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Antithrombin Cambridge II, 384 Ala to Ser

Further evidence of the role of the reactive centre loop in the inhibitory function of the serpins

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Four unrelated individuals have been identified with an identical antithrombin variant, associated in one of them with episodes of recurrent venous thromboses. In each case, the plasma antithrombin concentration was normal and the only functional abnormality was a minor but consistent decrease in the heparin-induced thrombin inhibition suggesting a mutation at or near the reactive centre of the molecule. Amplification and direct sequencing of exon 6 showed a G→T mutation at nucleotide 1246, which corresponds to a substitution of a serine for an alanine at residue 384. This is one of a series of conserved alanines that form the stalk to the reactive centre loop. The observed changes in this variant are compatible with recent structural studies that infer that mobility of this stalk with partial re-entry into the A-sheet of the molecule is necessary for optimal inhibitory activity.

Antithrombin; PCR; Active site variant

1. INTRODUCTION

Human antithrombin III (here referred to as antithrombin) is a 58 kDa plasma glycoprotein belonging to the Serpin superfamily. It is the major inhibitor of a number of serine proteases involved in coagulation, including factor IIa (thrombin) and factor Xa [1]. Under normal physiological conditions its inhibitory activity is relatively slow, but in the presence of heparin this activity is increased by 1000-fold [1]. The importance of amtithrombin in the regulation of normal haemostasis is emphasised by the recurrent thromboses that individuals with either a deficient or abnormal antithrombin are prone to develop [2].

We have used the polymerase chain reaction (PCR) [3] to amplify exon 6 [4] of the antithrombin gene in four patients with an antithrombin variant which exhibited heparin binding but defective inhibitory activity. The identified mutation provides support for the proposal that inhibitory activity of the serpins is dependent on a folding of the N-terminal region of the reactive centre loop.

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2. MATERIALS AND METHODS

2.1. *Materials*

All reagents unless otherwist stated were obtained from the Sigma Chemical Co., Poole, Dorset, UK. Deoxynucleotides and T4 polynucleotide kinase were obtained from Pharmacia-LKB Biotechnology, Milton Keynes, UK; Thermus Aquaticus (*Taq*) polymerase from Perkin-Elmer Cetus, Beaconsfield, UK; low melting point agarose from BioRad Labs Ltd., Watford, UK; Ultrapure urea, agarose and restriction enzymes from Gibco-BRL, Paisley, Scotland. DNA sequencing used Sequenase v2.0 obtained from United States Biochemical Corporation (Cambridge Bioscience), Cambridge, UK. Chromogenic substrate Chromozym TH was obtained from Boehringer Mannheim, Lewes, E. Sussex and S2222 from Kabi Vitrum, Uxbridge, Middx.

2.2. *Patients*

Patient 1 was a 68-year-old male who was presented at the age of 35 years with a deep vein thrombosis following an acute episode of gout and subsequently experienced two further spontaneous thromboses. There was no family history of thromboembolic disease.

Patients 2, 3 and 4 were unrelated to the first patient. In each case there was no history of thromboembolic disease and the abnormality was detected as part of a screening programme of normal blood donors to establish the incidence of antithrombin deficiency.

2.3. *Plasma antithrombin assays*

Antithrombin antigen was measured by competitive ELISA [5]. Antithrombin anti-IIa activity was measured in the absence (antithrombin progressive activity) or in the presence of heparin (heparin cofactor activity) according to Abildgaard [6] using the chromogenic substrate Chromozym TH. Anti-Xa activity was performed according to Odegard [7] in the presence of heparin (anti-Xa heparin cofactor) using the substrate S2222. Crossed immunoelectrophoresis was per-

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formed according to Sas [8] with and without the addition of heparin (25 U/ml) in the first dimension.

2.4. *Amplification and sequencing of exon 6 of the antithrombin gene*

DNA was isolated from peripheral blood leucocytes as previously described [9].

Synthetic oligonucleotides 5'-AATGAAGACAAGTGTTTGGT-TTTTAT and 5'-AAGCATTGAGGAATTGCTGTGTCTGT were designed to selectively amplify a 249-base pair (bp) region of the AT gene encompassing the entire coding region of exon 6 together with some 5'-intronic and 3'-untranslated sequence. The PCR product was gel-purified and directly sequenced using a ^{32}P-labelled 'nested' sequencing primer [10].

3. RESULTS

Plasma antithrombin assays (Table I) demonstrated normal antigenic levels of antithrombin, normal progressive inhibitory activity and normal heparin induced anti-Xa activity. However, anti-IIa activity in the presence of heparin was reduced to 76% in two cases and to 30% and 59% in the remaining two cases. Crossed immunoelectrophoresis showed an identical mobility of the normal and abnormal components both in the presence and absence of heparin.

DNA was isolated from peripheral blood leucocytes. In the four patients studied, amplification of exon 6 of the AT gene generated a single fragment of 249 bp. Sequencing of the PCR product demonstrated two bands at nucleotide position 1246 representing the normal guanine (G) and the mutant thymidine (T) bases. The sequences for codon 384 derived from the anti-sense strand are, therefore, GCA coding for alanine and TCA coding for serine.

This mutation is associated with the loss of a *Pvu*II site, which allows for the rapid screening of family members. Testing of related family members has identified the mutation in a further two asymptomatic siblings.

4. DISCUSSION

We report here an antithrombin variant, Cambridge II, found in four unrelated heterozygotes, in only one of whom there was a history of recurrent venous thromboses. The variant 384 Ala to Ser has properties in common with similar variants having mutations at or near the active site (Fig. 1), i.e. normal heparin binding but a loss of thrombin inhibitory activity. However, a unique feature of the Cambridge II variant (Table I) is that it retains anti-Xa activity whereas others, including the same site 384 Ala to Pro [10,11] lose both IIa and Xa inhibitory activity.

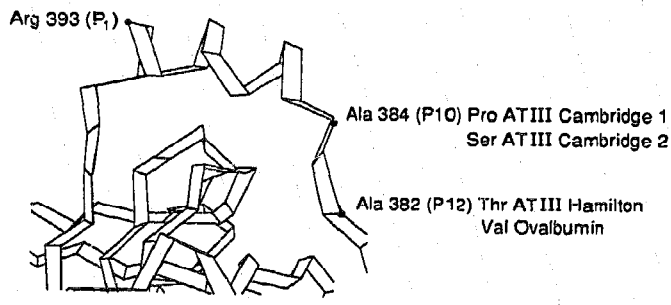

Fig. 1. Projection of antithrombin on the reactive centre of ovalbumin [13], a vertical view of that shown in Fig. 2a, indicating the position of the N-terminal stalk substitutions at alanine 384 (P10), ten residues from the reactive center (P1) and at alanine 382 (P12).

Although these results may seem puzzling, they fit well both with current proposals as to the structural basis of inhibition in the serpin family (reviewed by Huber and Carrell [12]) and with the structural model for a mobile reactive centre recently presented by Stein et al. [13]. There is now good evidence that the reactive centre of antithrombin, Arg-Ser 393–394, lies on an external peptide loop hinged on Glu-377 and extending to Arg-393. It is believed that a critical contribution of this loop is to hold the reactive centre in an available and strained conformation.

A proposed basis for the strain is the inability of the loop, in the intact antithrombin, to move fully back into the adjacent A sheet, where it would form a more stable conformation, as occurs spontaneously on cleavage of the loop [14]. The resultant conformation of the loop on incomplete re-entry into the sheet is likely to be close to that of the canonical form found in other

Table I

Alanine 382 and 384 variants

Variant	Mutation	Inhibitory Activity (%)			Antigenic conc (%) (70–126)	Reference
		+ Heparin		− Heparin		
		IIa (85–133)	Xa (98–115)	IIa (76–97)		
Patient 1	Ala→Ser 384	76	99	100	103	
Patient 2	Ala→Ser 384	76	101	120	106	
Patient 3	Ala→Ser 384	30	-	80	113	
Patient 4	Ala→Ser 384	59	147	104	108	
Cambridge I[a]	Ala→Pro 384	54	43	63	95	Perry et al. 1988 [9]
Hamilton[b]	Ala→Thr 382	55	59	50	98	Devraj-Kizuk et al., 1988 [15]

[a]ATIII Charleville - identical variant [11].
[b]Data refers to an AT Hamilton variant identified locally.

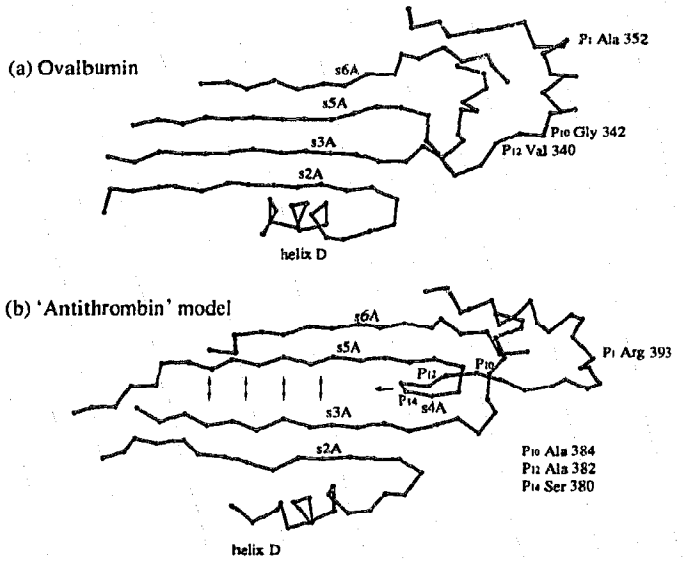

Fig. 2. Diagrammatic models of the A-sheet and reactive centre loop of (a) ovalbumin [13] and (b) of antithrombin modelled to show how partial re-entry of strand 4 into the A sheet would give a loop structure approximating to the optimal inhibitory conformation. Re-entry of the loop to give strand 4 (s4a) is incomplete and can be modelled beyond this point for perhaps one or two residues emphasising the need for flexibility at P10 and P12 (382 and 384 in antithrombin).

families of serine proteinase inhibitors [15] that matches the active site of the protease (Fig. 2). This need to maintain mobility provides an explanation for the conservation throughout the inhibitor members of the family, of a trio of amino acids with low-bulk side chains, usually -Ala-Ala-Ala-, at a position, near the proposed bend of the loop, 382–384 in antithrombin [12,13].

Alanine 382 is strongly conserved throughout the inhibitor serpins and its replacement by threonine in antithrombin Hamilton [16] results in a loss of inhibitory activity. Alanine 384 is less strictly conserved and hence likely to be less critical for function. This is in keeping with the observation that a major change such as the replacement of alanine 384 by proline [10] causes an apparently complete loss of activity whereas the less drastic substitution of the alanine by a serine causes only a selective loss of inhibition.

Although the presence of a row of alanines suggests a requirement for flexibility, there will also be steric and polarity requirements in order for these residues to fit in place as they fold back into the A-sheet. Evidence for the requirements of fit comes from binary complexing experiments in which a synthesised peptide with the sequence of the active centre loop of antithrombin 380–392 (P2–P14) can be shown to anneal in the A-sheet in the intact inhibitors [17]. Replacement of the 382 (P12) alanine by threonine as in AT Hamilton gives a much slower annealing indicating a decrease in the fit of the threonine in the A-sheet (D. Evans et al., unpublished). The same experiments have not been tried with the new AT Cambridge II mutant but it is difficult to see from the model (Fig. 2b) how reincorporation of residue 384 could occur in the intact protein even to give the fully stretched canonical form of the reactive centre. It is likely then that the AT Cambridge II mutation will primarily affect the mobility of the loop rather than its ability to fit sterically into the A-sheet.

The observed findings with these mutants of antithrombin fit with the changes that follow loosening of the loop by the insertion of an extra residue as in the natural variant antiplasmin Enschede [18] which loses its inhibitory activity, and with site-directed mutants of antitrypsin in which the addition of a residue alters the specificity, as well as activity of inhibition [19]. Thus, the overall results are compatible with a role for alanines 382–384 in the maintenance of the flexibility of the reactive centre as well as its ability to fit back into the A-sheet.

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