Volume 266, number 1,2, 163–166 FEBS 08526 June 1990

Antithrombin Rouen-IV 24 Arg→Cys

The amino-terminal contribution to heparin binding

J.-Y. Borg[1], S.O. Brennan[2], R.W. Carrell[3], P. George[2], D.J. Perry[3] and J. Shaw[4]

[1] *Laboratoire d'hemostase, Centre Hospitalier Universitaire, Rouen 67038 Cedex, France,* [2] *Department of Pathology, Christchurch Hospital, Christchurch, New Zealand,* [3] *University Department of Haematology, MRC Centre, Hills Road, Cambridge CB2 2QH, UK* and [4] *Massey University, Palmerston, New Zealand*

Received 22 March 1990

A variant antithrombin with reduced heparin affinity was shown by mass spectrometry sequencing and DNA amplification to have a substitution of a cysteine for an arginine at residue 24. The position of Arg-24 can be fixed within a 12 Å radius from the bridge at Cys-21. This is compatible with findings in the homologous protease nexin-1 which indicate an extension of the binding site of heparin from the D-helix to under the adjacent amino-terminal pole.

Antithrombin; Heparin binding site; Serpin; Protease nexin-1

1. INTRODUCTION

Antithrombin, the major inhibitor of thrombin in human plasma, is a member of the serpin family of serine proteinase inhibitors. It shares with two other serpins, protease nexin 1 and heparin cofactor II, the requirement to bind sulphated polysaccharides in order to activate inhibition, with antithrombin increasing its association constant for thrombin 10 000-fold in the presence of heparin [1]. Comparison of the structures of the three serpins shows a shared homologous binding site for heparin formed by basic residues and extending from the commencement of the A helix to along the distal third of the D-helix [2–4]. Although there is good evidence that this is the common teleological binding site, there is variation in the binding characteristics of the three serpins. In particular, there is strong evidence that the basic amino-terminal extension, uniquely present in antithrombin, is also involved in the primary binding of heparin [5]. The precise relationship of this N-terminal site in antithrombin to the shared site must await further crystallographic data, but preliminary evidence of the structural relationship of the two sites is provided from natural mutants of antithrombin. We present here a new low-heparin-affinity variant of antithrombin which indicates the involvement of residue Arg-24 in the N-terminal in the binding of heparin and we discuss the relationship of this residue to an extended common (D-helix) site.

Correspondence address: J.-Y. Borg, Laboratoire d'hemostase, Centre Hospitalier Universitaire, Rouen 67038 Cedex, France

2. MATERIALS AND METHODS

2.1. *Materials*

Antithrombin measurements in plasma, amplification of exon 2 of the antithrombin gene, and direct sequencing of the DNA product were carried out as recently detailed [6]. Oligonucleotides for exon 2,5′(ATCTTTTGTCCTTGCTGCTAATTGGC) and 5′(CCATCAGTTGCTGGAGGTGTCATTA), spanning positions 50–406 were synthesised on an Applied Biosystems Synthesiser and used in amplification reactions without purification.

2.2. *Heparin-Sepharose chromatography*

1 ml of 5 mM $CaCl_2$ and 1 ml of 10% dextran sulphate were added to 100 ml of plasma. The supernatant was applied to a 1.5 × 25 cm column of heparin-Sepharose equilibrated in 50 mM Tris-HCl, 10 mM citrate, pH 7.4 [3]. The column was eluted with a linear gradient to 1.3 M NaCl in the same buffer.

2.3. *Protein structure analysis*

Antithrombin was carboxyamidomethylated in 8 M urea [7] and digested with trypsin for 16 h at 37°C [8]. Reverse-phase HPLC mapping was carried out using a Waters 8 mm i.d. radial compression cartridge with a 10 μm C-18 packing. The initial solvent (A) was 49 mM KH_2PO_4 adjusted to pH 2.9 with orthophosphoric acid, while solvent B consisted of a 50:50 mixture of A in acetonitrile [8]. The flow rate was 1.5 ml/min and a linear gradient was run from 0% to 67% B over 46 min then to 100% B over the next 8 min. Preparative separations employed 1 mg (17 nmol) of digest. Individual peaks were collected and further purified by rechromatography in a volatile ammonium acetate/acetonitrile solvent system [8].

Amino acid analysis was performed on a Dionex D-300 Analyser with ninhydrin detector. Mass spectrometry was performed on a VG70-2505 double-focusing magnetic sector instrument using a liquid secondary ion mode with caesium ions forming the primary ion [9].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

3. RESULTS

3.1. *Case history*

Plasma was obtained from a 25-year-old man who developed an unexpected coronary thrombosis. Subsequent investigations showed two abnormalities; the presence of a heterozygous dysfunctional antithrombin (described here) inherited from his father, and a hypofibrinogenaemia inherited from his mother. The single other instance of thromboembolic disease in the family had occurred in the mother's kinship; there was no history of thromboembolism from the father or his kinship.

3.2. *Antithrombin measurements in plasma*

Plasma from the propositus gave normal antigenic levels of antithrombin (105%) and there was no impairment of slow (progressive) inhibitory activity against thrombin. However, the fast (heparin cofactor) activity against thrombin in the presence of heparin was reduced to 57%. Electrophoresis in agarose gel at pH 8.6 showed the presence of an abnormal band of antithrombin with increased overall negative charge.

3.3. *Heparin Sepharose chromatography*

Elution of plasma from heparin Sepharose gave two well-resolved peaks of anti-thrombin; one eluted in the normal position at 0.83 M NaCl, the other, the variant (Rouen-IV) peak, eluted at 0.45 M NaCl. Both peaks gave single bands M_r 58 000 on SDS-PAGE. The low-affinity (Rouen-IV) fraction separated anodally on agarose gel electrophoresis, indicating a decrease in net positive change.

3.4. *Protein structure analysis*

Reverse phase tryptic maps showed 3 clear and reproducible differences between digests of the normal and variant antithrombin components (Fig. 1). Antithrombin Rouen-IV lacked 3 of the tryptic peptides that were present in normal antithrombin at 8.5, 9.6 and 22 min. Amino acid analysis of these normal peaks showed that they represented adjacent and overlapping sequences encompassing residues 21–29, Cys-Ile-Tyr-Arg-Ser-Pro-Glu-Lys-Lys.

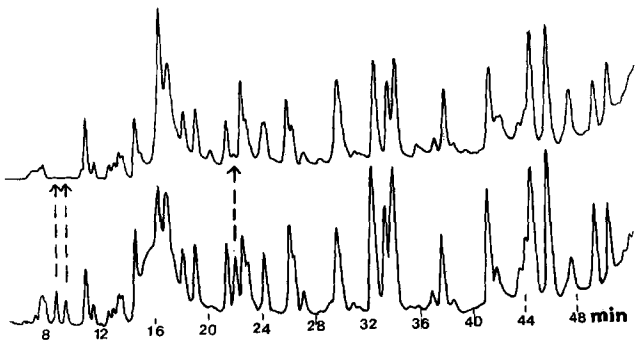

Fig. 1. Reverse-phase tryptic peptide maps of carboxamidomethylated anti-thrombin from the propositus. Lower trace, normal component; upper trace, antithrombin Rouen-IV.

The 3 missing peaks in the digests of Rouen-IV are readily explicable in terms of mutation of Arg-24 to a neutral residue. As no new abnormal peak could be readily discerned in the HPLC trace, total tryptic digests were analysed by liquid secondary ion mass spectrometry [11]. New peptide signals were detected at mass 1057 and 1185. These values correspond to the masses of the protonated peptides C I Y C S P E K and C I Y C S P E K K with two carboxamidomethylated cysteine residues. These results indicate the mutation 24 Arg → Cys.

3.5. *DNA sequence analysis*

Direct sequence analysis of amplified DNA showed a C → T mutation at nucleotide 166 giving a codon reading alteration 24 Arg (CGC) → Cys(TGC).

4. DISCUSSION

The DNA and protein sequence data show the patient to be the heterozygous carrier of a new variant antithrombin arising from a point mutation causing a substitution of arginine-24 by cysteine. This loss of an arginine explains the observed anodal shift on electrophoresis as well as the decreased affinity on heparin-Sepharose and the decreased heparin-induced inhibitory activity. Antithrombin Rouen-IV is thus an addition to the known heparin-affinity variants of antithrombin. By implication arginine-24 is therefore likely to be involved in the ionic bonding to the sulphate of the oligosaccharide and to form part of the heparin binding site.

This would initially appear to be in conflict with current proposals as to the position of the site. There is good evidence [3,4] that the primary binding site for heparin is centred on the upper third of the D-helix (Fig. 2). The strongest evidence is the unique conservation of this positively charged face of the D-helix in the three serpins activated by sulphated polysaccharides: antithrombin, protease nexin 1 and heparin cofactor II. However, the identified mutations of antithrombin affecting heparin affinity are mostly situated in the N-terminal portion of the molecule, particularly at Arg-47 and Pro-41, raising the question of a possible second site.

In fact, closer analysis of the results provides an explanation compatible with a unitary site. In the first place the frequency of observed mutants is a misleading index of the comparative contribution of different amino acids. The occurrence of CpG dinucleotides in the codons for Arg-47 and Pro-41 explains the frequency of mutants [10] at these residues as it does the mutation of the present variant at Arg-24. The comparative infrequency of variants of the basic residues of the D-

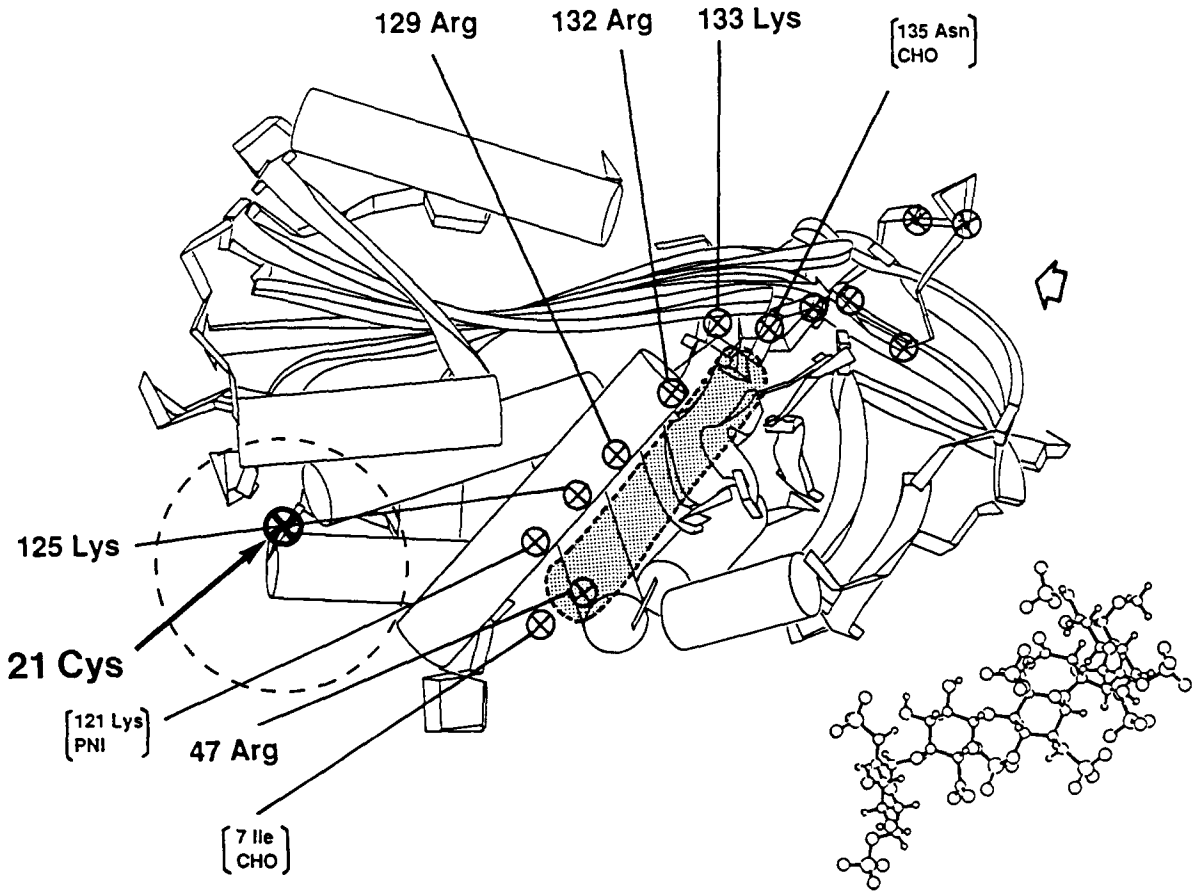

Fig. 2. Heparin binding site of antithrombin projected on the structure of cleaved antitrypsin; below right is schematic pentasaccharide heparin. The position of Arg-24 must lie within the 12 Å radius shown about Cys-21. Figure adapted from [4] which gives further details.

helix is explained by the absence in their codons of CpG dinucleotides which will give rise to expressed substitutions. A second point is that although Arg-47 is near the amino-terminus it is also closely situated to the proposed common binding site. Its position can be reliably calculated since it is at the commencement of the A-helix present in the archetypal tertiary structures. On the model Arg-47 is adjacent to the base of the D-helix and is clearly seen to form part of the contiguous basic binding site [4]. This, however, is the limit of our present knowledge of the tertiary structure as positions proximal, i.e. amino-terminal, to the base of the A-helix are not yet defined on the model. At this stage we cannot determine the position of Arg-24 but the area within which it must lie can be calculated as being within a 12 Å radius of the sulphydryl bridge formed by Cys-21, near the amino-terminal pole of the molecule (see Fig. 2).

A clue to the overall extent of the heparin site is provided by the model of protease nexin 1. This lacks a basic amino-terminal extension and also does not have an arginine at the base of the A-helix; protease nexin 1 does, however, have extra basic residues which are seen on the model to substitute for Arg-47 and to provide an extension of charge towards the pole of the molecule and within the region of Arg-24. These extra positively charged residues, all within 12 Å of Cys-21, are in the C-helix at positions 53 Arg, 55 and 56 Lys and 63 Arg in protease nexin-1. This last residue, 63 Arg is homologous to 107 Lys in antithrombin, implicated by Chang [11] as a binding site for heparin. In conclusion, we believe the weight of evidence favours a unitary primary heparin binding site extending from the lower third of the D-helix to under the adjacent (amino-terminal) pole of the molecule. This is compatible with the overall data from variant studies but the precise relationship of the folded N-terminus to the binding site in antithrombin must await crystallographic studies.

Acknowledgements: We gratefully acknowledge the support of the Wellcome Trust, the Medical Research Councils of Great Britain and New Zealand and the British Heart Foundation.

REFERENCES

[1] Rosenburg, R.D. and Damus, P.S. (1973) J. Biol. Chem. 248, 6490–6505.

[2] Carrell, R.W., Christey, P.B. and Boswell, D.R. (1987) Thrombosis and Haemostasis (Verstraete M., Vermylen J., Lijnen, H.R. and Arnout, J. eds) Leuven University Press, pp. 1–15.
[3] Borg, J.-Y., Owen, M.C., Soria, C., Soria, J., Caen, J. and Carrell, R.W. (1988) J. Clin Invest. 81, 1292–1296.
[4] Huber, R. and Carrell, R.W. (1989) Biochemistry 28, 8951–8966.
[5] Blackburn, M.N., Smith, R.L., Carson, J. and Sibley, C.C. (1984) J. Biol. Chem. 259, 939–941.
[6] Perry, D.J., Harper, P.L., Fairham, S., Daly, M. and Carrell, R.W. (1989) FEBS Lett. 254, 174–176.
[7] Nelson, C., Noelkan, M., Buckley, C., Tanford, C. and Hill, R. (1965) Biochemistry 4, 1418–1426.
[8] Brennan, S.O. (1985) Biochem. Biophys. Acta 830, 320–324.
[9] Owen, M.C., Boswell, D.R., Shaw, J. and Carrell, R.W. (1990) Thrombosis Res. in press.
[10] Perry, D.J. and Carrell, R.W. (1989) Mol. Biol. Med. 6, 239–243.
[11] Chang, J.Y. (1989) J. Biol. Chem. 261, 1174–1176.