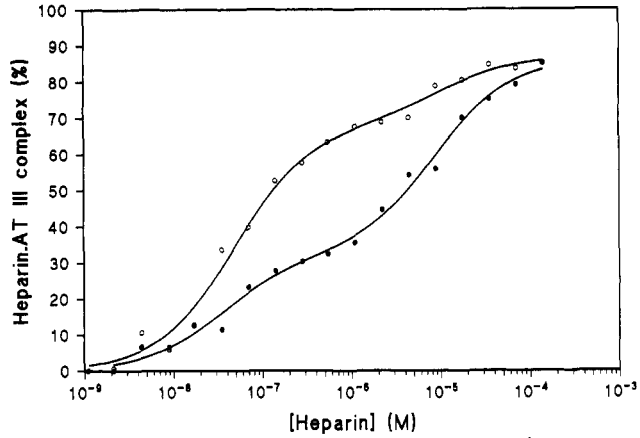

FIGURE 2: Binding curves, produced as described in the legend for Figure 1, for plasma from two patients with pleiotropic mutations: (○) Torino and (●) Budapest 1.

(*c*) *Pleiotropic Effect Mutants.* Table IV shows the K_d values determined for AT III in plasma listed in Table IC from five patients heterozygous and one homozygous for different mutations of amino acids in hydrophobic regions of the C terminus of AT III. A recent study has shown that the AT III in these patients has impaired function of the reactive site and an abnormal binding interaction with heparin (Lane et al., 1992). Furthermore, unlike type I AT III deficiency cases, which have antigen levels 50% of normal, or most type II deficiencies, which have 100% antigen levels, the patients in this group usually have antigen levels around 70% of normal. The binding experiments of the present study demonstrate that the plasmas show reduced heparin affinity. We found that improved fits to the experimental data could be obtained using eq 3 (Materials and Methods), where K_d and K_d' populations could be varied. In every case, there is a population of AT III with a K_d within the range expected for normal AT III and a population with a K_d that is reduced. Although the AT III Budapest 1 plasma is obtained from a patient who is homozygous for a substitution mutation, plasma from the propositus has previously been shown to contain two populations of AT III with different binding affinities for heparin–Sepharose (Sorensen et al., 1982; Olds et al., 1992b). In each case except that of AT III Budapest 1, the variant population is determined to be smaller than that of the normal. Figure 2 shows binding curves obtained for plasma from the Torino and Budapest 1 kindreds, highlighting the different binding isotherms that indicate both the different binding affinities and the different amounts of variant AT III present in the two plasmas. The affinities of all the variant AT III components

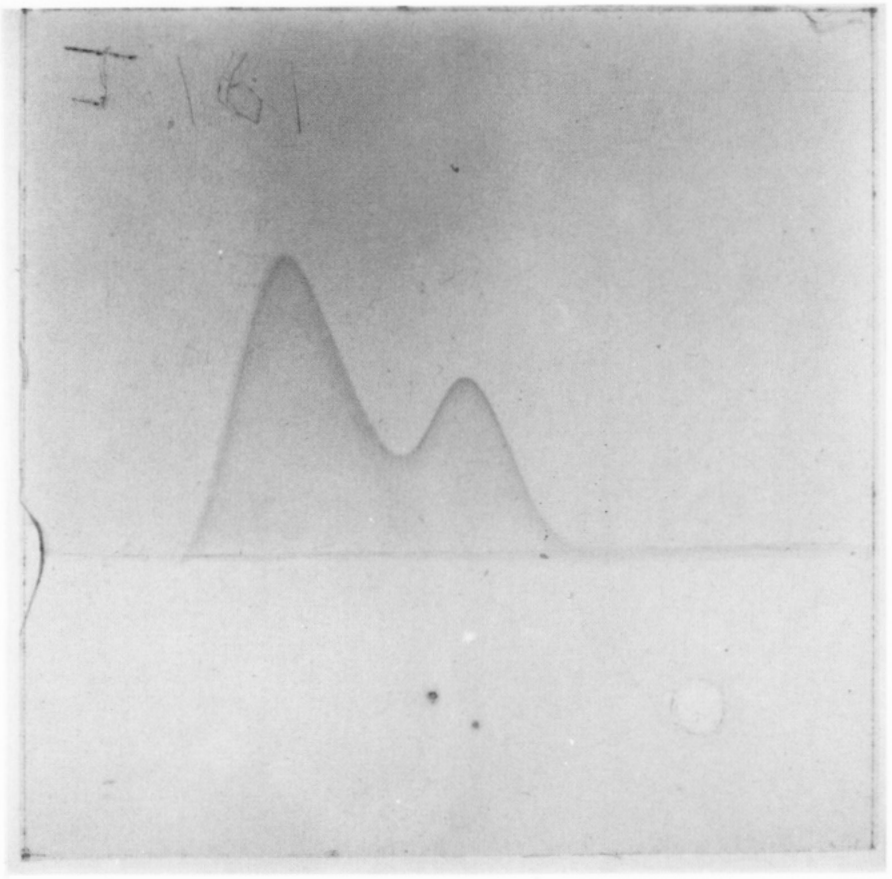

FIGURE 3: Crossed immunoelectrophoresis of an AT III concentrate with 15 IU/mL heparin in the first dimension (run from right to left).

in this group are similar, varying from 6 to 43 μM, which is from 194- to 1241-fold less than the normal molecule.

Investigation of the Heparin Binding Characteristics of Purified AT III from Concentrates and Patient Plasmas. Figure 3 shows crossed immunoelectrophoresis of a commercial purified AT III concentrate in the presence of heparin, revealing a significant proportion of slow moving material which has a reduced ability to bind heparin. This is thought to be AT III denatured during the heat treatment required for viral inactivation of the product. The existence of 2 populations of AT III was confirmed when the interaction of the concentrate with UFH was measured in the binding assay, giving K_d values of 51 nM (in excellent agreement with the K_d for plasma AT III) and 7 μM. Elution of these concentrates from a heparin–Sepharose column with a NaCl gradient (0.15 to 1.5 M) resulted in 2 fractions, one eluting at $\approx$0.4 M and the other at $\approx$0.9 M NaCl. The binding affinities measured in the IRMA for these 2 fractions were 61 nM and 9 μM, in good agreement with those determined on the starting material.

K_d values were also measured in the binding assay for 3 variant AT IIIs separated from their plasma milieux by elution from heparin–Sepharose at low NaCl concentrations. The mean K_d values for separated AT III Geneva was 10.2 $\pm$ 4.3 μM (n = 2) compared to 8.0 $\pm$ 2.23 μM (n = 6) in the plasma, for separated AT III Budapest 1 was 25.2 $\pm$ 7.2 μM (n = 2) compared to 29.5 $\pm$ 12.9 μM (n = 5) in the plasma, and for separated AT III Budapest 5 was 10.9 $\pm$ 5.5 μM (n = 2) compared to 6.09 μM (n = 1) in the plasma. The K_d values obtained for purified variant AT III agree well with the estimates obtained from determinations on their plasmas. This confirms the reliability of the method for measuring binding affinities on nonhomogeneous samples, including plasma, without purification, and for providing a quantitative measure of the heparin binding properties of samples for which only qualitative information is usually available.

DISCUSSION

We report a modification to the previously described IRMA (based on a MAb binding at or close to the heparin binding site of AT III), which has enabled quantitative investigation of the heparin–AT III interaction. This has resulted in a sensitive method for calculating the binding affinity (dissociation constant) for the interaction, based upon the competition between heparin and the MAb for the same binding site on AT III. This competition was suggested by the fact that the MAb blocks the catalytic enhancement of the inhibitory action of AT III induced by heparin and that the detection of AT III in an IRMA using this MAb as a capture antibody was reduced by increasing concentrations of heparin added with the AT III (Watton et al., 1990).

Unfractionated heparin (UFH), which has been used in this investigation, comprises a heterogeneous population of sulfated polysaccharide chains. High-affinity binding of heparin to AT III requires the presence of a specific pentasaccharide sequence (Lindahl et al., 1980; Choay et al., 1983), and the population of chains containing this sequence is termed high-affinity heparin (HAH). About 70% of UFH are low-affinity chains which lack this sequence (Andersson et al., 1976; Lam et al., 1976). Previous measures of the high-affinity heparin–purified AT III interaction have been by analysis of fluorescence or UV spectroscopy using HAH of molecular weights ranging from 6000 to 35 000 (Einarsson & Andersson, 1977; Danielsson & Björk, 1981; Nordenman & Björk, 1981; Olson & Shore, 1981). The K_d values reported vary widely between 12.5 nM and 1.7 μM, although most are between 20 and 100 nM at low ionic strength. The dissociation

constant we determined for purified AT III–heparin using fluorescence changes is at the high-affinity end of this range, with tighter binding at lower temperatures, in agreement with previous observations (Atha et al., 1985; Hogg, et al., 1991). As expected, the value derived from the binding assay using either the same materials or plasma AT III is slightly higher (48.8 nM) due to competition of MAb and heparin for the heparin binding site, but it remains well within the accepted range of K_d values for this interaction.

Further confirmation of the specificity of the MAb for the heparin binding site of AT III was obtained by measuring the K_d for low-affinity heparin (LAH) and a synthetic pentasaccharide. The total contribution of heparin to high-affinity binding appears to require some longer heparin chains, possibly providing ionic interactions, as well as the specific binding of the pentasaccharide sequence (Oosta et al., 1981). The slightly lower affinity of 175 nM measured for the pentasaccharide (MW 1714) is in agreement with other investigators reporting values of 140, 100, 192, and 300 nM (Choay et al., 1983; Atha et al., 1985; Ichikawa et al., 1986; Visser et al., 1991). The finding that the pentasaccharide successfully competes with the MAb for AT III binding suggests that the MAb recognizes an epitope located within or immediately adjacent to the pentasaccharide binding site on AT III. LAH contains no pentasaccharide sequence and thus has a much lower affinity—in this assay 270-fold reduced. The K_d of 13 μM is very close to other reports of 15–20 μM (Nordenman & Björk, 1978; Lindahl et al., 1984), although somewhat lower than another of 100 μM (Jordan et al., 1979). However, it should be noted that low-affinity interactions require high concentrations of heparin for complete binding curves, and problems due to ionic strength or protein destabilization can arise. This may also affect some K_d determinations for the low-affinity mutant AT III molecules. The spectroscopic methods used by other investigators for K_d measurements require purified materials. A major advantage of the binding assay is that the specificity of the MAb removes the need for purification; thus, heparin affinity for AT III in plasma or conceivably recombinant AT III prior to purification may be investigated in an undenatured state. K_d values have not previously been determined for genetically abnormal AT III, and studies of their heparin binding characteristics have been limited to crossed immunoelectrophoresis and binding to heparin–Sepharose. Crossed immunoelectrophoresis in the presence of heparin may reveal low-affinity AT III as a slow moving peak which does not bind to heparin under the experimental conditions but will give no indication of the affinity. Heparin–Sepharose chromatography may give some indication of relative affinities when AT III bound to the column in low ionic strength buffer is eluted by a salt gradient. However, AT III is often loaded in 0.4 M NaCl with the assumption that AT III with reduced affinity would appear in the breakthrough and normal AT III can be eluted by a step to 2 M NaCl. This would only be true with a large reduction in affinity. In this study we not only have determined the dissociation constant for normal plasma AT III but also have been able to measure, for the first time, both K_d values for the two populations of AT III present in plasma from patients heterozygous for known genetic abnormalities. The validity of the measurements on heterogenous samples was confirmed when the K_d values for high- and low-affinity AT III separated from plasma-derived concentrates were in good agreement with the values obtained on the unfractionated concentrates. The low-affinity AT III in these materials is likely to be denatured and thus different from the low-affinity AT III arising from point mutations. However, K_d values obtained for separated variant AT IIIs Geneva, Budapest 1, and Budapest 5 also agreed well with the values obtained from analysis of the plasma samples.

Predictably, no AT III with reduced affinity was found in plasmas from patients with substitution mutations in the reactive site loop as it is well separated from the heparin binding region in the tertiary structure. However, several investigators have suggested increased binding to heparin–Sepharose for AT III with a mutation at the P1 Arg393 residue, namely substitution with His in AT III Chicago (Bauer et al., 1983) and Glasgow (Owen et al., 1991), with Pro in AT III Pescara (Owen et al., 1991), and with Cys in AT III Northwick Park (Lane et al., 1987). The mechanism responsible for this possible increased heparin affinity is unknown. Furthermore, increased binding could not be shown for AT III Sheffield (Lane et al., 1989b) (also Arg393 to His). Where increased binding was reported, it usually manifested itself as a broader peak on gradient elution of the plasma from heparin–Sepharose, with an increased proportion of the abnormal AT III later in the peak. Only with plasma containing AT III Glasgow was there partial resolution of the peaks. Often the AT III with increased binding appeared to be less than 50% of the total. In the binding assay, plasma from Pescara and Chicago kindreds did show a tendency toward a second population with higher affinity, but as the changes were small and could not be quantitated, this was not conclusive. The present evidence suggests that any small increase in affinity resulting from these mutations would require many assays to determine reliable K_d values for the two populations, such that it may not be possible to measure such small *increases* in heparin affinity with this assay.

In contrast to the P1 mutants, the normal and variant AT III in plasma from patients heterozygous for mutations in the region proposed as the primary interaction site for heparin are clearly seen by crossed immunoelectrophoresis and readily separated by heparin–Sepharose chromatography. A computer model of the three-dimensional structure of reactive site cleaved AT III based upon the X-ray crystal structure of α_1-antitrypsin has been used to orient the specific amino acids likely to interact with heparin (Huber & Carrell, 1989). There is a concentration of positively charged Arg and Lys residues in helices A and D, encompassed by amino acids 35–50 and 114–154, which align across the surface of the folded molecule (Borg et al., 1988). The band of positive charges forms the heparin binding site, providing interactions with the negative sulfate groups of the pentasaccharide binding sequence. Evidence for the involvement in heparin binding of residues in these helices has come from chemical modification experiments (Blackburn et al., 1984; Chang, 1989; Sun & Chang, 1990) and from studying plasma from patients with known mutations. Studies have been reported on natural substitution mutations of amino acids in the heparin binding domain: the structurally important Pro41 in AT III Basel (Chang & Tran, 1986) and the positively charged Arg47 in AT III Padua 2 (Olds et al., 1990), Alger (Wolf et al., 1982), Paris (Fischer et al., 1986), and Rouen I (Owen et al., 1987) as well as Arg129 in AT III Geneva (Gandrille et al., 1990). In one of these studies, the variant AT III Geneva (Arg129 to Gln) was separated from plasma by its nonbinding to heparin–Sepharose in 0.4 M NaCl but was shown to have some residual heparin cofactor activity. This implied that while heparin binding was diminished by this mutation, it was not abolished. Investigations have compared, indirectly and by inference, the effect of this mutation to that of mutations at Arg47. AT

III Padua 2, Paris, and Alger have a Cys replacement which showed no binding to heparin–Sepharose at 0.15 M NaCl and no heparin cofactor activity. In contrast, families in which Arg47 is replaced by His showed considerably more binding to heparin–Sepharose; for example, AT III Rouen I was eluted at 0.45 M NaCl. The K_d values reported here confirm the importance of Arg47 and Arg129 for heparin binding and support the qualitative evidence for these mutations having different effects on heparin affinity. A 136-fold increase in K_d is seen for AT III Geneva, where substitution of Arg129 with an uncharged Gln impairs the interaction with the sulfate groups. The importance of positive charges is emphasized by the substitution of Arg47 with the neutral Cys in AT III Padua 2, reducing the affinity almost 1000-fold, as expected from previous work (Olds et al., 1990), giving a lower affinity than AT III Geneva. A His residue, however, is capable of carrying a positive charge, which may explain why it is much less disruptive to heparin binding and causes only an 11-fold decrease in affinity in AT III Padua 1.

Budapest 3 is characterized by a Leu99 to Phe substitution found in an individual homozygous for the mutation. This has been reported to elute from heparin–Sepharose as a single peak at 0.48 M NaCl and gives a single peak of slow moving material on crossed immunoelectrophoresis (Olds et al., 1992a). In contrast to the chromatography (but not the electrophoresis, which merely indicates the absence of normal AT III), the distribution of the data in the binding assay clearly favors the existence of two populations of AT III in this plasma. Although both have a reduced affinity for heparin, there appears to be a 14-fold difference in K_d between them. Apart from this, the affinities determined by K_d calculation agree well with the qualitative comparisons to other heparin binding mutants. Residue 99 is in helix C, which lies beneath helix D of the N-terminal heparin binding domain (Huber & Carrell, 1989). It has been suggested (Olds et al., 1992a) that the mutation with a bulky Phe may distort the residues close to it in the heparin binding domain or may affect the nearby glycosylation site of Asn96. This could alter the carbohydrate content, which is known to be important in heparin binding from studies of AT III β, where reduced glycosylation at Asn135 results in increased heparin affinity (Peterson & Blackburn, 1985). It is possible that variable glycosylation at Asn96 could provide an explanation for the apparent heterogeneity of the heparin binding of the AT III in this plasma. Our results confirm the importance of residue 99 in maintaining the structural integrity of the heparin binding site.

The final group of mutants studied in this report are in or around the strand 1C region, C-terminal to the reactive site: amino acids 402–407 as well as 429, which lies close to this region. Strand 1C is close to strands 4B and 5B, which are hydrophobic regions highly conserved in the serpin family (Carrell & Travis, 1985). Recent investigations on plasmas containing AT III with these mutations indicated that their effects are pleiotropic, affecting heparin binding, the reactive site, and circulating levels of AT III (Lane et al., 1992). Their proximity to the reactive site loop could explain the reduced thrombin inhibition, but crossed immunoelectrophoresis of the plasmas unexpectedly gave abnormal patterns indicating the presence of some material with reduced heparin affinity. Total antigen levels in plasma from patients heterozygous for such mutations were estimated to be between 50% and 100%. Heterozygotes are expected to have 50% of the usual antigen level of normal AT III, and antigen levels greater than 50% indicate the presence of variant AT III. The total antigen content of <100% implies that there is a reduced level of variant protein, and in most cases this was confirmed by the crossed immunoelectrophoresis pattern. Heparin binding has been postulated to occur in two stages (Olson et al., 1981): an initial interaction having an affinity of $\approx$40 μM, followed by an increase in affinity which may be due to a conformational change in the AT III molecule. This conformational change possibly affects the reactive site which is in the C-terminal region, enhancing the thrombin inhibition. Mutations in or around the hydrophobic strands 4B and 5B, C-terminal to the reactive site, could either prevent the conformational change necessary for high-affinity binding of heparin or induce general dysfunctional folding which directly alters the primary pentasaccharide binding site. For instance, the Pro at position 407 is at the turn from strand 1C to strand 4B, so its substitution with Thr in Budapest 5 is likely to alter the conformation. A similar structural change would be expected for the Pro429 to Leu substitution in Budapest 1. However, unlike mutations of the N terminus, the K_d values determined for this group of mutants are all of the same order, between 6 and 44 μM, and consistent with heparin forming an initial loose complex with AT III but not able to follow this with the high-affinity binding. The binding assay also showed that the plasma levels of variant AT III were affected (usually reduced), which could be due to a conformational change causing abnormal synthesis or turnover of the mutant AT III. The results of these binding assays taken together with the recent investigations (Lane et al., 1992) strongly suggest that the conserved hydrophobicity of this C-terminal region of AT III is important for the structural integrity of AT III. It appears that mutations in the strand 1C region result in conformational changes of the molecule which cause these pleiotropic effects on the inhibitory activity, heparin binding, and circulating levels of AT III. The result with Budapest 1 deserves comment. It has been noted before (Sorensen et al., 1982; Olds et al., 1992b) that two populations of AT III exist in the plasma of the propositus who is homozygous for the mutation. The present results confirm that two forms of AT III with different affinities for heparin coexist in the plasma but provide no further insight into an explanation for the two populations. The mutation is located next to the C-terminal Cys residue, which participates in a disulfide bond, and it is possible that a partial disruption of this bond may explain the heterogeneity observed.

The K_d values obtained with the binding assay provide the first quantitation of the heparin binding affinities of genetically abnormal AT III, extending previous studies on these materials. The assay should be a useful tool for continued quantitative investigations of the structural–functional relationships involved in heparin–AT III interactions.

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