Arginine residues are critical for the heparin-cofactor activity of antithrombin III

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A dilution/quench technique was used to monitor the time course of chemical modification on the heparin-cofactor (*a*) and progressive thrombin-inhibitory (*b*) activities of human antithrombin III. Treatment of antithrombin III (AT III) with 2,4,6-trinitrobenzenesulphonate at pH 8.3 and 25 °C leads to the loss of (*a*) at 60-fold more rapid rate than the loss of (*b*). This is consistent with previous reports [Rosenberg & Damus (1973) *J. Biol. Chem.* **248**, 6490–6505; Pecon & Blackburn (1984) *J. Biol. Chem.* **259**, 935–938] that lysine residues are involved in the binding of heparin to AT III, but not in thrombin binding. Treatment of AT III with phenylglyoxal at pH 8.3 and 25 °C again leads to a more rapid loss of (*a*) than of (*b*), with the loss of the former proceeding at a 4-fold faster rate. The presence of heparin during modification with phenylglyoxal significantly decreases the rate of loss of (*a*). Full loss of (*a*) correlates with the modification of seven arginine residues per inhibitor molecule, whereas loss of (*b*) does not commence until approximately four arginine residues are modified and is complete upon the modification of approximately eleven arginine residues per inhibitor molecule. This suggests that (the) arginine residue(s) in AT III are involved in the binding of heparin in addition to the known role of Arg-393 at the thrombin-recognition site [Rosenberg & Damus (1973) *J. Biol. Chem.* **248**, 6490–6505; Jörnval, Fish & Björk (1979) *FEBS Lett.* **106**, 358–362].

INTRODUCTION

Antithrombin III (AT III) inhibits thrombin and the other serine proteinases of the intrinsic pathway of blood coagulation (for reviews, see Rosenberg, 1977; Björk & Lindahl, 1982). Inhibition involves the formation of a 1:1 proteinase-AT III complex (Rosenberg & Damus, 1973; Owen, 1975) through recognition involving at least the active-site serine residue of the proteinase and Arg-393 (Petersen *et al.*, 1979; Bock *et al.*, 1982; Chandra *et al.*, 1983) of the inhibitor (Jörnval *et al.*, 1979). Heparin greatly accelerates the rate of AT III inhibition of these proteinases, but the detailed mechanism of this rate enhancement is still unresolved (Björk & Lindahl, 1982). It is known, however, that heparin binds tightly to antithrombin III and causes a conformational change (Villanueva & Danishefsky, 1977; Einarsson & Andersson, 1977; Björk *et al.*, 1979; Olson *et al.*, 1981) that is thought to assemble the proteinase binding/inhibitory site into its optimal configuration.

A number of attempts to determine which amino acid residues of AT III are required for heparin binding and/or thrombin-inhibitory activities have used chemical modification with group-specific reagents. Rosenberg & Damus (1973) showed that extensive treatment with cyclohexane-1,2-dione resulted in a loss of both thrombin-inhibitory and heparin-cofactor activities, which they attributed to modification of a critical arginine residue at the thrombin-binding site. They also (Rosenberg & Damus, 1973) used guanidination with *O*-methylisourea

and suggested that lysine residues are likely essential for heparin binding, but are not necessary for progressive thrombin-inhibitory activity in the absence of heparin. Pecon & Blackburn (1984), who used modification with pyridoxal 5'-phosphate, showed that one or two lysine residues may be critical for heparin binding. Several groups (Björk & Nordling, 1979; Blackburn & Sibley, 1980; Villanueva *et al.*, 1980) have shown that heparin binding, and thus heparin-cofactor activity, is abolished by chemical modification of a single tryptophan residue, and Blackburn *et al.* (1984) have tentatively identified Trp-49 as the essential residue. Reduction of the disulphide linkage between Cys-430 and Cys-247 of AT III causes a loss of heparin-binding and heparin-cofactor activity, but has little effect on progressive thrombin-inhibitory activity in the absence of heparin (Longas *et al.*, 1980; Ferguson & Finlay, 1983).

None of the previous reports on the chemical modification of AT III has looked at the time-dependence of modification on the heparin cofactor and thrombin-inhibitory activities. Instead, a single extensively modified sample is always studied. This static technique does not allow one to observe differential rates of loss of heparin cofactor then thrombin-inhibitory activities, or vice versa. Since the work of Rosenberg & Damus (1973), it has been tacitly assumed that lysine residues of AT III are the positively charged recognition sites for the heparin polyanion and that arginine is involved only at the proteinase-recognition site. However, given the well-established involvement of arginine residues at anion

Abbreviations used. MB, modification buffer (50 mM-Bicine/100 mM-NaHCO₃, pH 8.3); IB, incubation buffer (50 mM-Tris/HCl/100 mM-NaCl, pH 7.4, adjusted with NaOH); PEG, poly(ethylene glycol); S-2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide; TNBS, 2,4,6-trinitrobenzenesulphonate; AT III, antithrombin III.

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recognition sites in proteins (Riordan *et al.*, 1977; Riordan, 1979) and the polyanionic nature of heparin (Björk & Lindahl, 1982), it seems highly probable that arginine residues might also be involved in heparin binding. The present paper reports the time-dependent effects of chemical modification on the activities of AT III. The results suggest that TNBS, a lysine-selective reagent, gives the expected rapid abolition of heparin-cofactor activity followed by a dramatically slower loss of progressive thrombin-inhibitory activity. Somewhat surprisingly, phenylglyoxal, an arginine-specific reagent (Takahashi, 1968; Riordan, 1979), also inactivates the former more rapidly than the latter. These data strongly suggest that arginine residues are also essential for the binding of heparin to AT III. A preliminary report of some of this work has already appeared (Jorgensen *et al.*, 1985).

EXPERIMENTAL

Materials

Human AT III was a gift from Dr. Milan Wickerhauser of the Plasma Derivatives Laboratory of the American Red Cross, Bethesda, MD, U.S.A. The protein was > 95% AT III by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Stock solutions of AT III were prepared for each experiment by dialysing a concentrated aqueous solution at 4 °C against 2 × 400 vol. of MB. Bovine thrombin was a commercial product, Thrombinair, from Armour Pharmaceuticals, Kankakee, IL, U.S.A. The substrate was S-2238, a product of Kabi Diagnostica, Stockholm, Sweden, and was purchased from Helena Laboratories, Beaumont, TX, U.S.A. Heparin (sodium salt, from porcine intestinal mucosa), PEG (approximate M_r 8000) and buffer salts were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Phenylglyoxal monohydrate was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and TNBS (sodium salt) was a product of Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. All other chemicals were reagent grade.

Assays

Antithrombin activity was determined as the ability to inhibit the thrombin-catalysed hydrolysis of S-2238 (Björk & Nordling, 1979). A typical incubation mixture (1.00 ml), incubated at 37 °C, contained 0.10 mM-S-2238, 45 mM-Tris, 90 mM-NaCl and PEG (6.5 mg/ml) at pH 8.2. For heparin-enhanced thrombin-inhibitory activity (Ødegård *et al.*, 1975), heparin (8 µg/ml), AT III (~ 10 µg/ml), and thrombin (4 µg/ml) were incubated at 37 °C for exactly 20 s before addition of a 40 µl portion to the incubation mixture. For heparin-independent, or progressive, thrombin-inhibitory activity (Blomback *et al.*, 1974), the heparin was omitted, and AT III and thrombin were preincubated for 18 min before addition of a 40 µl portion to the incubation mixture. The values for heparin-independent antithrombin activities of the native AT III control were typically $67 \pm 2\%$ of those of the heparin-enhanced activities.

Chemical modifications

Modifications were carried out at 25 °C in MB as further described in the Figure legends. All modifications were initiated by addition of a freshly prepared stock

solution of the modifying reagent in MB. At appropriate times, portions of the reaction medium were quenched by 17- to 26-fold dilution into IB at 0 °C. The combination of dilution and lowered pH and temperature was deemed sufficient to stop the modification. In addition, Tris, which is a primary amine and which was present in the IB, reacts with α -dicarbonyls (Riordan, 1973) and TNBS. Thus its presence minimized the concentration of these modifying reagents in the diluted sample. A dilution protocol was first established so that sufficient native AT III was used to inhibit 98–99% of the thrombin activity as assayed by the heparin-dependent antithrombin assay. Modified AT III was then diluted by the same factor so that each incubation and assay contained the same amount of AT III. Residual AT III activity was expressed on a percentage basis as the thrombin-inhibitory activity of the modified protein, AT, divided by that of the control (which had been subjected to the same conditions but in the absence of modifying reagent), AT_c.

Modification of specific amino acid residues by phenylglyoxal was determined by analysis on either an LKB 4150 Alpha or a Durrum D-500 amino acid analyser after work-up analogous to those published previously (Borders & Riordan, 1975; Borders *et al.*, 1982). Portions (125 µl) of the modified samples were separated from remaining reactants by desalting on Sephadex G-25 with, as elution buffer, 10 mM-sodium phosphate/25 mM NaCl, pH 7.0. The concentration of AT III in each sample after gel chromatography was determined by the method of Lowry *et al.* (1951), with native AT III as a standard. An unmodified control was subjected to the same protocol, and the two thrombin-inhibitory activities of all samples were determined under conditions where all incubations contained the same amount of AT III. Multiple portions of each sample were diluted into an equal volume of 12 M-HCl/2% thioglycolic acid, sealed under N₂, and hydrolysed at 113 °C for 22 h before amino acid analysis. Values for individual amino acid residues in native AT III hydrolysates were obtained by assuming that:

$$\text{Glx} + \text{Gly} + \text{Ala} + \text{Leu} + \text{Lys} = 174$$

[determined from the amino acid sequence reported by Petersen *et al.* (1979), Bock *et al.* (1982), Chandra *et al.* (1983) and Prochownik *et al.* (1983)]. In this manner, values for histidine, lysine and arginine in native AT III were found to be 4.5, 34.9 and 22.3 mol of amino acid/mol. of protein respectively, for an average of eleven analyses. These values compare favourably with the corresponding values of 5, 35 and 22 that were calculated from the amino acid sequence.

RESULTS

Modification of AT III by 2,4,6-trinitrobenzenesulphonate

An initial goal of this work was to develop a dynamic assay system with which to monitor the time-dependence of chemical modification on the various biological activities of AT III. Since it had been reported (Rosenberg & Damus, 1973; Pecon & Blackburn, 1984) that lysine residues are critical for heparin-binding and heparin-mediated activities of AT III, but are not involved at the thrombin-inhibitory site, we modified AT III with TNBS, a lysine-selective reagent (Okuyama & Satake,

Fig. 1. Modification of AT III (1.08 mg/ml) with 2.5 mM-TNBS in MB at 25 °C

Samples were quenched at the times indicated and subjected to heparin-dependent (●) and heparin-independent (□) thrombin-inhibitory assays. (a) Plot of residual antithrombin activities as a function of the time of modification. (b) Semilogarithmic plot of the data shown in (a).

1960; Goldfarb, 1966; George & Borders, 1979; Lundblad & Noyes, 1984). Fig. 1(a) shows that when AT III is treated with 2.5 mM-TNBS at pH 8.3 and 25 °C, the heparin-dependent AT III activity (●) was lost much more rapidly than the heparin-independent thrombin-inhibitory activity (□). Thus the heparin-binding site is functionally modified much more rapidly than is the thrombin recognition site.

Fig. 1(b) shows that the loss of heparin-dependent activity is a pseudo-first-order process, as is the loss of heparin-independent activity after an initial induction phase. The pseudo-first-order rate constants for the loss of activity are 0.83 min⁻¹ and 0.014 min⁻¹ respectively. Thus the functional inactivation of the heparin-binding site proceeds approx. 60-fold more rapidly than the loss of heparin-independent thrombin-inhibitory activity in the absence of heparin. These data are consistent with the earlier suggestion (Rosenberg & Damus, 1973) that lysine residues are critical for heparin-cofactor activity but not for progressive thrombin-inhibitory activity. They also demonstrate that the assay system devised for this work can indeed sort out the effects of chemical modification on the various activities of AT III.

Modification of AT III by phenylglyoxal

Treatment of AT III with 20 mM-phenylglyoxal in MB at 25 °C caused a rapid loss of heparin-cofactor activity (Fig. 2a, ●). This loss was more rapid than the loss of progressive thrombin-inhibitory activity. From the data of Fig. 2(a) the pseudo-first-order rate constants for the loss of heparin-cofactor activity and progressive thrombin-inhibitory activities were 0.33 min⁻¹ and 0.084 min⁻¹ respectively.

The modification of the arginine guanidino group by phenylglyoxal has been reported to be slowly reversible at neutral to mildly alkaline pH (Takahashi, 1968, 1977). To test if modification reversal might be affecting the results obtained by our experimental design, AT III was modified with phenylglyoxal and quenched in the usual way by dilution into IB at 0 °C. The diluted sample was placed immediately in a 37 °C bath and a portion was

Fig. 2. Modification of AT III (1.35 mg/ml in a, 0.94 mg/ml in b) with 20 mM-phenylglyoxal in MB at 25 °C, in the absence (a) or presence (b) of heparin (5.0 mg/ml)

Samples were quenched at the times indicated and subjected to heparin-dependent (●) and heparin-independent (□) thrombin-inhibitory assays. Note that in (b) the progress curve for inactivation in the absence of heparin is reproduced from panel (a) (----).

immediately diluted and assayed. It was found to have 46% thrombin-inhibitory activity as determined by the heparin-dependent assay. After incubation of the originally quenched sample at 37 °C for 30 and 60 min, portions were again assayed by the same assay procedure. The thrombin-inhibitory activities were 48 and 47% after the 30 and 60 min of incubation. This indicates that reversal of the modified arginine residues did not occur under the conditions of our experiments.

Since the loss of heparin-cofactor activity precedes the loss of progressive thrombin-inhibitory activity, modification by 20 mM-phenylglyoxal was repeated in the presence of excess heparin (~ 20 mol of unfractionated heparin/mol of AT III). If one makes the assumption that the average M_r of commercial unfractionated heparin is 15000 (Björk & Lindahl, 1982), and that only one-third of the heparin is of the 'high-affinity' type (Björk & Lindahl, 1982), 20 heparin molecules per AT III molecule correspond to six to seven high-affinity heparin molecules per inhibitor molecule. Fig. 2(b) shows that the presence of heparin during modification greatly decreases the rate of loss of heparin-cofactor activity. The heparin-independent assay procedure was not performed in this experiment because of significant amounts of heparin in the quenched samples.

When AT III was treated for 200 s with various concentrations of phenylglyoxal under conditions identical with those of the experiment shown in Fig. 2(a), the results illustrated in Fig. 3 were obtained. Thrombin-inhibitory activity decreased as a function of increased phenylglyoxal concentration. Again, heparin-cofactor activity is lost more rapidly than is heparin-independent activity.

Fig. 3. Effect of phenylglyoxal concentration on the inactivation of AT III

The protein (1.34 mg/ml) was modified for 200 s in MB at 25 °C, with the indicated concentration of phenylglyoxal. Each quenched sample was subjected to heparin-dependent (●) and heparin-independent (□) thrombin-inhibitory assays.

Fig. 4. Correlation of the inactivation of AT III with the modification of arginine by phenylglyoxal

The protein (2.91 mg/ml) was incubated with 5.0 mM-phenylglyoxal in MB at 25 °C. Portions were withdrawn at approx. 5, 10, 15, 24 and 40 min and subjected to gel filtration and subsequent analyses as described in the text. The antithrombin activity of each sample was measured by the heparin-dependent (●) and the heparin-independent (□) assay procedures. The AT_c values are averages for four assays, whereas each activity for a modified sample is the average for two assays. For amino acid analyses, three hydrolysates for each of two different control samples were run in duplicate, for a total of twelve analyses; for each modified sample, two analyses of three hydrolysates were run.

Chemical consequences of phenylglyoxal modification

Amino acid analysis of AT III was performed in order to identify the residues modified by treatment with phenylglyoxal. Significantly, the only amino acid residue found to decrease on modification of AT III was arginine; the quantity of histidine [the *N*-terminal residue (Petersen *et al.*, 1979)] remained constant. This strongly suggests that arginine residues are involved in the

heparin-cofactor activity, since α -amino groups are the most likely alternative candidates for reaction with phenylglyoxal (Takahashi, 1968, 1977). As shown in Fig. 4, the complete loss of heparin-cofactor activity is accompanied by the modification of approx. 7 of the 22 arginines per polypeptide. The loss of progressive thrombin-inhibitory activity, apparently requires the modification of an additional three to five arginine residues. It is important to note that three to four arginine residues are apparently modified by phenylglyoxal, with a concomitant 40% decrease in the heparin-dependent antithrombin activity before the loss of heparin-independent thrombin-inhibitory activity commences. This suggests that the modification of one or more arginine residues at the heparin-binding site precedes, and is possibly required for, the modification of an arginine residue or residues at the thrombin-recognition site.

DISCUSSION

Our examination of the time-dependence of inactivation by TNBS of both heparin-dependent and heparin-independent thrombin-inhibitory activities of AT III supports earlier evidence, obtained by static modification techniques (Rosenberg & Damus, 1973; Pecon & Blackburn, 1984), that lysine residues are critical for the binding of heparin to AT III. Somewhat unexpectedly, however, is our observation that modification of arginine guanidino groups of AT III by phenylglyoxal also produces a differential loss of heparin-cofactor and thrombin-inhibitory activities (Fig. 2). As in the case of lysine modification, the loss of heparin-cofactor activity precedes the loss of thrombin-inhibitory activity. In addition, heparin exerts a protective effect on the rate of loss of heparin-cofactor activity by arginine modification (Fig. 2*b*). The loss of heparin-cofactor activity as a function of the number of arginine residues modified also follows a pattern different from that shown by the loss of progressive thrombin-inhibitory activity (Fig. 4). Thus, we conclude that one or more arginine residues play(s) an essential role in the binding of heparin to AT III, in addition to the known involvement of arginine at the proteinase-recognition site (Rosenberg & Damus, 1973; Jörnvald *et al.*, 1979).

The role of arginine in heparin binding is most assuredly to serve as a positively charged site or set of sites to interact with the anionic sulphate or carboxylate moieties of the functional oligosaccharide unit(s) which give heparin its high affinity for AT III. The recent report (Koide *et al.*, 1984) of a hereditary abnormal AT III in which Arg-47 is replaced by a cysteine residue with a consequent total loss of heparin-binding ability would seem to define Arg-47 as at least one essential site. Although Arg-47 may be essential for binding, it is highly unlikely that it alone is sufficient for binding. Accumulated evidence (Rosenberg & Lam, 1979; Lindahl *et al.*, 1979; Björk & Lindahl, 1982; Choay *et al.*, 1983) suggests that a pentasaccharide (Björk & Lindahl, 1982; Choay *et al.*, 1983) unit of heparin provides the high affinity for AT III and, allowing for minor structural variation, this means eight to nine negative charges would have to be accommodated by the heparin-binding site of AT III (Björk & Lindahl, 1982). A likely mode of accommodation of this large number of concentrated negative charges would be through positive-charge

clusters at the heparin-binding site of the inhibitor. Indeed, an examination of the primary sequence (Peterson *et al.*, 1979; Bock *et al.*, 1982) reveals this. In addition to an Arg-46/Arg-47 pair, there are at least eight other clusters of two or more arginine and/or lysine residues either adjacent or one residue removed.

As has been pointed out elsewhere (Bjerrum *et al.*, 1983), the concept of a residue essential for binding often appears to be misconstrued to mean that this residue alone is involved in binding. It is the structural integrity of the site that is essential, not of a specific residue that is a part of the site, and all side chains must act in concert for the accurate expression of the biological activity. Assuming that Arg-47 (Koide *et al.*, 1984) is one residue essential for binding, the observation that modification of Trp-49 causes a loss of heparin-binding and heparin-cofactor activity (Blackburn *et al.*, 1984) could either be due to the fact that this residue is directly involved in heparin binding, or that the general integrity of the binding site is altered to such an extent that other residues which are directly involved in binding can no longer function properly. We have no direct way of knowing how many of the seven arginines modified on complete loss of heparin-cofactor activity (Fig. 4) may be involved in heparin binding, but it is probably several. On the other hand, it is also likely that different molecules of phenylglyoxal-inactivated AT III have different 'essential' arginine residues modified and that an undefined number of the total of seven arginine residues play no role in heparin binding.

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