

# Antithrombin Milano, Single Amino Acid Substitution at the Reactive Site, Arg393 to Cys

H. Erdjument, D. A. Lane, H. Ireland, V. Di Marzo\*, M. Panico\*, H. R. Morris\*, A. Tripodi\*\*, and P. M. Mannucci\*\*

From the Department of Haematology, Charing Cross and Westminster Medical School, London, the Department of Biochemistry\*, Imperial College, London, UK and the A. Bianchi Bonomi Haemophilia and Thrombosis Centre and Institute of Internal Medicine\*\*, University of Milano, Italy

## Key words

Antithrombin – Albumin complex – Antithrombin variants – Functional antithrombin deficiency – Familial thrombosis

## Summary

Antithrombin Milano is an unusual antithrombin variant, exhibiting an abnormal, fast moving component on crossed immunoelectrophoresis (in the absence of heparin). Antithrombin isolated from the propositus could be resolved into two peaks on anion-exchange chromatography; antithrombin Milano peak 1 of  $M_r \sim 60,000$  which could inhibit thrombin, and antithrombin Milano peak 2 of  $M_r \sim 120,000$  which was inactive. The latter component also reacted with antisera to both antithrombin and albumin on immunoblotting. Under reducing conditions, the  $\sim 120,000 M_r$  component migrated on SDS-PAGE as two distinct bands with  $M_r \sim 60,000$ , one of which reacted with antiserum to antithrombin and the other (of slower mobility) of which reacted with antiserum to albumin only. These and other results established the  $\sim 120,000 M_r$  component to be an inactive, disulphide-linked variant antithrombin and albumin complex. The variant antithrombin was isolated, following reduction and S-carboxymethylation, by reverse-phase HPLC and then it was fragmented with CNBr. A major CNBr pool containing the sequence Gly339-Met423 was treated with trypsin, followed by V8 protease. The resulting peptides were analysed by fast atom bombardment mass spectrometry (Fab-MS) mapping. A peptide of molecular mass 1086, corresponding to the normal sequence Ala382-Arg393, was almost absent from the mass spectrum, but an additional peptide of mass number 1772 was present. These results are almost identical to those found in another variant antithrombin, Northwick Park (Erdjument et al., *J Biol Chem*, 262: 13381, 1987; Erdjument et al., *J Biol Chem*, 263: 5589–5593, 1988), indicating the same single amino acid substitution of Arg393 to Cys.

## Introduction

Antithrombin is a single chain glycoprotein which can inactivate thrombin and most of the other serine proteinases of the blood coagulation system (1). Protein and cDNA sequencing studies have established its amino acid sequence (2–5). It is composed of 432 amino acid residues and has three disulphide bridges and four carbohydrate side chains. Antithrombin is homologous with other serine proteinase inhibitors now collectively known as “serpins” (6, 7).

Reaction between antithrombin and serine proteinases is greatly accelerated by heparin (8) and endothelial cell heparin-like glycosaminoglycans (9), which may act as a template for

inhibitor and enzyme (10). There is now appreciable evidence from the study of congenital variants and chemical modification studies that locates the region involved in its binding to heparin to near its  $NH_2$  terminus (11–19).

It has been demonstrated that thrombin attacks a specific bond of antithrombin near its COOH-terminus during antithrombin-thrombin complex formation. This bond has been identified as Arg393-Ser394 (20). Structural studies carried out on antithrombin variants which are characterised by impaired ability to inhibit thrombin and which are associated with familial thrombosis have corroborated the location of the reactive site bond (21–23).

Antithrombin Milano has been shown to be characterised by an abnormal, fast moving peak on crossed immunoelectrophoresis in the absence of heparin, by Wolf et al. (24). Initial studies by SDS-PAGE of antithrombin purified by heparin-Sepharose from the propositus' plasma suggested the presence of abnormal “monomeric” and “dimeric” antithrombins (24). Further characterization studies suggested that these “monomeric” and “dimeric” antithrombins both lacked the ability to inhibit thrombin (25).

We have previously demonstrated that in the case of antithrombin Northwick Park, a similar fast moving abnormal electrophoretic component observed on crossed immunoelectrophoresis comprises an inactive, covalent disulphide-linked variant antithrombin-albumin complex of  $M_r \sim 120,000$  (26). Structural investigation of the isolated variant antithrombin demonstrated a single amino acid substitution of Arg393 to Cys (22). In this communication, we present evidence that antithrombin Milano has the same structural abnormality as antithrombin Northwick Park.

## Materials and Methods

Citrated plasma samples from the propositus and from normal volunteers were subjected to preliminary precipitation with dextran sulphate and applied to heparin-Sepharose (Pharmacia/LKB, England) equilibrated with 0.1 M Tris/HCl at pH 7.4, containing 0.14 M NaCl (27). Antithrombin was eluted from this column with 2.5 M NaCl in 0.1 M Tris/HCl, pH 7.4, after overnight washing with 0.4 M NaCl in 0.1 M Tris/HCl at pH 7.4 (28). Antithrombin was further purified by anion-exchange chromatography, using a monobead column, Mono Q HR 5/5 (Pharmacia/LKB, England) and fast protein liquid chromatography (FPLC) delivery system (Pharmacia/LKB, England) with NaCl gradient elution, as described (29).

SDS-PAGE and immunoblotting from these gels was performed as described previously (22), using rabbit anti-human antithrombin (Dako) and rabbit anti-human albumin (Behring Diagnostics) antisera.

Antithrombin was reduced, S-carboxymethylated and reverse-phase FPLC performed using C8 ProRPC 5 $\mu$  HR 5/5 column (Pharmacia/LKB, England) with FPLC delivery system as described previously (26).

For chemical fragmentation, S-carboxymethylated antithrombin preparations were dissolved in 70% formic acid in the presence of cyanogen bromide (CNBr), at CNBr:protein ratio of 3:1 (w/w) and incubated at room temperature for 17 h. The mixtures were diluted 10-fold with

Correspondence to: Dr. D. A. Lane, Dept. of Haematology, Charing Cross and Westminster Medical School, London W6 8RF, UK

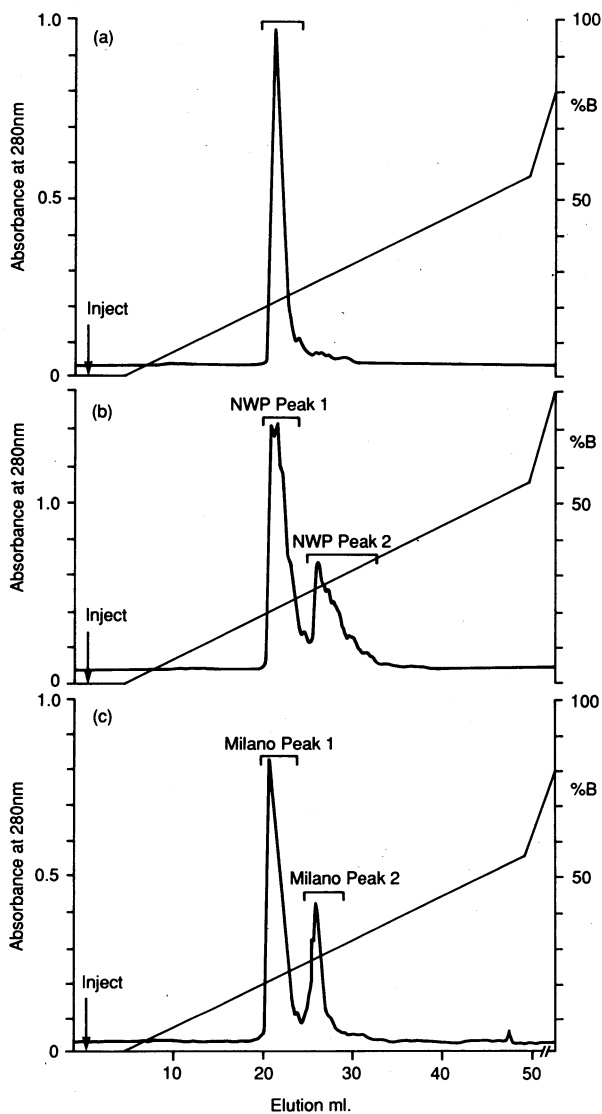
distilled water and injected onto a 15 $\mu$  C18 PepRPC column with FPLC delivery system (Pharmacia/LKB, England) as described (22).

Trypsin digestion and *Staphylococcus aureus* V8 protease digestion were performed as described (22, 26).

Fab-MS was performed using a VG ZAB HF instrument equipped with an M-Scan Fab gun operating at 20  $\mu$ A beam current at 8 keV. Results of molecular mass determinations are expressed as (M+H)<sup>+</sup> (26, 30).

## Results

Normal antithrombin prepared by heparin-Sepharose affinity chromatography eluted as a single peak during anion-exchange



**Fig. 1** Anion-exchange fast protein liquid chromatography of antithrombin. (a) ~4 mg normal antithrombin, (b) ~10 mg antithrombin from propositus with antithrombin Northwick Park, (c) ~4 mg antithrombin from propositus with antithrombin Milano. All preparations had previously been isolated by stepwise elution from heparin-Sepharose with NaCl. Separation by anion-exchange chromatography was achieved by a linear gradient of 0–55% buffer B in 50 ml after initial equilibration with 5 ml of elution buffer A, where A: 20 mM triethanolamine, pH 7.3 and B: 20 mM triethanolamine in 1 M NaCl, pH 7.3. A Mono Q HR 5/5 column with FPLC delivery system (Pharmacia/LKB) was used at a flow rate of 1 ml/min and detection at 280 nm. Pooled fractions of normal antithrombin and antithrombin Milano peaks 1 and 2, antithrombin Northwick Park peaks 1 and 2 (indicated by bars) were dialysed against 0.1 M  $\text{NH}_4\text{HCO}_3$ , pH 8.3 and lyophilised in preparation for further investigations

FPLC (Fig. 1 a). Antithrombin from propositus with antithrombin Milano resolved into two heterogeneous peaks, designated here as Milano peak 1 and Milano peak 2 (Fig. 1c) in order of elution from the column with NaCl gradient. Antithrombin Milano peak 1, but not antithrombin Milano peak 2, exhibited functional activity in heparin cofactor assay (results not shown). Both of these pooled peaks reacted with anti-antithrombin antiserum using immunodiffusion (results not shown). A similar elution profile to that of antithrombin Milano was also obtained with another variant, antithrombin Northwick Park (Fig. 1b), eluted under the same experimental conditions (see also reference 29).

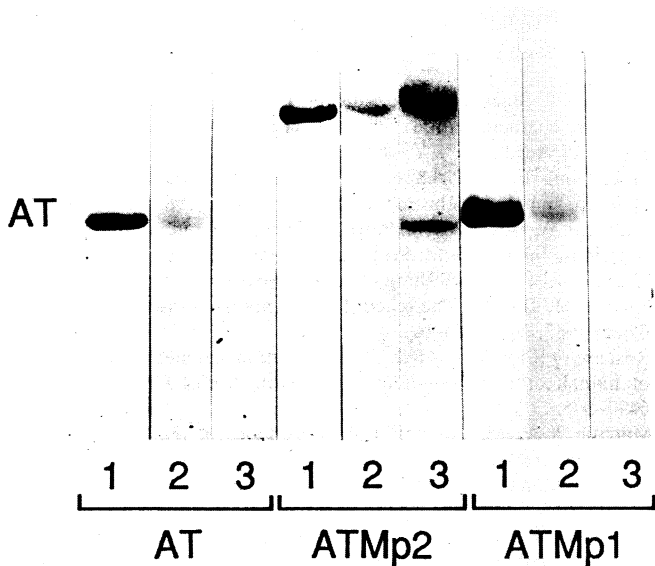
SDS-PAGE in combination with immunoblotting using antisera raised against human antithrombin and albumin was used to study the nature of antithrombin Milano peak 1 and 2. Under nonreducing conditions, antithrombin Milano peak 1 had similar electrophoretic mobility to that of normal antithrombin, migrating as a single protein band with Coomassie staining (Fig. 2), and reacting with anti-antithrombin antiserum and minimally with anti-albumin antiserum (Fig. 2). Antithrombin Milano peak 2 was comprised of a major band that migrated with  $M_r$  exceeding that of normal antithrombin (~120,000 cf ~60,000) on Coomassie staining (Fig. 2). This major band reacted with antisera specific to both antithrombin and albumin (Fig. 2).

The same antithrombin preparations were also studied by SDS-PAGE when electrophoresis was carried out in the presence of a reducing agent, DTT. As expected, reduced, normal antithrombin and Milano peak 1 both migrated as single major bands on Coomassie staining (Fig. 3), with indistinguishable mobility, which reacted only with anti-antithrombin antiserum (Fig. 3). In contrast, reduced antithrombin Milano peak 2 migrated as two closely spaced, nonidentical bands on Coomassie staining (Fig. 3). The most mobile of these bands had a  $M_r$  indistinguishable from that of normal antithrombin (Fig. 3). This band of antithrombin Milano peak 2 reacted only with anti-antithrombin antiserum (Fig. 3), while the higher  $M_r$  component reacted only with anti-albumin antiserum (Fig. 3).

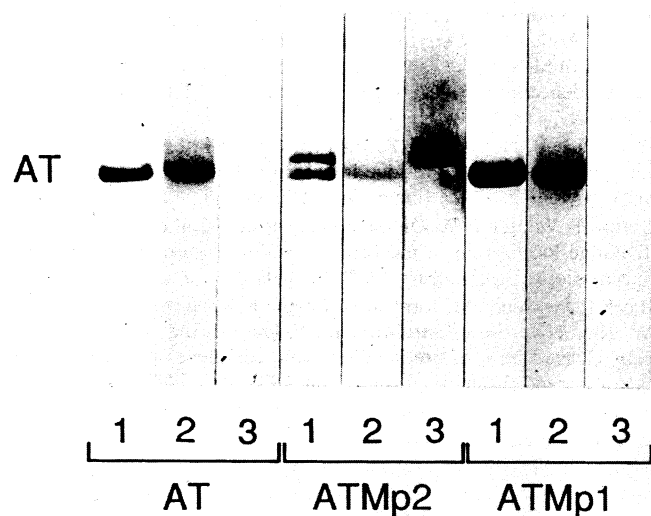
Reduced, S-carboxymethylated Milano peak 2 was subjected to reverse-phase HPLC in order to separate its antithrombin and albumin components. Normal antithrombin produced a single peak on a ProRPC 5/5 column with acetonitrile gradient; antithrombin Milano peak 2 resolved into two major components, designated as Milano peak 2.1 and 2.2 in order of elution from the column (results not illustrated).

Fab-MS of tryptic peptides of Milano peak 2.1 enabled mapping of more than 70% of the primary structure of albumin (results not shown). Taken together with the immunoblotting result above, this conclusively confirmed albumin to be covalently bound to antithrombin Milano in peak 2.

Structural investigation of the isolated component, antithrombin Milano peak 2.2, henceforth denoted as antithrombin Milano, was carried out by chemical fragmentation using CNBr, followed by reverse phase FPLC separation of CNBr peptides, as described previously (22). Each peak and pool was subjected to trypsin digestion and Fab-MS used to assign these peaks and pools to the known primary structure of normal antithrombin. A major pool of CNBr fragment, "pool 4", contained the amino acid sequences of antithrombin, Gly339-Met423, Glu104-Met251 and Val282-Met314, identified on the basis of the molecular masses, (M+H)<sup>+</sup>, of their tryptic peptides. [We have previously carried out one step of Edman degradation followed by Fab-MS on tryptic peptide contained in CNBr pool 4 from normal antithrombin in order to further substantiate the assignments made of these tryptic peptides to the known sequence of antithrombin (22).] The masses, (M+H)<sup>+</sup> 2290 and 700, assigned to normal antithrombin amino acid sequences Ala371-Arg393 and Ser394-Arg399, respectively, could only be detected in small amounts in CNBr pool 4 of



**Fig.2** SDS-PAGE of antithrombin isolated by anion-exchange chromatography, performed under non-reducing conditions. Normal antithrombin (AT), antithrombin Milano peak 2 (ATMp2), and antithrombin Milano peak 1 (ATMp1). SDS-PAGE was performed with 10–15% gels (Pharmacia/LKB). Electrophoresis buffer strips contained 0.2 M Tris, 0.55% SDS, pH 7.5. The gels initially contained 0.112 M acetate, 0.112 M Tris, pH 6.4. Lanes 1 contain gels stained for protein with Coomassie Blue. Lanes 2 contain protein transferred onto nitrocellulose and incubated with anti-antithrombin antiserum. Lanes 3 contain protein transferred onto nitrocellulose and incubated with anti-albumin antiserum. For details of blotting procedure, see (26)

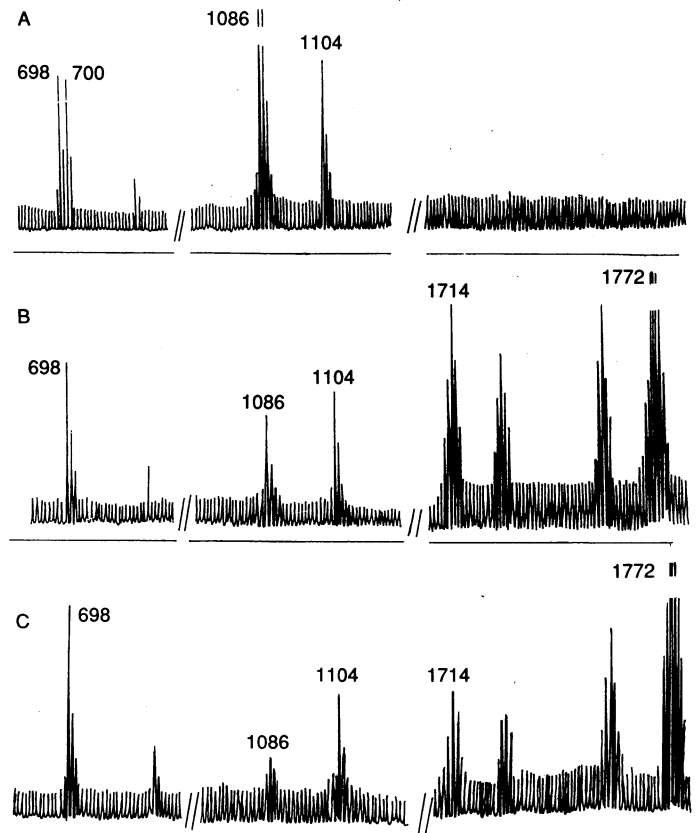


**Fig.3** SDS-PAGE of antithrombin isolated by anion-exchange chromatography, performed under reducing conditions (with DTT). Normal antithrombin (AT), antithrombin Milano peak 2 (ATMp2) and antithrombin Milano peak 1 (ATMp1). SDS-PAGE was performed with 10–15% gels as described in the legend to Fig.2. Lanes 1 contain gels stained for protein with Coomassie Blue. Lanes 2 contain protein transferred onto nitrocellulose and incubated with anti-antithrombin antiserum. Lanes 3 contain protein transferred onto nitrocellulose and incubated with anti-albumin antiserum. For details of the blotting procedure, see (26)

antithrombin Milano. V8 protease subdigestion was performed on the tryptic digest of CNBr pool 4 of antithrombin Milano, and Fab-MS was used to identify new, shortened, additional peptides which resulted from cleavages at glutamic acid residues. In Fig. 4, selected regions of the Fab-MS map of VIII protease subdigests of the tryptic digests of CNBr pool 4 from normal antithrombin (A), antithrombin Northwick Park (B) and antithrombin Milano (C) are illustrated. It is interesting to note the reduced signal for  $(M+H)+1086$  in both variant antithrombins Northwick Park and Milano; this mass signal corresponds to the peptide with amino acid sequence Ala382-Arg393 in normal antithrombin. A novel peptide with  $(M+H)+1772$  previously found in antithrombin Northwick Park peak 2.2 (B), was also identified in the subdigest of antithrombin Milano peak 2.2 (C). This peptide has been shown previously to comprise the amino acid sequence Ala382-Arg399 and to result from a substitution at the reactive site, Arg393 to Cys (22), which prevents enzymatic cleavage of the 393–394 bond.

### Discussion

Antithrombin Milano, a reactive site variant, has been previously described to be present in “monomeric” ( $M_r \sim 60,000$ ) and “dimeric” ( $M_r \sim 120,000$ ) forms (24, 25). In this communication,



**Fig. 4** Comparison of the Fab mass spectra of the V8 protease subdigests of the tryptic digests of CNBr pool 4 from normal antithrombin (A), antithrombin Northwick Park (B), and antithrombin Milano (C). Only regions of the spectra where differences were observed between the three digests are illustrated, i.e., the region corresponding to quasimolecular ions at  $m/z$  700, 1086 and 1772. Unlabelled signals are due to anticipated satellites of  $-15$ ,  $-44$ , and  $+22$ . V8 protease digestion and Fab-MS analysis were conducted as described (26, 30)

we demonstrate that "monomeric" and "dimeric" forms of antithrombin in the propositus' plasma are in fact antithrombin and a covalent, disulphide-linked variant antithrombin-albumin complex, respectively.

Wolf et al., using heparin-Sepharose chromatography (25), prepared two fractions of antithrombin Milano, "Fraction I" and "Fraction II", in which the major components were apparently  $M_r \sim 60,000$  and  $M_r \sim 120,000$  forms of this variant, respectively. We have shown that early and late eluting fractions of antithrombin Northwick Park (from heparin-Sepharose) are enriched with its  $M_r \sim 60,000$  and  $M_r \sim 120,000$  components, respectively (29). In contrast to the results of Wolf et al. (25), we have been unable to isolate a completely inactive  $\sim 60,000$  antithrombin Northwick Park component by heparin-Sepharose chromatography. The finding of an Arg393 to Cys substitution in peak 2 from both variants provides an explanation for the  $\sim 120,000$   $M_r$  components, as a disulphide bridge forms between this Cys and a free Cys of albumin (26). The presence of this substitution also provides an explanation for the lack of reactivity of the  $\sim 120,000$   $M_r$  complex with thrombin, as it would not be expected that the Cys393-Ser394 bond would provide an appropriate substrate for thrombin. The finding of a functionally inactive  $\sim 60,000$  variant component would imply that the variant could circulate in a form in which its substituted Cys residue is not complexed to albumin. To date, we have been unable to identify the Arg393 to Cys substitution in the  $\sim 60,000$  components of either antithrombins Northwick Park or Milano, but that does not exclude the possibility of copurification of small amounts of uncomplexed variant with normal antithrombin.

The results presented here and elsewhere (29) indicate that anion-exchange chromatography is a much more efficient separation method than heparin-Sepharose chromatography of these two antithrombin components of  $M_r \sim 120,000$  and  $M_r \sim 60,000$ . The question remains, however, as to why there is an apparent difference between the  $M_r \sim 60,000$  and  $M_r \sim 120,000$  forms in their affinity towards heparin-Sepharose. In both of these variant antithrombins, the characterisation of the  $M_r \sim 120,000$  component as a variant antithrombin and albumin complex may provide an explanation for its apparently enhanced heparin binding properties. After all, it is this complex which has been shown to be preferentially retained on heparin-Sepharose. Albumin has been attributed weak heparin binding properties (31) and the covalent complex may undergo a cooperative binding interaction with heparin-Sepharose such that the affinity of the complex exceeds that of the individual proteins. An alternative explanation may be that substitution by the cysteine residue in both variant antithrombins may cause a conformational change, exposing hidden basic amino acids and thus facilitating their heparin binding.

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