THROMBOSIS RESEARCH
Printed in the United States

Vol. 8, pp. 99-109, 1976
Pergamon Press, Inc.

THE ANTITHROMBIN ACTIVITY OF α-1-PROTEASE INHIBITOR: THE ANTITRYPSIN ACTIVITY OF ANTITHROMBIN III

L. A. Learned, J. W. Bloom, and M. J. Hunter
Institute of Science and Technology, Biophysics Research Division,
The University of Michigan and, Department of Biological Chemistry,
The University of Michigan Medical School, Ann Arbor, Michigan 48109

(Received 1.10.1975; in revised form 31.10.1975.
Accepted by Editor R.F. Doolittle.
Received by Executive Editorial Office 9.12.1975)

ABSTRACT

Antithrombin III and α-1-protease inhibitor (α-1-antitrypsin) were purified from human plasma and the cross-reactivities of the two inhibitors were examined. The α-1-protease inhibitor was found to have no antithrombin III or antithrombin II (heparin cofactor) activity nor did it modify in any way the antithrombin activity of antithrombin III. Antithrombin III was found to possess some antitrypsin activity but was a much less efficient inhibitor than was α-1-protease inhibitor.

INTRODUCTION

Three α-globulins have been implicated in the progressive antithrombin activity of human plasma: antithrombin III (AT-III), α-2-macroglobulin and α-1-protease inhibitor (α-1-PI), formerly called α-1-antitrypsin. While there is general agreement that AT-III provides the major antithrombin activity of the plasma (1,2,3) and that α-2-macroglobulin also contributes significantly to this inhibition of thrombin (1,2,4), there are conflicting reports as to the ability of α-1-PI to function in this capacity (2,5,6,7,8,9).

Although Heimburger et al. (8,9) have reported that purified α-1-PI does not inhibit thrombin, several other investigators have suggested that α-1-PI functions as an antithrombin in plasma. In investigations on normal and α-1-PI deficient plasmas Gans and Tan (6) as well as Borsodi et al. (7) have observed lower progressive antithrombin activity in patients with a genetic α-1-PI deficiency and have attributed this deficiency to the lower α-1-PI level in the plasma. Rimon et al. (5) have purified an α-1-proteolytic inhibitor from human plasma which comprised about 2% of the plasma proteins and which inhibited plasmin, trypsin, chymotrypsin and thrombin. This inhibitor appears to be identical with α-1-PI

In view of these conflicting reports it was decided to reinvestigate the

antithrombin activity of human α-1-PI. Some preliminary investigations on the antitrypsin activity of AT-III have also been undertaken.

MATERIALS AND METHODS

Antithrombin assay

The two-stage AT-III and AT-II (heparin cofactor) assays which were developed for this investigation were modifications of a plasma AT-III assay which had been developed by Dr. J. A. Penner, Department of Internal Medicine, The University of Michigan Medical Center.

Dried human fibrinogen, Lot 223, Michigan Department of Health, Lansing, Michigan, was used as the source of fibrinogen in these assays. The thrombin used in the assay was bovine thrombin (topical), 5000 NIH units, obtained from Parke Davis Co., Detroit, Michigan. A stock solution was prepared by adding 10 ml of 50% glycerol to the powdered thrombin. The resultant solution was mixed well and divided into one hundred 0.1 ml samples in plastic tubes and frozen to prevent inactivation. Samples were melted and used as required. The thrombin assay solution was prepared by adding 1.5 ml each of a 0.2 M imidazole buffer solution, pH 7.4, and a 0.1 M calcium chloride solution to a 0.1 ml sample of the stock thrombin solution. The thrombin assay solution served as a source of thrombin in the antithrombin assays and was stored in ice prior to use. A stock heparin solution, "Panheparin", 1000 units/ml, was obtained from Abbott Laboratories, North Chicago, Illinois. The heparin assay solution was prepared by diluting 0.1 ml of the stock solution with 6 ml of 0.15 M NaCl.

In the standard assay procedure human serum was used as a source of antithrombin. Samples (0.2 ml) of a 1% solution of fibrinogen in 0.15 M NaCl, pH 6.8, were prewarmed to 37° in glass test tubes. A 0.2 ml sample of the thrombin assay solution was pipetted into a plastic tube and warmed for 3 minutes at 37°. Serum, 0.2 ml, was added to the warmed thrombin solution, the solution mixed well and incubated at 37° for exactly 5 minutes. At the end of the incubation period, 0.1 ml of the serum-thrombin solution was added to the prewarmed 0.2 ml sample of the fibrinogen solution and the clotting time of the mixture was determined.

Control clotting times were obtained by substituting 0.2 ml of the imidazole buffer for the 0.2 ml of serum. To ensure that the logarithm of the clotting time bore a linear relationship to the antithrombin concentration, 0.1 ml of serum and 0.1 ml of imidazole buffer were added to the thrombin solution and a third clotting time was determined.

The assay procedure for AT-II (heparin cofactor) activity was identical with that described for AT-III except that 2.0 µl of the heparin assay solution was added to 1.0 ml of the thrombin assay solution just prior to the addition of serum. The concentration of heparin in the final clotting mixture was 5.5×10^{-3} units/ml. At these heparin concentrations the clotting time of the 0.2 ml serum sample was approximately 1.2 times that of the heparin-free serum sample.

Antitrypsin assay

The antitrypsin activity of human serum and serum fractions was determined by inhibition of the hydrolysis of α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) by trypsin as described by Dietz *et al.* (10). Trypsin,

TRTPCK, was obtained from Worthington Biochemical Corporation and the BAPNA was a product of Sigma Chemical Co.

Immunodiffusion analyses on serum and serum fractions were performed on microscope slides in 0.8% agarose gels using antisera to whole plasma and to various specific plasma proteins. The antisera were obtained from Behring Diagnostics. Disc gel electrophoresis was performed as described by Davies (11) and protein concentrations were determined by the microbiuret method (12). All procedures involved in the purification of the protease inhibitors were conducted at +2°.

Purification of α-1-PI

The α-1-PI was purified from human serum by a) adsorption on $Al(OH)_3$ and ammonium sulfate precipitation as described in the heparin cofactor preparation of Wickerhauser and Sgouris (13) followed by b) blue dextran-Sepharose 4B affinity chromatography by the method of Travis and Pannell (14), and then c) chromatography on DE-52 cellulose columns at pH 7.2 or 8.8 and at pH 6.5 by methods similar to those described by Pannell, Johnson and Travis (15). Details of the purification procedure will be reported elsewhere (16).

Purification of AT-III

AT-III was purified by heparin-Sepharose 4B affinity chromatography by the method described by Miller-Andersson et al. for the purification of AT-III from human plasma (17). One of the major contaminants which these investigators observed in their AT-III preparation after chromatography was a plasma lipoprotein. Since lipoprotein-free serum, prepared by density gradient centrifugation of human serum in NaBr solution (18), was readily available as a result of other investigations in our laboratory on serum lipoproteins (18), this lipoprotein-free serum was used as the starting material in the AT-III purification in the hope of improving the purity of the AT-III preparation. The high salt centrifugation did not prove deleterious to the AT-III: the antithrombin activity of the lipoprotein-free serum was as great as that of the untreated serum. All lipoprotein-free sera were dialyzed extensively against 0.15 M NaCl and their pH adjusted to 7.4 prior to fractionation of the sera.

RESULTS

Homogeneity of α-1-PI preparation

The α-1-PI preparation obtained by the methods outlined earlier in this paper was almost homogeneous as determined by electrophoresis (Figure 1) and by immunodiffusion against antisera to whole plasma and to AT-III, antichymotrypsin, inter-α-trypsin inhibitor, acid glycoprotein, prealbumin, α-2-macroglobulin, α-1-T-glycoprotein and albumin. Two trace contaminants could be detected: albumin and α-1-T-glycoprotein. The specific activity of the preparation was 45-fold that of the original serum and the yield about 30%.

Heparin-Sepharose 4B chromatography of lipoprotein-free serum

The serum fraction which did not bind to the heparin-Sepharose affinity column still exhibited a reduced but substantial antithrombin activity. Unlike the original serum however, the clotting times observed on assay of this fraction were the same in the presence and absence of heparin. Immunodiffusion of the fraction against antisera specific to AT-III and α-2-macroglobulin

showed both proteins to be present in the fraction, the AT-III being present at a much lower concentration than in the original serum. If the presence of AT-III in the fraction was due to the leaching of heparin-AT-III complex from the affinity column, the antithrombin activity of the AT-III, like that of the α-2-macroglobulin, should be independent of the presence of added heparin in the assay solution.

The proteins which had been bound by the affinity column were eluted by raising the ionic strength of the eluting buffer (17). The resulting protein fractions were dialyzed against 0.15 M NaCl. Only low levels of antithrombin activity could be detected in the AT-III eluate fraction. The protein concentration of this fraction was very low and it was suspected that the much reduced level of antithrombin activity might be due to denaturation of the AT-III during the incubation period. Accordingly, samples of the AT-III fraction and the serum were diluted with equal volumes of 1% bovine serum albumin in 0.15 M NaCl prior to assay. The presence of the albumin greatly increased the antithrombin activity of the AT-III fraction. No further increase in activity was observed at higher albumin concentrations. The clotting time of the serum which had been diluted with an equal volume of the albumin solution was the same as that obtained when the serum had been diluted with an equal volume of imidazole buffer. Representative data are given in Tables 1 and 2.

The logarithm of the clotting time was plotted against the volume of serum or AT-III fraction (0.0, 0.1 and 0.2 ml). By inspection of the resultant two straight lines, the volume of the AT-III fraction which would give the same clotting time as the 0.2 ml serum sample was found to be 0.13 ml. By combining these data with the concentrations of protein in the serum and the AT-III fraction (6.29 and 0.0134 g/100 ml respectively), the specific activity of the AT-III fraction was found to be 720 times that of the serum. Since an almost identical increase in specific activity was obtained in the presence of added heparin (AT-II assay), no significant contamination of the AT-III fraction by heparin which had leached from the affinity column can have occurred and the observed high relative specific activity of the fraction represents a real purification of the AT-III.

The volume of serum applied to the affinity column was 65 ml and the volume of the AT-III fraction was 23 ml. The concentration of AT-III in the AT-III fraction was 0.2/0.13 times that in the serum. The yield of AT-III in the AT-III fraction was therefore about 55%.

No α-1-PI, α-2-macroglobulin, α-1-T-glycoprotein, α-1-antichymotrypsin or inter-α-trypsin inhibitor could be detected after immunodiffusion of a 0.2% solution of the AT-III fraction against antisera that were specific for these proteins. Disc gel electrophoresis of the AT-III fraction (Figure 1) showed one major band with only a few very minor contaminants.

The antithrombin activity of α-1-PI

AT-III and AT-II (heparin cofactor) assays were conducted on the purified α-1-PI preparation (0.353 g/100 ml), on the AT-III fraction (0.0134 g/100 ml) and on serum. All three solutions were diluted with an equal volume of 1% bovine serum albumin before assay. The resulting clotting data are given in Tables 1 and 2. The data clearly show that α-1-PI does not act as an antithrombin in the presence or absence of heparin.

TABLE 1

AT-III ACTIVITY OF SERUM AND OF AT-III AND α-1-PI SERUM FRACTIONS

Volume of serum or fraction (ml)	Clotting time (sec) serum	AT-III	α-1-PI
0.0	14.5	14.5	14.5
0.1	16.4	17.0	14.5
0.2	18.4	20.0	14.5

TABLE 2

AT-II (HEPARIN COFACTOR) ACTIVITY OF SERUM AND OF AT-III AND α-1-PI SERUM FRACTIONS

Volume of serum or fraction (ml)	Clotting time (sec) serum	AT-III	α-1-PI
0.0	14.5	14.5	14.5
0.1	17.8	22.0	14.5
0.2	22.0	33.9	14.5

The clotting time of a solution which contained equal volumes of the α-1-PI solution (0.353 g/100 ml) and the AT-III solution (0.0134 g/100 ml) was determined in the presence and absence of heparin. The data are given in Table 3 and show that α-1-PI does not appear to modify the action of AT-III on thrombin.

TABLE 3

ANTITHROMBIN ACTIVITY OF AT-III IN THE PRESENCE AND ABSENCE OF α-1-PI

Fraction	Clotting time (sec) without heparin	with heparin
0.1 ml AT-III + 0.1 ml buffer	17.0	22.0
0.1 ml AT-III + 0.1 ml α-1-PI	17.0	22.1

FIG. 1

Disc gel electrophoresis of AT-III and α-1-PI fractions and AT-III fraction + trypsin at pH 8.9.

A. AT-III fraction.

B. AT-III fraction + bovine trypsin; AT-III:active trypsin molar ratio of 6:1.

C. α-1-PI fraction.

The antitrypsin activity of AT-III

The rate of hydrolysis of BAPNA by trypsin was measured in the presence of α-1-PI and of AT-III and in the absence of both inhibitors. Various concentrations of both inhibitors were added to the trypsin solution prior to the addition of the BAPNA. Bovine serum albumin was also added to the trypsin solution to stabilize the inhibitors during the incubation step of the assay procedure. The bovine trypsin used in these investigations was determined to be 63% active by the active site titration method of Chase and Shaw (19).

The AT-III fraction was found to possess some antitrypsin activity but was a much less efficient inhibitor than was α-1-PI (Figure 2). When the trypsin activity was plotted against AT-III:trypsin molar ratio and the data points linearly extrapolated to zero trypsin activity, the intercept value of the AT-III:trypsin molar ratio was 8-9.

Evidence for the interaction of AT-III with trypsin was obtained by disc gel electrophoresis (Figure 1). At AT-III:active trypsin ratios of 6:1 essentially all the AT-III was converted to a slower migrating species.

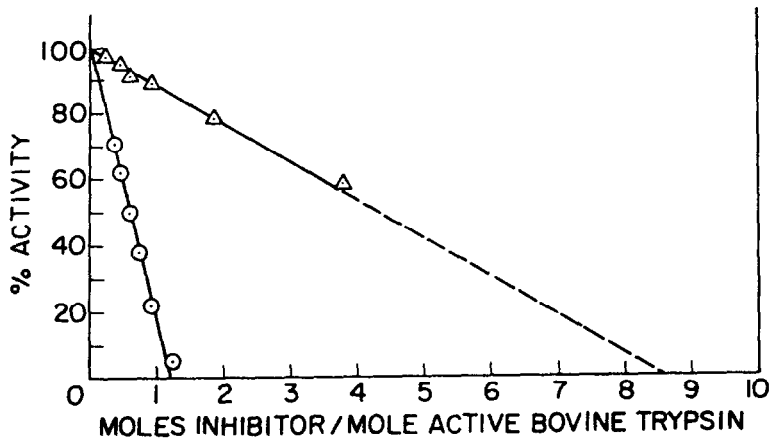

FIG. 2. The antitrypsin activity of α-1-PI and AT-III as a function of inhibitor concentrations. ⊙ trypsin + α-1-PI. active trypsin, 5.3×10^{-7}M; α-1-PI, $2.0 - 6.7 \times 10^{-7}$M. Literature values for the molecular weight of α-1-PI vary substantially and a mean value of 53,000 was used for the molecular weight of α-1-PI. △ trypsin + AT-III. active trypsin, 4.92×10^{-7}M; AT-III, $1.2 - 18.3 \times 10^{-7}$M. The molecular weight of AT-III was taken as 65,000 (3,17).

DISCUSSION

Purification of AT-III and α-1-PI

Miller-Andersson et al. (17) achieved a 500-fold purification of AT-III by fractionation of human plasma on a heparin-Sepharose affinity column. The 700-fold purification which was obtained in this study by a similar fractionation of lipoprotein-free serum is in good agreement with that obtained by Miller-Andersson et al. if allowance is made for the lipoprotein present in their AT-III fraction.

Other plasma fractions have been shown to bind to insolubilized heparin. Gentry and Alexander (20) have found that Factors IX and XI and thrombin were bound by heparin in which had been coupled to agarose beads and Machovich et al. (21) have observed that thrombin as well as AT-III was adsorbed by a heparin-Sephadex G-200 column. The serum used in the present investigations contained no measurable thrombin activity but the presence or absence of the other two factors in the coagulation cascade was not determined and small amounts of these minor plasma components may be present in the AT-III fraction. The absence of detectable amounts of protease inhibitors other than AT-III in the AT-III fraction made the removal of the small amounts of contaminating proteins unnecessary for the purposes of this investigation and no further purification of the AT-III fraction was undertaken.

The 45-fold purification of α-1-PI which was achieved is comparable to values reported by other workers (22,23) but considerably lower than that reported by Pannell et al. (15). The reason for this difference is difficult to assess since the two preparative procedures are very similar and both preparations appear to be essentially homogeneous when examined by electrophoresis or by immunodiffusion against antisera to whole plasma and to specific plasma proteins. Investigations as to the cause of this disagreement are in progress. The only two contaminants which could be detected in the purified α-1-PI preparation were albumin and α-1-T-glycoprotein, neither of which are protease inhibitors. Any inhibition of trypsin activity by the α-1-PI preparation could therefore by ascribed to α-1-PI.

The antithrombin activity of α-1-PI

Thrombin is remarkably specific for certain arginyl peptide bonds (24, 25). Walz et al. (25) have recently found that a distinguishing characteristic of the proteolytic function of thrombin is the cleavage of an arginyl and, to a much lesser extent, lysyl peptide bond when a glutamic or aspartic acid residue or one of their respective amides occurs 6, 7 or 8 positions from the carboxyl side of the cleavage bond. Furthermore, Rosenberg and Damus (3) have shown that at least one arginyl residue of AT-III is essential for effective thrombin-AT-III complex formation.

Like the serine proteases with which they combine, AT-III and α-1-PI have many similar physico-chemical and functional properties (7). It has been suggested that they share common structural features and that they may even be derived from a common ancestor (7). However, despite this similarity, α-1-PI may lack a critically oriented arginyl residue and thus be unable to complex effectively with thrombin. This lack of reactivity of α-1-PI will probably not be paralleled by a similar unreactivity to other less specific proteases of the coagulation cascade. Indeed, recent observations by Heck and Kaplan (26) suggest that α-1-PI is the major inhibitor of Factor XI_a in human plasma.

The observed inability of α-1-PI to inhibit the conversion of fibrinogen to fibrin by thrombin is in agreement with the findings of Heimburger et al. (8,9). The concentration of α-1-PI (0.353 g/100 ml) in the α-1-PI solution used in the clotting assay is quite high but within the normal range of plasma levels and 25 times greater than that of a AT-III solution which markedly inhibited the ability of thrombin to convert fibrinogen to fibrin. Therefore it would appear that α-1-PI does not measurably contribute to the progressive antithrombin activity of plasma under normal physiological conditions.

Heparin is bound by thrombin (20,21) as well as by AT-III and it was thought that the thrombin-heparin complex might be inhibited by α-1-PI. However the lack of thrombin inhibition by α-1-PI in the presence of heparin (AT-II or heparin cofactor assay) indicates that α-1-PI does not contribute to the immediate antithrombin activity of the plasma.

Finally, the inability of a 25-fold excess of α-1-PI to modify the inhibition of thrombin by AT-III provides conclusive evidence that α-1-PI does not contribute, directly or indirectly, to the antithrombin activity of the plasma.

It is difficult to reconcile these data with the reports of the antithrombin activity of α-1-PI in plasma (6,7). If, as has been suggested, α-1-PI and AT-III are closely related genetically (7), the low antithrombin activities observed by Gans and Tan (6) and by Borsodi et al. (7) in the plasmas of individuals with a genetic α-1-PI deficiency may reflect a parallel lower level of AT-III in the plasma and be unrelated to the lower α-1-PI level. No plasma AT-III levels were reported by either group of investigators. It is impossible at present to interpret the data on the α-1-PI deficient plasmas. The situation is further complicated by a report by Abildgaard (27) of individuals with genetic α-1-PI deficiency but normal antithrombin activity.

Abildgaard (2) has also observed that patients whose conditions were associated with low progressive antithrombin activity (cirrhosis of the liver, thrombotic states, hereditary antithrombin deficiency) showed a complete lack of correlation between antithrombin activity and α-1-PI concentration. This observation however does not exclude the participation of α-1-PI in contributing in a minor role to the total progressive antithrombin activity of human plasma.

The antitrypsin activity of AT-III

A characteristic feature of the reaction of serine proteases with their macromolecular inhibitors is the high affinity of the inhibitor for the protease and the stability of the protease-inhibitor complex even after the initial covalent linkage between the inhibitor and the seryl residue of the protease has been broken. Such complexes as have been investigated are either equimolar in protease and inhibitor (3) or are composed of two moles of protease per mole of inhibitor (28).

The data reported in this paper would indicate that this simple stoichiometry does not apply in the case of the trypsin-AT-III complex. Linear extrapolation of the BAPNA assay data obtained at various ratios of AT-III to active trypsin would indicate that complete inhibition of the trypsin can only be achieved in the presence of an 8-fold excess of inhibitor. Disc gel electrophoresis of a solution containing a 5-fold excess of AT-III demonstrated that essentially all of the AT-III had been modified by the trypsin.

It is difficult to explain these observations. Any explanation of the trypsin inhibition data in terms of contamination by another protease would require that the protease comprise over 10% of the proteins in the fraction and contamination of this magnitude would have been detected by immunodiffusion or electrophoretic analyses. It might be argued that some minor denaturation of much of the AT-III had occurred during the purification procedure and that approximately 90% of the AT-III, while still identifiable as such electrophoretically and antigenically, was no longer capable of acting as a

protease inhibitor. This explanation however cannot be valid since the antithrombin activity of the AT-III fraction represented 55% of the activity of the serum: at least 55% of the AT-III in the fraction therefore must represent active inhibitor. Furthermore, the altered mobility of essentially all the AT-III in the presence of trypsin, as determined by disc gel electrophoresis, also argues against this explanation. Were approximately 90% of the AT-III molecules incapable of reacting with trypsin and acting as an antitrypsin, approximately 90% of the AT-III should have the same mobility in the presence and absence of trypsin.

While the nature of the AT-III-trypsin interaction seems atypical and cannot be satisfactorily explained at present, AT-III has been shown to be a valid, if relatively ineffective, inhibitor of trypsin. Further investigations on the mechanism of this inhibition are in progress.

ACKNOWLEDGMENTS

The authors would like to thank Dr. John Penner, Ms. Judy Rohwedder and Ms. Angelina di Francesco for their invaluable help in the development of the clotting assay which was used to determine the antithrombin activity of the inhibitors. This research was supported in part by grant HL-09739, and by Biophysics Training Grant, GM-1355, from the NIH.

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