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Antithrombin Glasgow, 393 Arg to His: a P_1 reactive site variant with increased heparin affinity but no thrombin inhibitory activity

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Antithrombin Glasgow is a hereditary abnormal antithrombin that has lost thrombin inhibitory activity. It was isolated from the plasma of a 41-year-old male with a history of thrombotic events. Antithrombin Glasgow was purified from plasma using heparin-Sepharose chromatography at pH 7.4 eluting with increasing concentrations of NaCl. The normal protein eluted with 0.9 mol/l NaCl and Glasgow with 1.05 mol/l NaCl. Electrophoresis in agarose at pH 8.6 showed the variant to migrate more anodally than normal. The C-terminal small fragment resulting from catalytic cleavage with elastase between P_3 and P_4 of the reactive loop was isolated and sequenced. This showed the replacement of the arginine at residue 3 by a histidine. This is residue 393 in the intact molecule. The findings suggest that heparin, on binding, interacts indirectly with the reactive centre region of antithrombin.

Antithrombin; Pathological variant; Heparin binding; Reactive site mutant

1. INTRODUCTION

Antithrombin (AT) is the major physiological inhibitor of thrombin. The rate of inhibition of its target enzymes is greatly increased through interaction with heparin [1]. A syndrome of recurrent thrombosis was reported by Egeberg in 1965 [2] to be related to a deficiency of AT. Since then, there have been many reports of quantitative deficiencies and an increasing number of reports of qualitative abnormalities of AT.

The molecular characterisation of these variants has revealed that the substitutions involve two major areas on the molecule: the N-terminal heparin binding region with substitutions at residues 47 and 41 accounting for four characterised variants [3–7] and the other region being the reactive centre with variants, such as AT Denver, residue 394 [8] and Hamilton, residue 382 [9]. The precise location of the areas of interaction between heparin and antithrombin and details of the resultant mechanism of activation are as yet unclear.

We report here a new variant of antithrombin where the P_1 reactive centre arginine residue has been replaced by a histidine. This has, somewhat surprisingly, resulted in an increase in heparin affinity. The mutant has lost both progressive and heparin cofactor activity.

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2. MATERIALS AND METHODS

2.1. *Antithrombin assays*

The plasma concentration of immunoreactive antithrombin was determined by Laurell rockets [10]. Functional studies were performed with and without heparin using the chromogenic substrate Chromozym-TH for thrombin [5].

2.2. *Isolation of antithrombin*

Antithrombin was isolated from plasma on heparin-Sepharose [11]. To 100 ml of plasma was added 1 ml each of 5 mol/l calcium chloride and 10% dextran sulphate (M_r 500000) solution. The supernatant was loaded onto the heparin-Sepharose column (2.5 × 30 cm) equilibrated with 0.05 mol/l Tris, 0.01 mol/l citrate, 0.1 mol/l NaCl, pH 7.4. The an-

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tithrombins were eluted with a linear NaCl gradient to 1.5 mol/l.

2.3. *Heparin binding at pH 6.0*

The supernatant from plasma treated, as above, with calcium chloride and dextran sulphate was adjusted to pH 6.0 with HCl and loaded onto a heparin-Sepharose column equilibrated with 0.01 mol/l sodium citrate, 0.01 mol/l disodium EDTA, pH 6.0. The bound antithrombins were eluted with a linear NaCl gradient to 1.5 mol/l.

2.4. *Complex formation with thrombin*

A fixed amount (25 μg) of AT, dissolved in 10 μl of barbitol buffer, pH 8.3, was allowed to react in the presence of 1 unit of heparin with varying amounts (1.0, 2.5, 5.0, 12.5 μg) of bovine thrombin for 10 min at 25°C. These were then run on a reduced 10% SDS-polyacrylamide gel.

2.5. *Electrophoresis on agarose and sodium dodecyl sulphate polyacrylamide gels*

Electrophoresis was carried out on 1% agarose at pH 8.6 in 0.075 mol/l barbital buffer [12]. M_r values were determined from electrophoretic mobilities in 10% SDS-polyacrylamide gels according to the method of Laemmli [13].

2.6. *Catalytic cleavage with elastase and isolation of small fragment*

AT was catalytically cleaved with elastase [14], and the fragments separated by ethanol precipitation [15]. Purified antithrombin, 1 mg, was dissolved in 200 μl of phosphate buffered saline to which was added 500 IU of heparin (mucous, Evans Medical, Liverpool, England) and 10 μg of human neutrophil elastase (gift from Dr C.C. Winterbourn). This was incubated at 37°C for 40 min, taking 2 μl aliquots to monitor thrombin inhibitory activity at 10 min intervals. The sample was then concentrated and dialysed against distilled water using an Amicon stirred concentrating cell. The sample was immediately frozen (volume about 200 μl) and 30 mg of sodium dodecyl sulphate, and 50 μl of mercaptoethanol were added and immediately heated in a boiling water bath for 10 min. The large fragment was precipitated by adding 5 vols of ethanol and the supernatant was dried down under N_2 and redissolved in 300 μl of water. This was then added dropwise to 4 ml of 1% HCl in acetone while vortex mixing. The supernatant was discarded and the small fragment dried under N_2.

2.7. *Amino acid sequencing*

The manual Edman degradation method was used. The PTH-amino acids were identified by reverse-phase HPLC using a Waters Nova pac column.

2.8. *Case report*

A 41-year-old man with a history of spontaneous venous thromboembolism underwent laboratory studies for coagulation abnormalities. These showed his antithrombin activity in the presence of heparin to be 52% of a normal pool. Both his sisters showed low antithrombin functional activity, and had suffered thrombotic events. His three brothers also had low functional activity with one suffering from deep venous thrombosis. The propositus' father died (aged 47 yr) from a coronary thrombosis as did one of the father's two brothers (at age 31 yr). All three of the propositus' children have low heparin cofactor activity.

3. RESULTS

The antigenic level of antithrombin in the propositus' plasma was 96% of a normal pool whereas the thrombin inhibitory activity in the presence of heparin was 52% of a normal pool. Chromatography of the patient's plasma on heparin-Sepharose at pH 7.4 showed the presence of an antithrombin of increased heparin affinity (fig.1). Normal antithrombin eluted with 0.9 mol/l of NaCl whereas antithrombin Glasgow eluted with 1.05 mol/l NaCl. At pH 6.0 normal AT and AT Glasgow coeluted from heparin-Sepharose at 1.05 mol/l NaCl.

The M_r values of the proteins were identical at 58000. Electrophoresis on agarose gel showed antithrombin Glasgow to migrate more anodally than the normal at pH 8.6 (fig.2). Immunofixation with antiserum to antithrombin following electrophoresis at pH 8.6 of plasma from the propositus showed two bands, confirming that the abnormal band had not arisen as an artifact during purification (fig.2).

Normal AT reacted with thrombin to form a complex with a band at M_r 83000 and a minor band at M_r 92000 (fig.3). With increasing amounts of thrombin there was a further band at M_r 78000, which is presumably a degradation product of the complex. In contrast the abnormal AT did not react with thrombin to form a complex (fig.3).

Following catalytic cleavage by elastase both the

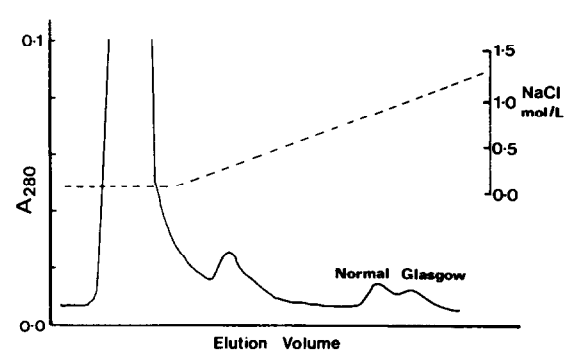

Fig.1. Elution profile of heparin-Sepharose chromatography of patient's plasma at pH 7.4 with increasing linear NaCl gradient.

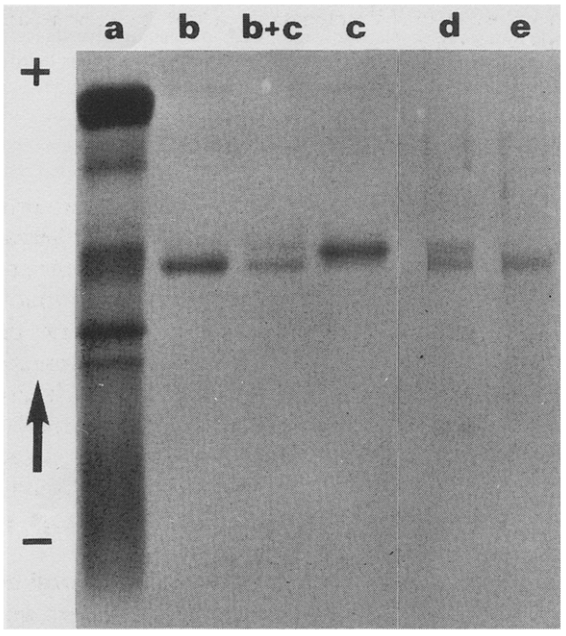

Fig.2. Electrophoresis in agarose gel at pH 8.6 of normal human serum (a), purified normal antithrombin (AT) (b), and purified AT Glasgow (c). The results of immunofixation with antiserum to AT following electrophoresis are shown of the patient's plasma (d) and normal plasma (e).

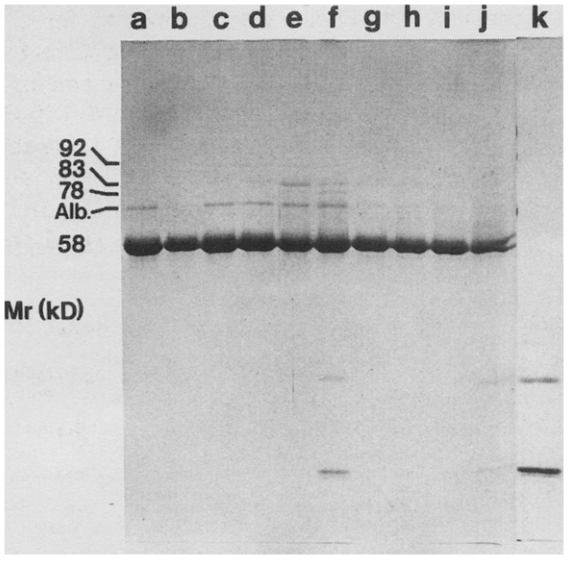

Fig.3. SDS-polyacrylamide gel of normal AT (a) and AT Glasgow (b). Lanes c–f show the result of incubation of increasing amounts of thrombin with normal AT, and lanes g–j with AT Glasgow. Track k has bovine thrombin. The gel was run under reducing conditions.

large fragment (that precipitated on addition of 5 vols of ethanol), and the small fragment were sequenced for 5 residues on both the normal and abnormal proteins. The sequences obtained for the large fragment from both normal and Glasgow were identical, His-Gly-Ser-Pro-Val, and correspond to the N-terminal of the intact protein. The first residues of the small fragment from Glasgow had the sequence Ala-Gly-His-Ser-Leu. This differed from that obtained from the normal small fragment at residue 3 which had the expected arginine. This corresponds to residue 393, the P_1 position, in the intact protein.

4. DISCUSSION

We report here a new variant of antithrombin, antithrombin Glasgow, that has increased heparin binding but has lost thrombin inhibitory activity. This has arisen from the replacement of the normal P_1 reactive site arginine (residue 393) by a histidine. Somewhat surprisingly this binds heparin more avidly. The majority of variants with altered heparin binding affinity have involved a region in the vicinity of the start of helix A [5] with arginine at residue 47 being replaced by cysteine [3,4], histidine [5] and serine [6], and the proline at 41 being replaced by leucine [7]. All these variants bind heparin less avidly than normal. The mechanism of heparin interaction and activation of antithrombin is not clear. We have shown that it is unlikely to involve a direct shielding of the active site by the N-terminal tail [5], and have suggested a positively charged groove comprising residues 47, 125, 129, 132 and 133 to be the likely site of heparin binding [16]. On charge considerations alone, it would be expected that the replacement of the positively charged arginine residue at the P_1 position by a histidine that would be uncharged at pH 7.4 [17] should, if there were direct interaction with heparin, result in a decrease in heparin affinity. However, the reverse occurs. Protonation of the new P_1 histidine by reducing the pH to 6.0 results in a return to normal heparin binding. This is in contrast to AT Rouen-I (47 Arg to His) where protonation of the new histidine did not restore the variant to normal heparin affinity [5]. This implies that the P_1 arginine is involved in an indirect manner in heparin binding; presumably

by a salt bridge to some other region of the molecule. Loss of the positive charge at the P_1 residue results in an altered conformation which is more favourable for heparin binding. This is also seen with AT Northwick Park where there is a substitution of the P_1 arginine for a non-charged cysteine residue [18]. In contrast to the N-terminal variants which are activated on binding to heparin, the Glasgow variant although it binds more avidly than normal is not activated by heparin. In this case binding per se is not a sufficient condition for activation.

In the serpins the P_1 residue is one of the prime determinants of inhibitor specificity [19]. Antitrypsin-Pittsburgh is an example of a dramatic alteration of specificity resulting from the substitution of the residue [20]. Replacing the P_1 methionine by arginine converted antitrypsin-Pittsburgh from an elastase inhibitor to a potent inhibitor of thrombin. AT Glasgow, as a result of the replacement of the important P_1 arginine by histidine has lost the ability to inhibit thrombin. We were unable to show the formation of a complex with thrombin. This is not surprising since the only serpin reported to date with a histidine at the P_1 residue is the chicken gene-Y protein which has no reported inhibitory activity [21,22].

The thrombotic episodes suffered by the propositus are related to the presence of this variant of antithrombin. Not only is AT Glasgow not activated by heparin but the fact that it binds heparin more avidly than normal means that, at sub-optimal levels of heparin, the heparin will preferentially bind the variant with no resultant increase in plasma antithrombin activity. These two factors, the loss of heparin cofactor activity and the increased avidity for heparin, combine to produce a situation that is potentially more serious than the heterozygous quantitative deficiency state.

It was our initial intention to call this variant AT Waikato. It has come to our attention that a cousin of the propositus has been independently investigated by Lane et al. [18] who have named the variant AT Glasgow. Because of their prior publication [23], and to avoid confusion in the literature we accept the name AT Glasgow.

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REFERENCES

[1] Bjork, I. and Danielsson, A. (1986) in: Proteinase Inhibitors (Barrett, A. and Salvesen, G. eds) pp.489–513, Elsevier Science Publishers, Amsterdam, New York.
[2] Egeberg, O. (1965) Scand. J. Clin. Lab. Invest. 17, 92.
[3] Koide, T., Odani, S., Takahashi, K., Ono, T. and Sukuragawa, N. (1984) Proc. Natl. Acad. Sci. USA 81, 289–293.
[4] Duchange, N., Chasse, J.-F., Cohen, G.N. and Zakin, M.M. (1987) Thrombosis Res. 45, 115–121.
[5] Owen, M.C., Borg, J.-Y., Soria, C., Soria, J., Caen, J. and Carrell, R.W. (1987) Blood 69, 1275–1279.
[6] Owen, M.C., Borg, J.-Y. and Carrell, R.W. (1987) N.Z. Med. J. 100, 566.
[7] Chang, J.-Y. and Tran, T.H. (1986) J. Biol. Chem. 261, 1174–1176.
[8] Stephens, A.W., Thalley, B.S. and Hirs, C.H.W. (1987) J. Biol. Chem. 262, 1044–1048.
[9] Devraj-Kizuk, R., Prochownik, E.V., Chui, D.H.K. and Blajchman, M.A. (1986) Blood 68, suppl.1, 345a.
[10] Laurell, C.-B. (1972) Scand. J. Clin. Lab. Invest. 29, suppl.124, 21–37.
[11] McKay, E.J. (1981) Thromb. Res. 21, 375–382.
[12] Johansson, B.G. (1972) Scand. J. Clin. Lab. Invest. 29, suppl. 124, 7–19.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Carrell, R.W. and Owen, M.C. (1986) Nature 322, 730–732.
[15] Brennan, S.O., George, P.M. and Jordan, R.E. (1987) FEBS Lett. 219, 431–436.
[16] Borg, J.Y., Owen, M.C., Soria, C., Soria, J., Caen, J. and Carrell, R.W. (1988) J. Clin. Invest., in press.
[17] Perutz, M.F., Groneborn, A.M., Clore, G.M., Fogg, J.H. and Shih, D.T. (1985) J. Mol. Biol. 183, 491–498.
[18] Lane, D.A. personal communication of: Erdjument, H., Lane, D.A., Panico, M., Di Marzo, V. and Morris, R. (1988) J. Biol. Chem., in press.
[19] Boswell, D.R. and Carrell, R.W. (1987) Recent Adv. Clin. Immunol. 4, 1–17.
[20] Owen, M.C., Brennan, S.O., Lewis, J.H. and Carrell, R.W. (1983) N. Engl. J. Med. 309, 694–698.
[21] Heilig, R., Muraskowsky, R., Kloepfer, C. and Mandel, J.L. (1982) Nucleic Acids Res. 10, 4363–4382.
[22] Carrell, R.W. and Boswell, D.R. (1986) in: Proteinase Inhibitors (Barrett, A. and Salvesen, G. eds) pp.403–420, Elsevier Science Publishers, Amsterdam, New York.
[23] Lane, D.A., Lowe, G.D.O., Flynn, A., Thompson, E., Ireland, H. and Erdjument, H. (1987) Br. J. Haematol. 66, 523–527.