The *N*-terminal domain of antithrombin-III is essential for heparin binding and complex-formation with, but not cleavage by, α -thrombin

Richard C. AUSTIN,* William P. SHEFFIELD,* Richard A. RACHUBINSKI† and Morris A. BLAJCHMAN*††

*Canadian Red Cross Blood Transfusion Service and the †Departments of Biochemistry and Pathology, McMaster University, Hamilton, Ont. L8N 3Z5, Canada

Normal and mutant forms of human antithrombin-III (AT-III) were synthesized in a cell-free system in order to identify putative functional domains required for heparin binding and complex-formation with α -thrombin. Heparin–Sephacrose chromatography resulted in the elution of approx. 70 % of cell-free-derived normal AT-III-(1–432)-polypeptide as a peak between 0.2 M- and 0.7 M-NaCl. The cell-free-derived normal AT-III also reacted with α -thrombin. Approx. 15 % of this AT-III formed covalent complexes with α -thrombin in 2 min. Unfractionated heparin accelerated the rate of formation of such complexes. Two truncated forms of AT-III (amino acid residues 219–432 and 251–432), containing only the putative thrombin-binding domain, were synthesized independently in this cell-free system. These truncated AT-III polypeptides did not bind heparin and were unable to form stable covalent complexes with α -thrombin. However, both of these AT-III polypeptides were cleaved by α -thrombin, presumably at the reactive centre Arg-393–Ser-394. The formation of the disulphide bond between Cys-247 and Cys-430 in AT-III-(219–432)-polypeptide had no effect on the results obtained. Mutations in full-length AT-III at Cys-430 had no effect on the ability of AT-III to bind heparin. There was, however, a slight decrease in the formation of stable inhibitory complexes with α -thrombin. A cell-free-derived AT-III mutant, devoid of amino acid residues 41–49, which comprise heparin-binding region 1 of AT-III, had slightly decreased heparin binding compared with cell-free-derived normal AT-III-(1–432)-polypeptide. This mutant AT-III polypeptide was unable, however, to form a stable complex with α -thrombin. We conclude therefore that the *N*-terminal domain of AT-III is essential for both heparin binding and complex-formation with α -thrombin, but not for the cleavage of AT-III at its reactive centre by α -thrombin.

INTRODUCTION

The serine-proteinase inhibitor (serpin) antithrombin-III (AT-III) is a single-chain plasma glycoprotein that inactivates thrombin as well as other serine proteinases involved in the blood coagulation cascade (Rosenberg & Damus, 1973; Petersen *et al.*, 1979). Proteinase inactivation is through the formation of a 1:1 stoichiometric covalent complex with the reactive centre Arg-393 of AT-III and the reactive-site serine residue of the proteinase (Björk *et al.*, 1982). With the addition of heparin the inhibitory activity of AT-III is accelerated by as much as 10000-fold (Jordan *et al.*, 1980). The characterization of natural mutations of AT-III along with chemical-modification data has allowed for the identification of two functional domains in AT-III (Manson *et al.*, 1989). The *C*-terminal domain of AT-III contains the reactive centre Arg-393–Ser-394 involved in proteinase inactivation whereas the *N*-terminal domain of AT-III mediates heparin-enhanced AT-III inhibitory activity. However, the extent to which these domains interact is still unknown.

In the present paper we report the cell-free expression of normal and mutant forms of human AT-III and their ability to bind heparin and form stable covalent complexes with α -thrombin. On the basis of the data obtained we conclude that the *N*-terminal domain of AT-III is essential for both heparin binding and complex-formation with α -thrombin. It is not, however, required for the cleavage of AT-III at its reactive centre by α -thrombin.

MATERIALS AND METHODS

Materials

The cDNA encoding human AT-III was generously provided by Dr. E. V. Prochownik (University of Michigan, Ann Arbor, MI, U.S.A.). Human α -thrombin (> 3000 NIH units/mg; > 99 % active) was kindly provided by Dr. J. Fenton (New York State Division of Biologicals, Albany, NY, U.S.A.). Restriction endonucleases were purchased from Bethesda Research Laboratories (Burlington, Ont., Canada). T4 DNA ligase, T7 RNA polymerase, ribonucleotides and 7-methylguanosylguanosine triphosphate were purchased from Pharmacia LKB Biotechnology (Baie d'Urfé, Quebec, Canada). The cell-free expression vector pGEM-3Zf(+) and rabbit reticulocyte lysate (RRL) were purchased from Promega-Biotec (Toronto, Ont., Canada). D-Phe-L-Pro-L-Arg-CH₂Cl was purchased from Calbiochem (Mississauga, Ont., Canada). Protein molecular-mass markers and GSSG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α -[³²P]dATP (3000 Ci/mmol) was purchased from Amersham (Oakville, Ont., Canada), and translation-grade L-[³⁵S]methionine (> 800 Ci/mmol) from New England Nuclear (Lachine, Quebec, Canada). All other chemicals and reagents were of the highest quality available.

Construction of cell-free expression vectors containing AT-III cDNA

The pGEM-3Zf(+)-AT-III-(1–432) and pGEM-3Zf(+)-AT-

Abbreviations used: AT-III, antithrombin-III; HBR, heparin-binding region; RRL, rabbit reticulocyte lysate; serpin, serine-proteinase inhibitor. † To whom correspondence should be addressed, at Department of Pathology, Room 2N31, McMaster University Medical Centre, 1200 Main Street West, Ont. L8N 3Z5, Canada.

III-(251–432) constructs were synthesized as described previously (Austin *et al.*, 1990). The pGEM-3Zf(+)-AT-III-(219–432) construct was synthesized in the following manner. pGEM-3Zf(+)-AT-III-(1–432) (4.8 kb) was digested to completion with *Sac*I to yield fragments of 4.1 and 0.7 kb. The 4.1 kb fragment which contains approx. 700 bp coding for Leu-210–Lys-432 of AT-III was isolated from polyacrylamide, re-ligated with T4 DNA ligase and transformed into *Escherichia coli* DH5 α F' cells. Bacterial cells harbouring this construct were infected with M13K07 helper bacteriophage to yield single-stranded DNA for site-directed mutagenesis (see below).

Site-directed mutagenesis

Site-directed mutagenesis was performed by the method of Taylor *et al.* (1985) with the reagents and protocol of the Amersham mutagenesis kit. The AT-III-(219–432) variant was synthesized with the use of the mutant oligodeoxyribonucleotide primer 5'-GTTAACACCATGTACTTC-3', which contains a single base substitution that converts Ile-219 (ATT) into Met-219 (ATG). This change allows for initiation of translation to occur at residue 219 of AT-III. Single-stranded pGEM-3Zf(+)-AT-III-(1–432) DNA was also used with the appropriate mutant oligodeoxyribonucleotide primers to synthesize mutants at Cys-430 as well as the heparin-binding region-1 (HBR-1) deletion mutant. A mixed oligodeoxyribonucleotide primer 5'-GCCAACCCTTGTGTTAAGTAA-3' was synthesized such that all three nucleotides corresponding to Cys-430 (TGT) could be altered. In addition, an oligodeoxyribonucleotide primer 5'-GAACAGAAGATCGAACTGTCCAA-GGC-3' was synthesized such that the nucleotides encoding Pro-41–Trp-49 were deleted to form the heparin-binding region-1 (HBR-1) deletion mutation. After duplex formation and phosphothioate enrichment, plasmid DNA was used to transform *E. coli* DH5 α cells. Authenticity of the mutations was confirmed by double-stranded DNA sequencing by the chemically modified T7 DNA polymerase method of Tabor & Richardson (1987).

Expression and characterization of cell-free-derived normal and variant forms of AT-III

Cell-free transcription–translation was performed as previously described (Austin *et al.*, 1990). The cDNA encoding human AT-III was inserted into the cell-free expression vector pGEM-3Zf(+) under the transcriptional control of the T7 promoter and designated pGEM-3Zf(+)-AT-III-(1–432). This construct was used for the synthesis of mutant forms of AT-III by employing standard cloning techniques as well as site-directed mutagenesis. Cell-free expression constructs were then linearized with *Hind*III and transcribed with T7 RNA polymerase. The mRNA obtained from the cell-free transcription of these constructs was translated in RRL in the presence of [³⁵S]methionine. The ability of the cell-free-derived normal and mutant forms of AT-III to bind heparin was assessed by heparin–Sepharose chromatography according to the method of Miller-Andersson *et al.* (1974). Standard pig heparin (> 150 USP units/mg; Sigma Chemical Co.) was coupled to CNBr-activated Sepharose (Pharmacia). Before heparin–Sepharose chromatography the translation mixtures were diluted 2-fold in 0.2 M-NaCl/0.04 M-Tris/HCl buffer, pH 7.5, and centrifuged at 150 000 *g* for 1 h at 4 °C to pellet insoluble material. The translation mixture was then dialysed against 0.1 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, overnight at 4 °C and applied to heparin–Sepharose equilibrated in the same buffer. The ability of cell-free-derived normal and variant forms of AT-III to react with α -thrombin in the presence of 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, was investigated as previously described (Austin

et al., 1990). Heparin binding and α -thrombin complex-formation of the various cell-free-derived AT-III polypeptides was assessed by SDS/PAGE (Laemmli, 1970) under both reducing and non-reducing conditions (Bulleid & Freedman, 1988). Quantitative analysis was determined by scanning the autoradiograms with the Hoefer GS300 transmittance/reflectance scanning densitometer and the GS350 data system (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

RESULTS

Cell-free expression and synthesis of normal and truncated forms of human AT-III

The major polypeptide synthesized in the presence of AT-III-(1–432)-polypeptide mRNA migrated with an apparent molecular mass of 47 kDa as determined by SDS/PAGE under reducing conditions (Fig. 1, lane 1). This radiolabelled polypeptide also migrated faster than plasma-derived AT-III (Fig. 1, asterisk). Densitometric scanning of the autoradiogram revealed that this non-glycosylated mature form of AT-III constituted greater than 95% of the total translated products synthesized in this cell-free system. Two truncated forms of AT-III were also synthesized. The major polypeptides synthesized in the presence of either AT-III-(219–432) or AT-III-(251–432) mRNA had apparent molecular masses of 24 and 21 kDa respectively, as determined by SDS/PAGE under reducing conditions (Fig. 1, lanes 2 and 3). Densitometric scanning revealed that cell-free-derived AT-III-(219–432)-polypeptide and AT-III-(251–432)-polypeptide constituted greater than 90 and 95% respectively of the total translated products.

Fig. 1. SDS/PAGE analysis of cell-free-derived normal and truncated AT-III polypeptides

Cell-free-transcribed AT-III mRNAs were translated in RRL containing [³⁵S]methionine and analysed by SDS/PAGE on a 12% (w/v) polyacrylamide gel under reducing conditions, followed by fluorography. Lane 1, products of translation in the presence of AT-III-(1–432) mRNA; lane 2, products of translation in the presence of AT-III-(219–432) mRNA; lane 3, products of translation in the presence of AT-III-(251–432) mRNA. The asterisk represents the migration position of plasma-derived AT-III. m, molecular-mass markers (kDa): 66, BSA; 45, egg albumin; 36, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; 29, bovine erythrocyte carbonic anhydrase; 20, soyabean trypsin inhibitor; 14, bovine milk α -lactalbumin.

Fig. 2. Effect of disulphide bond formation on the electrophoretic mobility of cell-free-derived truncated AT-III polypeptides

Products of translation in the presence of cell-free-transcribed AT-III-(219–432) (a and b) or AT-III-(251–432) (c) mRNA were analysed by SDS/PAGE on a 10–20% (w/v) polyacrylamide gradient gel under reducing (R) and non-reducing (N) conditions. Cell-free-derived polypeptides were prepared for SDS/PAGE under the following conditions: in (a) and (c), GSSG at a concentration of 2.5 mM was added to the translation system at the initiation of translation; in (c), iodoacetamide at a concentration of 100 mM was added in the translation system at the end of translation and incubated for 30 min at 22 °C. After 1 h at 30 °C the translation mixtures (lanes 1 and 2) were diluted 2-fold in 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, and dialysed overnight at 4 °C in the same buffer (lanes 3 and 4). The arrowhead represents the migration position of AT-III-(219–432)-polypeptide under non-reducing conditions (a). m, molecular-mass markers (kDa).

Effect of disulphide bond formation on the electrophoretic mobility of cell-free-derived truncated AT-III polypeptides

In human plasma-derived AT-III, Cys-247 and Cys-430 form a disulphide bond within the C-terminal domain, which contains the reactive centre of the inhibitor. Fig. 2 shows the electrophoretic mobilities of the cell-free-derived truncated forms of AT-III resolved by SDS/PAGE under reducing and non-reducing conditions, before and after dialysis. The truncated AT-III polypeptides synthesized were translated in the presence (Figs. 2a and 2c, lanes 1 and 2) or absence of 2.5 mM-GSSG (Fig. 2b, lanes 1 and 2) followed by dialysis in 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5 (Fig. 2, lanes 3 and 4). Non-reduced AT-III-(219–432)-polypeptide with intact disulphide bonds (Fig. 2a, lane 4, arrowhead) migrated faster, with an apparently lower molecular mass, than its reduced and *S*-carboxyamidomethylated counterpart (Fig. 2a, lane 3). Densitometric scanning revealed that greater than 96% of the AT-III-(219–432)-polypeptides were disulphide-linked. The addition of GSSG at final concentrations of 5 mM and 10 mM respectively resulted in disulphide bond formation without overnight dialysis (results not shown). However, the efficiency of translation was markedly inhibited, as determined by scanning of the autoradiograms and by the level of incorporation of [³⁵S]methionine into cell-free synthesized polypeptides (results not shown). AT-III-(219–432)-polypeptides synthesized in the absence of GSSG followed by *S*-carboxyamidomethylation were not able to form disulphide bonds (Fig. 2b, lanes 1 and 2) even after overnight dialysis (compare lanes 3 and 4, Fig. 2b). When AT-III-(251–432)-polypeptide was translated in the presence of 2.5 mM-GSSG, followed by overnight dialysis, no evidence of disulphide bond formation was seen as this truncated AT-III contains only Cys-430 (Fig. 2c). In addition,

Fig. 3. Heparin-Sepharose chromatography of cell-free-derived normal and mutant AT-III polypeptides

Cell-free translation mixtures containing [³⁵S]methionine-labelled AT-III polypeptides were diluted 2-fold in 0.1 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, and dialysed overnight at 4 °C in the same buffer. GSSG was added at a concentration of 2.5 mM to the translation mixture at the initiation of translation. The translation mixtures were dialysed overnight in 0.1 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, and applied to heparin-Sepharose followed by incubation for 60 min at room temperature. The cell-free-derived AT-III polypeptides were eluted from heparin-Sepharose in 0.02 M-Tris/HCl buffer, pH 7.5, containing 0.1–2.0 M-NaCl and the fractions were analysed by SDS/PAGE on a 12% (w/v) polyacrylamide gel under reducing conditions, followed by fluorography. The amount of cell-free-derived AT-III that does not bind to the heparin-Sepharose is shown as an NaCl concentration of 0 M. The molar concentration of this heparin-binding buffer is, in fact, 0.1 M. The percentage of bound and unbound AT-III polypeptides was determined by densitometric scanning of the autoradiograms. (a) shows the heparin-binding affinity of cell-free-derived AT-III-(1–432)-polypeptide (○); (b) shows the heparin-binding affinity of cell-free-derived AT-III-(219–432)-polypeptide (■); (c) shows the heparin-binding affinity of AT-III-(251–432)-polypeptide (▲); (d) shows the heparin-binding affinity of the cell-free-derived HBR-1 deletion polypeptide (●).

Fig. 4. Time-dependent interaction between α -thrombin and cell-free-derived normal and HBR-1-deleted AT-III polypeptides

Cell-free translation mixtures containing [35 S]methionine-labelled AT-III polypeptides were dialysed overnight in 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, and incubated with α -thrombin at 37 °C. At specific time points from 15 s to 40 min (lanes 2–8) portions of the reaction mixture were terminated with D-Phe-L-Pro-L-Arg-CH₂Cl at a final concentration of 5 μ M and analysed by SDS/PAGE on a 10% (w/v) polyacrylamide gel under reducing conditions, followed by fluorography. (a) Normal AT-III-(1–432)-polypeptides. (b) HBR-1-deleted AT-III polypeptides. The arrowhead indicates the migration position of the complex between α -thrombin and cell-free-derived normal AT-III. The dot (●) indicates the migration position of α -thrombin-dependent cleaved AT-III. m, molecular-mass markers (kDa).

the autoradiograms revealed no evidence of intermolecular disulphide bonding between the truncated polypeptides or any of the RRL proteins. These results indicate that, under the correct conditions, AT-III-(219–432)-polypeptide can be synthesized with an intact Cys-247–Cys-430 disulphide bond. The results also indicate that the formation of this disulphide bond in AT-III-(219–432)-polypeptide is not dependent on the presence of pancreatic microsomal fraction as has been suggested for other secretory proteins (Kaderbhai & Austen, 1985; Bulleid & Freedman, 1988).

Heparin–Sephacel chromatography of cell-free-derived normal and truncated forms of AT-III

Densitometric scanning of the fractions collected off heparin–Sephacel revealed that approx. 70% of the normal cell-free-derived AT-III-(1–432)-polypeptides bound to heparin–Sephacel and was eluted as a major peak between 0.2 M- and 0.7 M-NaCl (Fig. 3a). In addition, more than 90% of plasma-derived AT-III (in the absence of RRL) bound to heparin–Sephacel and was eluted as a major peak between 0.6 M- and 1.0 M-NaCl (results not shown). However, greater than 98% of cell-free-derived AT-III-(219–432)-polypeptide (Fig. 3b) and greater than

Fig. 5. Time-dependent interaction between human α -thrombin and cell-free-derived truncated AT-III polypeptides

Cell-free translation mixtures containing [35 S]methionine-labelled AT-III polypeptides were prepared for SDS/PAGE as described in the legend to Fig. 2 and incubated with α -thrombin at 37 °C. At specific time points from 15 s to 40 min (lanes 2–8) portions of the reaction mixture were terminated with D-Phe-L-Pro-L-Arg-CH₂Cl at a final concentration of 5 μ M and analysed by SDS/PAGE on a 10–20% (w/v) polyacrylamide gradient gel under reducing conditions, followed by fluorography. (a) Disulphide-linked AT-III-(219–432)-polypeptides; (b) reduced and carboxymethylated AT-III-(219–432)-polypeptides; (c) AT-III-(251–432)-polypeptides. ● indicates the expected migration position of the complex between α -thrombin and the truncated AT-III polypeptides. m, molecular-mass markers (kDa).

94% of the cell-free-derived AT-III-(251–432)-polypeptides (Fig. 3c) had no affinity for heparin.

Interaction between cell-free-derived normal or truncated forms of AT-III and α -thrombin

When incubated with a 100-fold molar excess of α -thrombin, normal cell-free-derived AT-III-(1–432)-polypeptide formed covalent complexes of a molecular mass of approx. 75 kDa (Fig. 4a, arrowhead). Densitometric scanning of the autoradiogram revealed that 15% of these radiolabelled AT-III polypeptides could form covalent complexes with α -thrombin in 2 min. Beyond this time point the amount of stable complexes detected declined slowly, through either degradation or dissociation. Unfractionated heparin was also able to accelerate the rate of formation of such complexes (results not shown). In addition, cell-free-derived AT-III-(1–432)-polypeptide was able to compete with plasma-derived AT-III for α -thrombin as previously described (Austin *et al.*, 1990). The cell-free-derived truncated forms of AT-III did not form covalent complexes of a higher molecular mass with α -thrombin (Fig. 5). Instead, AT-III-

Fig. 6. Effect of disulphide bond formation on the electrophoretic mobility of truncated AT-III polypeptides after time-dependent interaction with α -thrombin

AT-III-(219–432)- (a) and AT-III-(251–432)- (b) polypeptides were prepared as described in the legend to Fig. 2 and incubated with α -thrombin. At 1 min or 20 min (lanes 2 and 3) portions of the reaction mixture had D-Phe-L-Pro-L-Arg-CH₂Cl added and were subsequently analysed by SDS/PAGE on a 10–20% (w/v) polyacrylamide gel under non-reducing conditions, followed by fluorography. m, molecular-mass markers (kDa).

(219–432)-polypeptide was cleaved into two products of relative molecular mass 20 and 4 kDa (Figs. 5a and 5b) and AT-III-(251–432)-polypeptide was cleaved into two products of relative molecular mass 17 and 4 kDa (Fig. 5c), when analysed by SDS/PAGE under reducing conditions. Thus the two truncated forms of AT-III had cleavage products of molecular mass that corresponded to products derived from cleavage at the reactive centre Arg-393–Ser-394 of AT-III. Autoproteolysis of these truncated AT-III polypeptides was ruled out in control experiments in which incubation without α -thrombin did not result in any degradation, even after 80 min. The addition of heparin at 0.2 unit/ml had no effect on the rate of cleavage of these truncated AT-III polypeptides. Correctly disulphide-linked (Fig. 5a) or reduced and S-carboxyamidomethylated (Fig. 5b) AT-III-(219–432)-polypeptides had identical cleavage patterns in reactions with α -thrombin, indicating that disulphide bond formation is not essential for cleavage by α -thrombin. The rate of cleavage of AT-III-(251–432)-polypeptide by α -thrombin (Fig. 5c) was approx. 5-fold lower than that observed with AT-III-(219–432)-polypeptide. The Cys-247–Cys-430 bond was shown to be intact within AT-III-(219–432)-polypeptide after reaction with α -thrombin when the samples were analysed by SDS/PAGE under non-reducing conditions (Fig. 6a). In addition, AT-III-(251–432)-polypeptide was shown not to be disulphide-linked, since it was cleaved into two distinct products by α -thrombin when analysed by SDS/PAGE under non-reducing conditions (Fig. 6b).

Characterization of the HBR-1 deletion mutant

Cell-free expression of the HBR-1 deletion mutant of AT-III resulted in the synthesis of an unglycosylated mature form of AT-III in which amino acid residues 41–49 were missing. Cell-free-derived HBR-1-deleted AT-III showed decreased binding to heparin–Sepharose when compared with normal cell-free-derived AT-III-(1–432)-polypeptide (Fig. 3d). Densitometric scanning revealed that approx. 50% of cell-free-derived HBR-1-deleted AT-III bound to heparin–Sepharose and was eluted as a major peak between 0.1 M- and 0.5 M-NaCl (Fig. 3d). When incubated

with up to 100-fold molar excess of human α -thrombin cell-free-derived HBR-1-deleted AT-III was not able to form covalent complexes of higher molecular mass (Fig. 4b). However, like that seen with normal cell-free-derived AT-III-(1–432)-polypeptide, an α -thrombin-dependent cleaved form of this mutant was generated that increased in amount over time (Fig. 4b; high-lighted by dot). The addition of plasma-derived AT-III at a 2:1 molar ratio to α -thrombin did not affect the results except that the cleavage of AT-III was not observed after 2 min (results not shown).

Characterization of Cys-430 mutant AT-III polypeptides

With the use of site-directed mutagenesis, the Cys-430 residue of AT-III was mutated to alanine, glycine or serine. Fig. 7 shows the electrophoretic mobility of the cell-free-derived normal and Cys-430 mutants resolved by SDS/PAGE under both reducing and non-reducing conditions. Under reducing conditions there was no difference in electrophoretic mobility between the cell-free-derived normal AT-III-(1–432)-polypeptide and the Cys-430 mutants (Fig. 7a). However, under non-reducing conditions, only cell-free-derived normal AT-III-(1–432)-polypeptide migrated faster with an apparently lower molecular mass (Fig. 7b, arrowhead). Densitometric scanning of the autoradiogram revealed that approx. 50% of the cell-free-derived normal AT-III-(1–432)-polypeptide migrated faster. Each of the

Fig. 7. SDS/PAGE analysis of cell-free-derived AT-III-(1–432)-polypeptides mutated at Cys-430

Total translation mixtures containing cell-free transcribed AT-III mRNA were labelled with [³⁵S]methionine, dialysed overnight in 0.15 M-NaCl/0.02 M-Tris/HCl buffer, pH 7.5, and analysed by SDS/PAGE on a 12% (w/v) polyacrylamide gel under reducing (a) and non-reducing (b) conditions, followed by fluorography. Lane 1, products of translation in the presence of AT-III-(1–432) mRNA. Lanes 2–4, products of translation in the presence of AT-III-(1–432) mRNA with Cys-430 mutated to alanine, glycine and serine respectively. The arrowhead represents the migration position of AT-III-(1–432)-polypeptides under non-reducing conditions.

Table 1. Properties of AT-III-(1–432)-polypeptide Cys-430 variants

Cell-free-derived AT-III Cys-430 variants were incubated with a 100:1 molar excess of human α -thrombin at 37 °C. Portions of the reaction mixture were terminated with 5 μ M-D-Phe-L-Pro-L-Arg-CH₂Cl and the radiolabelled protein bands analysed by SDS/PAGE under reducing conditions followed by fluorography. Thrombin–AT-III (TAT) formation at 2 min incubation was quantified by densitometric scanning of the autoradiogram and normalized to wild-type (wt) cell-free-derived thrombin–AT-III complexes also determined at 2 min. Hydrophobicity is based on the data from Rose *et al.* (1985): –, hydrophobic; 0, moderately polar.

Amino acid at residue 430	Side-group structure	Hydrophobicity ^a	Heparin binding (NaCl, M)	Relative TAT formation
Cys (wt)	–CH ₂ –SH	–	0.2–0.7	1
Ala	–CH ₃	–	Same as wt	0.51
Gly	–H	0	Same as wt	0.23
Ser	–CH ₂ –OH	0	Same as wt	0.43

cell-free-derived AT-III mutants of Cys-430 was able to bind to heparin-Sepharose and had elution patterns identical with cell-free-derived normal AT-III-(1-432)-polypeptide (Fig. 3a). The relative α -thrombin-AT-III formation after 2 min for the Cys-430 mutants, compared with normal cell-free-derived AT-III, is shown in Table 1. The results indicate that normal cell-free-derived AT-III-(1-432)-polypeptide was the most efficient at forming α -thrombin-AT-III complexes (after 2 min) than the three Cys-430 mutants studied. Cys430Ala had 0.51 times the amount of normal whereas Cys430Gly and Cys430Ser had 0.23 and 0.43 times the amount of normal after 2 min respectively.

DISCUSSION

The functional domains of AT-III have been mapped by studying kindreds with natural point mutations (Manson *et al.*, 1989), by site-directed mutagenesis of the AT-III cDNA (Stephens *et al.*, 1988; Austin *et al.*, 1990, 1991a,b), by chemical modification of the natural protein (Blackburn *et al.*, 1984; Liu & Chang, 1987; Chang, 1989; Sun & Chang, 1990) and by comparing the three-dimensional structures of other native and post-complex serpins (Loeberman *et al.*, 1984; Stein *et al.*, 1990; Wright *et al.*, 1990). The reactive centre Arg-393-Ser-394 of AT-III has been localized to the C-terminal domain and two heparin-binding regions, consisting of two clusters of basic amino acids, have been localized to the N-terminal domain. Naturally occurring mutations (Manson *et al.*, 1989) and chemical modification of the tryptophan residue at position 49 (Blackburn *et al.*, 1984) imply that the region within AT-III from amino acid residues 41 to 49 is involved in heparin binding (HBR-1). In addition, amino acid residues 114-156 appear to constitute a second heparin-binding region (HBR-2) (Smith & Knauer, 1987; Smith *et al.*, 1990). Three-dimensional models of AT-III, based on the crystal structure of cleaved α_1 -antitrypsin, suggest that regions 41-49 and 114-156 in AT-III comprise the putative heparin-binding domain (Carrell *et al.*, 1987, 1989; Huber & Carrell, 1989). Villanueva (1984), using Chou-Fassman models of denatured AT-III, proposed that the C-terminal region from residues 282 to 289 also contained a heparin-binding region. In a recent paper we described the expression of biologically active AT-III in a cell-free system (Austin *et al.*, 1990). We have now used this cell-free expression system, together with site-directed mutagenesis, to determine the role of the N-terminal domain of AT-III in both heparin binding and complex-formation with α -thrombin.

Approx. 70% of the cell-free-derived AT-III-(1-432)-polypeptides bound heparin and were eluted as a peak between 0.2 M- and 0.7 M-NaCl. However, more than 90% of plasma-derived AT-III bound heparin and was eluted as a peak between 0.6 M- and 1.0 M-NaCl. This is comparable with previous data (Austin *et al.*, 1990) suggesting that the decrease in heparin affinity observed for cell-free-derived AT-III is due to either the presence of endogenous factors in RRL that compete for heparin (and is consistent with a decrease in heparin binding observed for plasma-derived AT-III in the presence of RRL; results not shown) or the formation of a subpopulation of aberrantly folded AT-III polypeptides having no affinity for heparin. Two cell-free-derived truncated forms of AT-III, one consisting of amino acid residues 219-432 and the other of residues 251-432, were also synthesized and exposed to heparin-Sepharose. In contrast with either cell-free-derived or plasma-derived AT-III, both truncated variants had no affinity towards heparin, providing evidence that the location of the heparin-binding domain in AT-III does not extend beyond amino acid residue 219.

The cell-free-derived normal AT-III-(1-432)-polypeptides were

capable of forming approx. 15% covalent complexes with α -thrombin after 2 min, which is consistent with previous observations (Austin *et al.*, 1990). The inability of a significant portion of these cell-free-derived AT-III-(1-432)-polypeptides to form stable complexes with α -thrombin may have been due to one or a combination of the following: (1) the formation of an aberrantly folded inactive subpopulation of AT-III molecules; (2) the presence of endogenous factors in RRL that inhibit the formation of complexes; (3) the dissociation of α -thrombin-AT-III complexes during SDS/PAGE. These data are also consistent with the recent observation that completely reduced native AT-III only recovered about 30% of its α -thrombin-binding activity after air oxidation (Sun & Chang, 1990). In contrast with that observed with cell-free-derived AT-III-(1-432)-polypeptide, cell-free-derived AT-III-(219-432)-polypeptide, when incubated with α -thrombin, with or without heparin, did not form a complex, but was cleaved into two distinct products of molecular mass 20 and 4 kDa. The molecular masses of these products provided strong evidence that the site of cleavage was at the reactive centre Arg-393-Ser-394 of AT-III. The rate of cleavage by α -thrombin was essentially identical between the Cys-247-Cys-430 linked and unlinked forms of the AT-III-(219-432)-polypeptide. This suggests that the presence of an intact disulphide bond between Cys-247 and Cys-430 has no effect on cleavage by α -thrombin. This also indicates that the presence of this disulphide bond is not sufficient to allow for complex-formation to occur between this truncated form of AT-III and α -thrombin. When incubated with α -thrombin, in the presence or in the absence of heparin, cell-free-derived AT-III-(251-432)-polypeptide also did not form a complex with α -thrombin but was cleaved, presumably at the reactive centre, to form products of molecular mass 17 and 4 kDa. Results of the interaction of these truncated AT-III polypeptides with α -thrombin indicate that additional amino acid residues between positions 1 and 218 may be required for complex-formation to occur between AT-III and α -thrombin.

Horton *et al.* (1989) identified an N-terminal polyanionic segment of heparin cofactor II consisting of amino acid residues 54-75 that inhibits the ability of thrombin to cleave fibrinogen by interacting with a non-catalytic site in the enzyme. This segment has also been shown to be analogous to the highly polyanionic C-terminal domain of hirudin (Chang *et al.*, 1990). In addition, interaction of another serpin, α -antiplasmin, with its target proteinase, plasmin, required the binding of a segment of the inhibitor to non-catalytic lysine-rich binding sites in plasmin (Sugiyama *et al.*, 1988). Most of the proteinase-specificity of serpins has been localized to amino acid residues at or near the reactive centre (Carrell *et al.*, 1987). However, the results obtained with these truncated variants of AT-III, together with the examples of heparin cofactor II and α_2 -antiplasmin, provide evidence in which primary amino acid segments distant from the reactive centre appear to have critical roles in inhibitor function.

Recent evidence has suggested that interactions between the side chains of residues P₁₄, P₁₂ and P₁₀ are essential in the movement of reactive-centre loop sequences from the surface of the molecule into the five-stranded A sheet (Skriver *et al.*, 1991). With respect to AT-III, the alanine residue at position P₁₂, which is highly conserved among the inhibitory serpins, interacts with a hydrophobic pocket consisting of three phenylalanine residues (located at positions 77, 221 and 422) and an isoleucine residue located at position 412 (Austin *et al.*, 1991a). When the P₁₂ residue cannot be incorporated into this hydrophobic pocket, AT-III is converted into a substrate rather than an inhibitor of α -thrombin (Austin *et al.*, 1991a,b; Skriver *et al.*, 1991; Asakura *et al.*, 1989). Thus it is conceivable that the absence of stable inhibitory complexes between α -thrombin and these truncated variants of AT-III may in fact be due to the absence of a portion

of the A sheet domain, which would also include Phe-77 of the hydrophobic pocket.

AT-III has been shown to be folded in a stressed conformation so as to allow for complex-formation with α -thrombin to occur (Carrell & Owen, 1985; Carrell *et al.*, 1989; Huber & Carrell, 1989). This implies that the presence of the disulphide bond at Cys-247–Cys-430 may be important for maintaining this stressed conformation. However, this is not supported by data from the cell-free-derived AT-III-(1–432)-polypeptide variants at Cys-430. These data indicate that each of the three Cys-430 mutants (Cys430Ala, Cys430Gly and Cys430Ser) could bind heparin and form complexes with α -thrombin. Interestingly, α_1 -antitrypsin, which is a member of the serpin family and has 30% identity of sequence with AT-III, does not contain a C-terminal cysteine residue, analogous to Cys-430 of AT-III, but can still exist in a stressed conformation before cleavage by elastase (Carrell *et al.*, 1980; Carrell & Owen, 1985). This suggests that the C-terminus of α_1 -antitrypsin must interact with additional regions of the molecule in order to fold into its stressed conformation.

The cell-free-derived HBR-1 AT-III mutant, which has amino acid residues 41–49 deleted, showed decreased heparin binding when compared with normal cell-free-derived AT-III-(1–432)-polypeptide. However, the loss in heparin binding was not as dramatic as that for the truncated AT-III-(219–432)- and AT-III-(251–432)-polypeptides. This may have been due, in part, to the presence of the second heparin-binding region located between amino acid residues 114 and 156. When this HBR-1-deleted mutant was incubated with α -thrombin, complex-formation was not seen. However, the HBR-1-deleted mutant was cleaved to a product of lower molecular mass. This observation strongly suggests that the 41–49 region may be necessary for complex-formation between AT-III and α -thrombin to occur. It is not known at present how the deletion of this heparin-binding region might affect folding of the AT-III molecule such that stable complex-formation with α -thrombin might be impaired.

In conclusion, the results obtained in this study suggest that the N-terminal domain of AT-III from amino acid residues 1 to 218 is essential for both heparin binding and complex-formation with α -thrombin. However, the absence of these residues does not affect the ability of AT-III to be cleaved by α -thrombin. The present data also indicate that residues 41–49 may be crucial for thrombin–AT-III complex-formation. However, further studies are required to define precisely the minimal structure of AT-III required for complex-formation with α -thrombin.

We thank Dr. Fred A. Ofofu for his many perceptive comments and suggestions. We also thank Kevin R. Yarosh for his assistance with computer analysis and graphics. This study was supported by Grant HAM-04-89 from the Canadian Red Cross Society Research and Development Fund. R.C.A. is the recipient of a Studentship from the Heart and Stroke Foundation of Ontario and W.P.S. is the recipient of a Career Fellowship award from the Canadian Red Cross Society.

REFERENCES

- Asakura, S., Matsuda, M., Yoshida, N., Terukina, S. & Kihara, H. (1989) *J. Biol. Chem.* **264**, 13736–13739
- Austin, R. C., Rachubinski, R. A., Fernandez-Rachubinski, F. & Blajchman, M. A. (1990) *Blood* **76**, 1521–1529
- Austin, R. C., Rachubinski, R. A. & Blajchman, M. A. (1991a) *FEBS Lett.* **280**, 254–258
- Austin, R. C., Rachubinski, R. A., Ofofu, F. A. & Blajchman, M. A. (1991b) *Blood* **77**, 2185–2189
- Björk, I., Jackson, C. M., Jörnvall, H., Lavine, K. K., Nordling, K. & Salsgiver, W. J. (1982) *J. Biol. Chem.* **257**, 2406–2411
- Blackburn, M. N., Smith, R. L., Carson, J. & Sibley, C. C. (1984) *J. Biol. Chem.* **259**, 939–941
- Bulleid, N. J. & Freedman, R. B. (1988) *Biochem. J.* **254**, 805–810
- Carrell, R. W. & Owen, M. C. (1985) *Nature (London)* **317**, 730–732
- Carrell, R. W., Boswell, D. R., Brennan, S. O. & Owen, M. C. (1980) *Biochem. Biophys. Res. Commun.* **93**, 399–402
- Carrell, R. W., Christey, P. B. & Boswell, D. R. (1987) in *Thrombosis and Haemostasis* (Verstrate, M., Vermeylen, J., Lijnen, R. & Arnout, J., eds.), pp. 1–15, Leuven University Press, Leuven
- Carrell, R. W., Aulak, K. S. & Owen, M. C. (1989) *Mol. Biol. Med.* **6**, 35–42
- Chang, J.-Y. (1989) *J. Biol. Chem.* **264**, 3111–3115
- Chang, J.-Y., Ngai, P. K., Rink, H., Dennis, S. & Schlaeppi, J.-M. (1990) *FEBS Lett.* **261**, 289–290
- Horton, G. L., Tollefsen, D. M. & Benutto, B. M. (1989) *J. Biol. Chem.* **264**, 13979–13982
- Huber, R. & Carrell, R. W. (1989) *Biochemistry* **28**, 8951–8966
- Jordan, R. E., Oosta, G. M., Gardner, W. T. & Rosenberg, R. D. (1980) *J. Biol. Chem.* **255**, 10081–10090
- Kaderbhai, M. A. & Austen, B. M. (1985) *Eur. J. Biochem.* **153**, 167–178
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Liu, C.-S. & Chang, J.-Y. (1987) *Eur. J. Biochem.* **167**, 247–252
- Loeberman, H., Tokuoka, R., Deisenhofer, J. & Huber, R. (1984) *J. Mol. Biol.* **177**, 531–556
- Manson, H. E., Austin, R. C., Fernandez-Rachubinski, F., Rachubinski, R. A. & Blajchman, M. A. (1989) *Transfus. Med. Rev.* **4**, 264–281
- Miller-Andersson, M., Borg, H. & Andersson, L.-O. (1974) *Thromb. Res.* **5**, 439–452
- Petersen, T. E., Dudec-Wojciechowska, G., Sottrup-Jensen, L. & Magnusson, S. (1979) in *Physiological Inhibitors of Blood Coagulation and Fibrinolysis* (Collen, D., Wiman, B. & Verstraete, M., eds.), pp. 43–54, Elsevier/North-Holland Biomedical Press, Amsterdam
- Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H. & Zehfus, M. H. (1985) *Science* **229**, 834–838
- Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem.* **248**, 6498–6505
- Skriver, K., Wikoff, W. R., Patson, P. A., Tausk, F., Shapira, M., Kaplan, A. P. & Bock, S. C. (1991) *J. Biol. Chem.* **266**, 9216–9221
- Smith, J. W. & Knauer, D. J. (1987) *J. Biol. Chem.* **262**, 11964–11972
- Smith, J. W., Dey, N. & Knauer, D. J. (1990) *Biochemistry* **29**, 8950–8957
- Stein, P., Leslie, A., Finch, J., Turnell, W., McLaughlin, P. & Carrell, R. (1990) *Nature (London)* **347**, 99–102
- Stephens, A. W., Siddiqui, A. & Hirs, C. H. W. (1988) *J. Biol. Chem.* **263**, 15849–15852
- Sugiyama, N., Sasaki, T., Iwamoto, M. & Abiko, Y. (1988) *Biochim. Biophys. Acta* **952**, 1–7
- Sun, X.-J. & Chang, J.-Y. (1990) *Biochem. J.* **269**, 665–669
- Tabor, S. & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4767–4770
- Taylor, J. W., Ott, J. & Ekstein, F. (1985) *Nucleic Acids Res.* **13**, 8764–8785
- Villanueva, G. B. (1984) *J. Biol. Chem.* **259**, 2531–2536
- Wright, H. T., Qian, H. X. & Huber, R. (1990) *J. Mol. Biol.* **213**, 513–528

Received 4 February 1991/4 July 1991; accepted 16 July 1991