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P1 variant antithrombins Glasgow (393 Arg to His) and Pescara (393 Arg to Pro) have increased heparin affinity and are resistant to catalytic cleavage by elastase

Implications for the heparin activation mechanism

Maurice C. Owen[1], Peter M. George[1], David A. Lane[2] and D. Ross Boswell[1]

[1]*Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand* and [2]*Department of Haematology, Charing Cross and Westminster Medical School, London*

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The heparin affinity of normal and two P1 variants of antithrombin-III (AT) was studied by gradient elution with NaCl in Tris buffer on heparin-Sepharose. At pH 7.4 normal AT eluted at [Na^+] 0.78 mol/l and the variants both showed increased affinity with AT Pescara eluting at [Na^+] 0.86 mol/l and AT Glasgow at [Na^+] 0.92 mol/l. We have earlier proposed a model for heparin activation in which the native state of AT maintains a salt bridge involving the P1 Arg-393 residue. Binding of heparin induces a higher heparin affinity conformation in which the salt bridge is disrupted to reveal the reactive centre for inhibition of thrombin. The Glasgow and Pescara variants, lacking a reactive centre P1 basic residue, would be unable to form this salt bridge, and we suggested that the high affinity conformation which they adopt as their native state would resemble the heparin induced conformation. To examine this model, we measured the heparin induced fluorescence of two P1 variants and tested the susceptibility of their reactive loops to catalytic cleavage. Both variants had fluorescence spectra indistinguishable from normal AT. In the absence of heparin, neither variant was more susceptible than normal to catalytic cleavage by human neutrophil elastase. These findings suggest that the conformation of these P1 variants is different to that of fully heparinized normal AT.

Antithrombin; Heparin binding; Reactive site mutant

1. INTRODUCTION

AT is the major physiological inhibitor of thrombin. The rate of inhibition is slow in the absence of heparin, but on binding to heparin AT undergoes a conformational change to give a configuration that rapidly reacts with target proteases and inactivates them.

Three main approaches have been taken to define the region of AT that is involved in heparin binding: (i) the characterisation of the mutations in AT variants that have reduced heparin binding affinity [2,3]; (ii) the identification of uniquely conserved basic residues in the homologous heparin binding serpins, heparin cofactor-II, AT, and protease nexin-1 [4,5]; (iii) the labelling of lysine residues in the presence and absence of heparin [6]. The cumulative results of these experiments show the primary heparin binding site to be a zone extending along the underside of the D-helix up towards the large β-sheet structure [3–5].

Somewhat less clear is the mechanism by which binding of heparin to antithrombin results in the activation of the inhibitor. Heparin is presumed to induce a conformational change in AT from an inactive to an active form. The evidence for this has come from studies such as heparin-induced changes in the fluorescence spectra [7], or circular dichroism [8], and the heparin affinity of P1 variant antithrombin Glasgow 393 Arg to His [1].

At pH 7.4, AT Glasgow has an increased affinity for heparin but at pH 6.0 with the protonation of the histidine this reverts to normal. On the basis of these studies, we have suggested that an ionic bond is formed with the positively charged Arg-393 and that this plays a role in maintaining AT in a constrained, non-activated configuration. When heparin binds at the binding site, the induced conformational change that is transmitted to the reactive centre, results in the disruption of this ionic bond. Thus, as a consequence, the reactive loop is freed to interact with target proteases in the heparin activated conformation. It was also suggested that the loss of this charged residue in P1 variants of AT results in a conformation more like the heparin activated form. This model predicts that all the P1 variants which are unable to form this ionic bond will have an increased heparin affinity. To date two other P1 variants have been identified, AT Northwick Park (393 Arg to Cys) [9] and AT Pescara (393 Arg to Pro) [10]. The first of these has been reported to have an increased affinity. Here we report the heparin affini-

Correspondence address: M.C. Owen, Department of Clinical Biochemistry, Christchurch Hospital, Christchurch, New Zealand

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ty, at pH 7.4, of AT Pescara relative to normal AT and AT Glasgow.

It is also known that heparin accelerates inactivation of AT by catalytic cleavage of its reactive loop. Thus in the presence of heparin normal AT is very susceptible to proteolytic cleavage with human neutrophil elastase which occurs either side of residue 391 (P4) [11]. If, the absence of heparin, the P1 variants of AT are already in the heparin activated conformation due to their inability to form the P1 ionic bond, they would be expected to be more susceptible than normal AT to catalytic cleavage of the reactive loop. This paper reports the results of experiments to test this prediction.

2. MATERIALS AND METHODS

2.1. *Heparin-Sepharose chromatography at pH 7.4*

To 10 ml of plasma was added 0.1 ml of 10% dextran sulphate (M_r 500 000) and 0.1 ml of 5 mol/l $CaCl_2$. The supernatant was loaded onto a heparin-Sepharose column (0.9 × 15 cm) equilibrated with 0.05 mol/l Tris, 0.01 mol/l citrate, 0.15 mol/l NaCl, pH 7.4. The ATs were eluted with a linear NaCl gradient to 1.65 mol/l, starting with 210 ml of each buffer [2].

2.2. *DEAE-Sephadex A-50, pH 8.6*

Further purification of AT and the removal of trace amounts of eluted heparin were accomplished by chromatography on DEAE-Sephadex A-50, pH 8.6, eluting with a linear gradient to 0.45 mol/l NaCl [2].

2.3. *Electroimmuno assay of column fractions*

A 1 mm thick plate was poured from 30 ml of 1% agarose in 75 mmol/l Tris/Glycine/Barbitone buffer pH 8.6, containing 150 μl of Dako antiserum to AT. A 3 μl volume from each fraction was loaded into the wells and electrophoresis carried out at 150 V for 6 h. Plates were pressed, dried and stained with Coomassie blue [12].

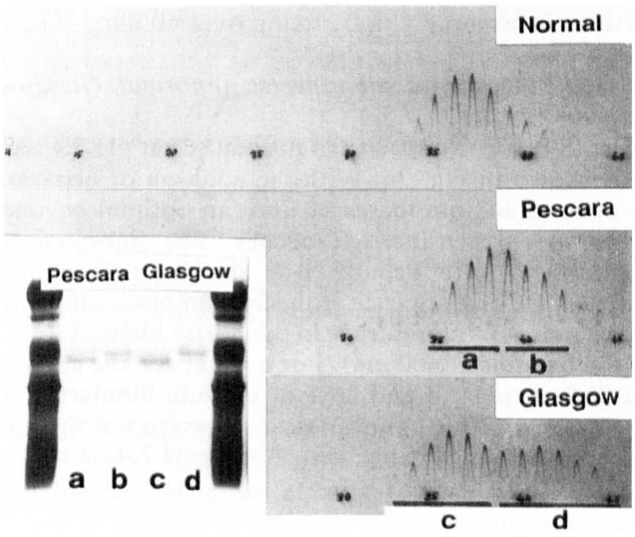

Fig. 1. Electroimmunoassay for antithrombin (AT) in fractions from chromatography of plasma from patients with normal AT, AT Pescara and AT Glasgow on heparin-Sepharose at pH 7.4. The insert shows electrophoresis in agarose gel at pH 8.6 of fractions a, b, c and d pooled from the heparin-Sepharose chromatography of AT Pescara and AT Glasgow patients' plasma. Normal human serum is run as a reference in the first and last lanes.

2.4. *Agarose gel electrophoresis at pH 8.6*

This was carried out as described previously [2].

2.5. *Sodium ion concentrations*

These were performed on pooled peaks from heparin-Sepharose chromatography on a Hitachi analyzer, model 717, using an ion specific electrode.

2.6. *SDS-Polyacrylamide gel electrophoresis*

This was carried out in 10% gels using the method of Laemmli [13].

2.7. *Catalytic cleavage of AT with human neutrophil elastase*

Purified AT (normal, Glasgow and Pescara variants) was incubated at 37°C with human neutrophil elastase (a gift from Dr M. Vissers) at a mass ratio of 1:250 (elastase/AT), with and without heparin, and samples were taken at timed intervals. The aliquots were immediately boiled with an equal volume of SDS sample buffer containing mercaptoethanol, and analysed by SDS-polyacrylamide gel electrophoresis.

In detail, the incubation mixture with heparin comprised 17 μg antithrombin, 0.068 μg human neutrophil elastase and 5 U of heparin all dissolved in 100 μl of 0.01 mol/l Tris, 0.03 mol/l NaCl, 1.5 mmol/l K_2EDTA, pH 8.4. 15 μl aliquots were taken at 0, 1, 2, 3, 5, 10 and 20 min. The mixture without heparin contained the same amounts of AT and elastase and buffer, but in place of heparin contained 5 μg of protamine sulphate. 15 μl aliquots were taken at 0, 1, 2, 5, 10, 20 and 60 min. The possible presence of contaminating proteinases in the elastase preparation was tested for by incubating the elastase firstly with a specific elastase inhibitor, Methoxy succinyl-Ala-Ala-Pro-Val-chloromethyl ketone at a molar ratio of 1:100. AT was then incubated with this preparation in the presence of heparin and aliquots taken at timed intervals as above.

2.8. *Fluorescence emission spectra of normal, Glasgow and Pescara AT with and without heparin at pH 7.4 and 6.0*

The method follows that of Olson [7]. Measurements were performed in an Aminco Bowman spectrofluorimeter with excitation at 280 nm and emission from 310 to 410 nm. Measurements at pH 7.4 were performed in phosphate buffered saline, and at pH 6.0 in 0.01 mol/l EDTA, 0.01 mol/l citrate buffer.

AT was added to 2 ml of buffer to give a final concentration of about 50 μg/ml. Spectra were performed with no added heparin, and with 1.5 U, 3 U, 10 U and 20 U. Spectra were also produced for buffer blanks.

3. RESULTS

3.1. *Heparin binding affinity*

The fractions from the heparin-Sepharose columns of normal, Pescara and Glasgow plasmas were analysed by electroimmuno assay. These results (Fig. 1) show that for the Glasgow sample AT elutes as two distinct peaks, whereas Pescara plasma has a broadened peak with a trailing edge, and normal plasma an almost symmetical peak. Agarose gel electrophoresis of the pooled first half of the Pescara peak (Fig. 1a), and the pooled second half (Fig. 1b) demonstrates that the variant protein is electrophoretically faster running, and elutes at a higher ionic strength than the normal component. The resolution for Glasgow is almost complete, with the early peak (Fig. 1c) identified by electrophoresis as the normal AT component, and the later peak (Fig. 1d) as the variant. The sodium ion concentration of the fraction

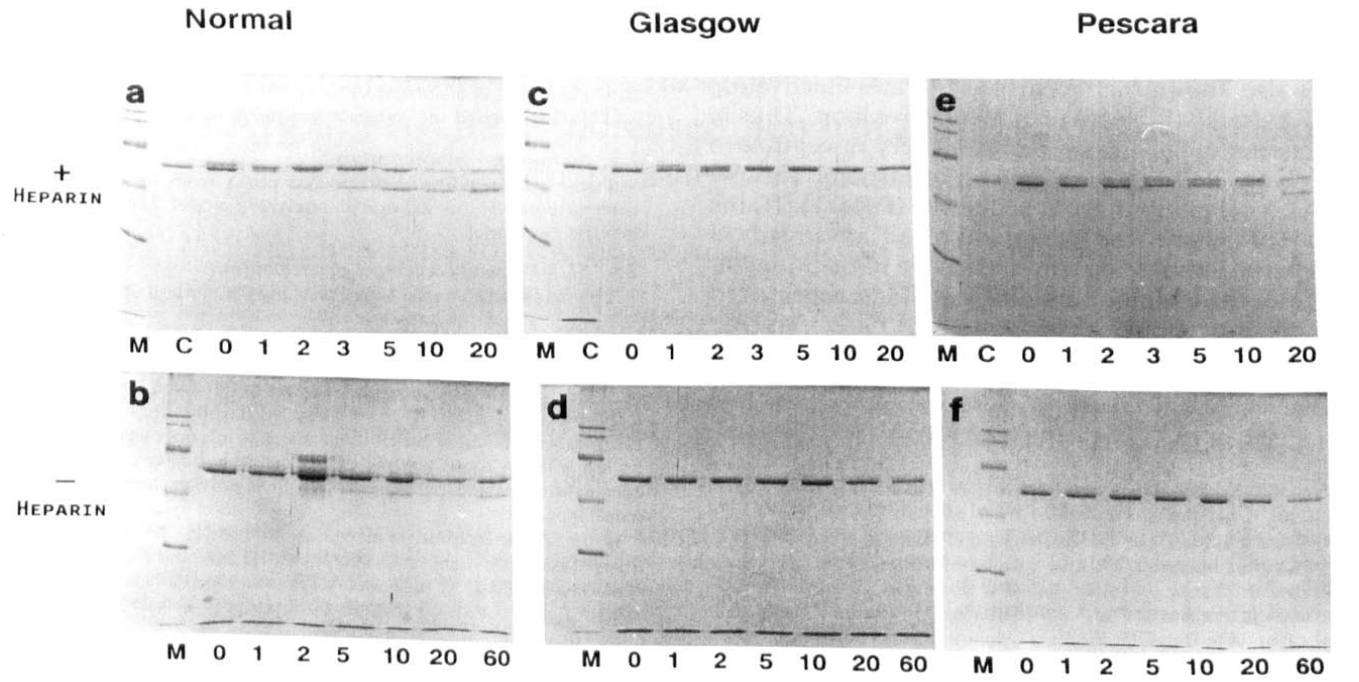

Fig. 2. SDS-polyacrylamide of purified normal AT (a and b), and Glasgow variant (c and d) and Pescara variant (e and f) as a control (C) and as incubated with human neutrophil elastase and sampled at times 0, 1, 2, 3, 5, 10, 20 and 60 min as shown. Gels a, c and e were from incubations in the presence of heparin, and gels b, d and f in the absence. Molecular weight markers (M) correspond to proteins of M_r (in kDa) 116, 97, 68, 45 and 29 from top to bottom of gel respectively. Lane 2 in gel b is ignored as an artifact.

containing normal AT was 0.78 mol/l; the Pescara variant (b) 0.86 mol/l, and the Glasgow variant (d) 0.92 mol/l.

3.2. *Cleavage with human neutrophil elastase*

The human neutrophil elastase contained negligible contaminating proteinases. Incubation of AT and heparin with elastase pretreated with the inhibitor showed very little reaction after 20 min, with maybe 2–3% of the protein being cleaved.

3.2.1. *Normal AT.* In the presence of heparin (Fig. 2a) proteolytic inactivation proceeds rapidly. There is often an initial formation of a higher M_r band which by 1 min comprises some 70% of the AT. This is then further degraded to give a lower M_r product. At 20 min about 70% of the AT has been degraded to the low M_r product. In contrast, the reaction is very much slower in the absence of heparin (Fig. 2b) and proceeds with a slow production of the low M_r product. At 20 min only about 10% of the AT has been degraded to the low M_r product.

3.2.2. *AT Glasgow.* In the presence of heparin (Fig. 2c) there is conversion to a higher M_r band which is complete by about 5 min (cf. normal, about 2 min). However, there is no further degradation to the lower M_r band. In the absence of heparin (Fig. 2d) there is very little reaction, or change, over the 60 min.

3.2.3. *AT Pescara.* In the presence of heparin (Fig. 2e) there is the formation of a higher M_r which then degrades to a low M_r band, as in normal AT. However, the reaction is much slower, probably about 20% that of normal. AT 10 min Pescara has a similar pattern to normal AT at 1–2 min. In the absence of heparin (Fig. 2f) there is essentially no reaction over 60 min.

3.3. *Fluorescence emission spectra of normal, Glasgow and Pescara*

The fluorescence spectra of normal AT at pH 7.4 and 6.0 is shown in Fig. 3a. With the addition of heparin, the peak at 347 nm increases until an optimal beyond which no further increase occurs. The fluorescence enhancement with heparin was calculated from the change in the fluorescence at the 347 nm peak, allowing for the non-AT fluorescence in the buffer blank, (defining the baseline at 400 nm). For normal AT the change was 58% at pH 7.4 and 26% at pH 6.0. Similarly for AT Glasgow (Fig. 3b) and Pescara (spectra not shown) the corresponding change was 58% at pH 7.4 and 25% at pH 6.0.

4. DISCUSSION

We set out to test 3 predictions that stem from our earlier proposal [1] concerning the role of the P1 active site residue in the activation of AT by heparin. Heparin

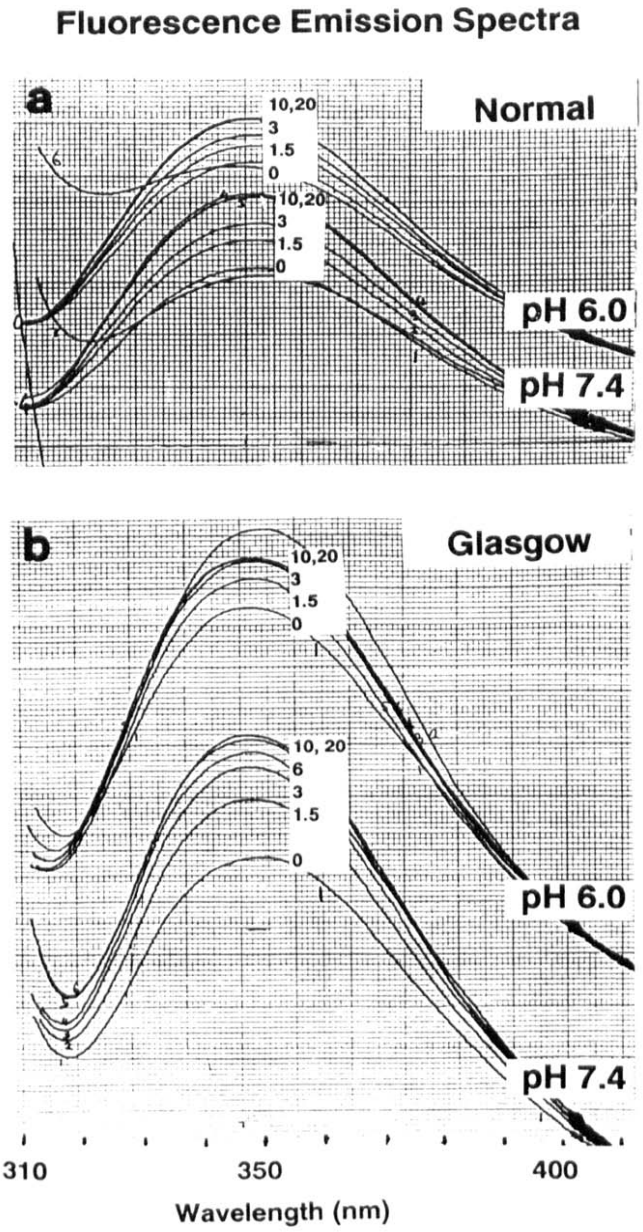

Fig. 3. Fluorescence emission spectra of purified normal (a) and AT Glasgow (b) at pH 6.0 and 7.4. Excitation was at 280 nm and spectra were run in the absence of heparin and with the addition of 1.5, 3, 6, 10 and 20 U, as shown on the traces.

binds to AT and induces a conformational change from an inactive to an active form. AT Glasgow, 393 Arg to His, has an increased affinity for heparin, but this reverts to normal at pH 6.0. Thus, we suggested, the positively charged Arg-393 plays a role in maintaining AT in a constrained, lower heparin affinity conformation. This conformation is stabilized by an ionic bond involving the P1 Arg residue. So, we reasoned, when heparin binds to AT at its primary binding site along the D-helix, there is a conformational change that is transmitted to the reactive centre disrupting this ionic bond. This would then free the residues in the reactive centre loop for interaction with proteases. The loss of the charged P1 ARg-393 was predicted to result in a conformation more like the heparin activated form. This model would predict firstly, that all P1 variants which are unable to form this ionic bond will have an increased heparin affinity. Secondly, it follows that even in the absence of heparin these P1 variants should be susceptible to catalytic cleavage by elastase. Thirdly, if the conformation of these variants was like the activated form, then they should display a reduced fluorescence enhancement on the addition of heparin.

4.1. *Heparin binding of P1 variants*

Both AT Glasgow and AT Pescara have increased heparin binding affinity. Compared with normal AT which elutes at 0.78 mol/l [Na^+], Glasgow elutes at 0.92 mol/l [Na^+] and Pescara at 0.86 mol/l [Na^+]. Although we have not been able to measure the heparin affinity of AT Northwick Park, 393 Arg to Cys, under these conditions, it has been reported to have an increased heparin affinity [14]. Thus, all the reported P1 variants have an increased heparin affinity. Further, we have previously shown that protonation of the P1 histidine in AT Glasgow at pH 6.0 results in the heparin affinity reverting to normal. Collectively, these results imply that the P1 residue plays a critical role in maintaining the normal non-activated conformation. A role that is consistent with a residue requiring a positive charge is its involvement in an ionic bond. β-AT lacks a carbohydrate side chain at Asn-135 which results in an increase in heparin binding affinity. However, the higher heparin affinity of AT Pescara and Glasgow can not be explained by the lack of a carbohydrate side chain since they have molecular weights identical to normal AT.

4.2. *Catalytic cleavage by elastase*

In the absence of heparin, AT is relatively resistant to inactivation by cleavage of the reactive centre loop by human neutrophil elastase (Fig. 2b). This is consistent with our proposal that in this conformation an ionic bond is formed with the positively charged Arg-393 and the adjacent residues would also be protected from proteolysis. Similarly in this conformation the P1 residue is unavailable, or inappropriately orientated, for interaction with the target enzymes, thrombin or Factor Xa. In the presence of heparin, the resultant conformational change gives a form in which the reactive centre loop is both available for interaction with thrombin or Xa and is very susceptible to catalytic cleavage by elastase (Fig. 2a). If this ionic bond contributes to maintaining the non-activated conformation, then variants unable to form the ionic bond could be expected to be susceptible to catalytic cleavage of the reactive loop in the absence of heparin. However, the results of incubating AT

Glasgow and AT Pescara with elastase in the absence of heparin do not show increased susceptibility to cleavage. Although this may mean that the model proposed is inappropriate, interpretation of the data is complicated by the lack of proteolytic degradation even in the presence of heparin. Possibly these mutations have a different effect on the conformation of the reactive centre loop. This situation can easily be conceived as applying for AT Pescara where the replacement of an arginine for a proline, an amino acid, would be expected to constrain the preceding loop in such a way that the effects are not easy to predict. However, AT Glasgow, with a histidine, would not be expected to produce a similar structural constraint. We have found that AT Glasgow with or without heparin does not inhibit elastase even at the catalytic ratios used in the catalytic cleavage experiments. Hence the lack of proteolytic degradation of Glasgow is not explicable in this way.

4.3. *Fluorescence enhancement with heparin*

As AT Glasgow at pH 7.4 is in a high heparin affinity conformation, it might be expected to have a reduced fluorescence enhancement with heparin, compared with normal AT. However, the results of these experiments show, at pH 7.4, identical fluorescence enhancement of 58% with normal and Glasgow. At pH 6.0, with the P1 residue charged in Glasgow, as expected from the model, the results are essentially identical; normal 26% enhancement and Glasgow 25%. These results may be interpreted as showing that the change in fluorescence induced by heparin binding is a direct consequence of heparin being bound adjacent to a critical tryptophan (e.g. residue 49). This is unlikely because other evidence [7] suggests the enhancement is due to a buried trytophan, probably 225. The enhancement then would be due to the same conformation change occurring in Glasgow and normal. The results do suggest that the high affinity conformation of AT Glasgow and Pescara (i.e. in the absence of heparin) is not similar to the heparin induced conformation of normal AT. The results do not preclude there being a localized conformational difference, between Glasgow and normal, that is not reflected in a change in fluorescence enhancement.

Heparin binding to AT results in the P1 residue changing from a relatively inaccessible position, with respect to its target protease, to a very accessible position. With this change, the reactive loop becomes accessible to cleavage by elastase. The resistance of the P1 variants to elastase in the absence of heparin does not preclude the involvement of a P1 ionic bond, but shows that the conformation of these P1 variants is not the same as the normal heparin activated conformation. The changes which result in the increase heparin binding affinity of P1 variants are not identical to the major change in conformation that occurs on heparin binding.

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