Physiological variant of antithrombin-III lacks carbohydrate sidechain at Asn 135

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Both normal antithrombin-III (AT-IIIα) and the high heparin affinity form (AT-IIIβ) were isolated from pooled human plasma. AT-IIIβ had a lower negative charge and lower molecular mass than AT-IIIα. Sialidase and endo-F digestion indicated that the inherent difference resided in the oligosaccharide component of the molecule. CNBr fragmentation showed there was an oligosaccharide sidechain missing between residues 104 and 251, subdigestion with trypsin indicated that Asn 135 was not glycosylated in AT-IIIβ. Chromatography of total tryptic digests on concanavalin A-Sepharose confirmed that the high heparin affinity form of antithrombin lacked an oligosaccharide moiety at Asn 135.

Antithrombin-III; Physiological variant; Carbohydrate attachment; Asparagine; (Human)

1. INTRODUCTION

Antithrombin-III (AT-III, molecular mass 58 kDa) is the prime plasma inhibitor of the serine proteases involved in the final steps of the coagulation pathway. Its rate of inhibition of thrombin and factor Xa are greatly increased through interaction with heparin [1,2]. Two forms of antithrombin-III (α and β) exist in normal plasma [3], the predominant species, AT-IIIα, has four biantennary oligosaccharide sidechains attached at asparagines 96, 135, 155 and 172 [4]. The minor species, AT-IIIβ, is present as 5–10% of the total antithrombin [3]. It has a lower apparent molecular mass and higher heparin affinity than AT-IIIα and, in addition, contains a lesser proportion

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Abbreviations: AT-III, antithrombin-III; endo-F, endo-β-N-acetyl glucosaminidase F; CMn, carboxamidomethyl

of constituent monosaccharide units [3].

We show here that AT-IIIβ has an apparently normal primary sequence, but lacks an oligosaccharide sidechain at asparagine 135.

2. MATERIALS AND METHODS

2.1. Materials

Sialidase Type VI *(C. perfringens)* was obtained from Sigma. Endo-β-N-acetylglucosaminidase-F (endo-F, *F. meningosepticum*) was obtained from New England Nuclear.

2.2. Fractionation of antithrombin

AT-IIIβ was isolated from an unused trailing fraction in the commercial production of AT-III on heparin-Agarose during elution with 2 M NaCl [5,6]. This material, enriched in high heparin affinity antithrombin, was re-chromatographed on a 2.5×30 cm column of heparin-Sepharose and eluted with a linear gradient of 0.4 to 1.5 M NaCl in 20 mM Tris-HCl, pH 7.5 [7], to give two fractions containing AT-IIIα and AT-IIIβ, respectively.

2.3. Isolation of CNBr fragments

Twelve mg of each antithrombin species was carboxyamidomethylated [8] and cleaved with CNBr [9]. Digests were redissolved in 0.8 ml 6 M urea/2% SDS and applied to separate preparative SDS polyacrylamide slab gels (12.5%). Bands were visualised by staining with 0.25 M KCl [10] and were eluted by diffusion in water. Peptides were recovered by precipitation with four volumes of acetone ($-20°C$).

2.4. Peptide mapping

Tryptic digestion was carried out as described by Brennan [11] and two-dimensional peptide mapping was performed on Whatman no. 1 paper with electrophoresis at pH 6.5 and chromatography in the upper phase of pyridine/isoamyl alcohol/water (6:6:7) [12]. Maps were stained with 0.002% fluorescamide. Mobilities and molecular masses were calculated as described by Offord [13].

2.5. Glycopeptide isolation

Eight mg of carboxyamidomethylated anti-antithrombin was digested with trypsin. Digests were chromatographed on a 1.5×9 cm column of concanavalin A-Sepharose in 20 mM NH_4HCO_3, 20 μM with respect to Mg^{2+}, Ca^{2+} and Mn^{2+}. Unbound peptides were collected directly in this buffer while bound peptides were eluted with 25 mM methyl-α-D-glucoside/20 mM NH_4HCO_3 and collected in a minimal volume.

2.6. Structural studies

Peptides were hydrolysed ($110°C$ for 16 h) under vacuum, using vapour from 6 M HCl/1% phenol [14]. Hydrolysates were coupled with phenylisothiocyanate and the phenylthiocarbamyl derivatives of the amino acids were quantified by reverse-phase HPLC using a Waters Nova pac column [12]. Peptides were sequenced by a manual micro Edman technique [12,15]. PTH amino acids were identified by reverse-phase chromatography on a Waters Nova pac column [12].

3. RESULTS

3.1. Preliminary characterisation

AT-IIIα was eluted from the heparin-Sepharose column at 0.8 M NaCl. AT-IIIβ had a higher heparin affinity eluting at 1.2 M NaCl, it also had a lower net negative charge as demonstrated by its electrophoretic mobility (fig.1, lanes 2 and 3). Incubation with sialidase abolished the charge difference between the two forms of antithrombin (fig.1) suggesting that their inherent difference resided in their carbohydrate structure rather than in their amino acid sequence. AT-IIIβ had a lower apparent molecular mass than AT-IIIα (fig.2, lanes 2 and 1). As expected the molecular mass of both proteins decreased on sialidase treatment, but the molecular mass difference between them was retained (fig.2).

On incubation with endo-F which cleaves the oligosaccharide component to leave a single asparagine-linked N-acetylglucosamine residue, most (70%) of the AT-IIIα was converted to a band of similar molecular mass to native AT-IIIβ, with apparently one carbohydrate sidechain missing (fig.2, lane 7). AT-IIIβ, on the other hand, was comparatively unaffected by endo-F. Both antithrombins showed additional minor lower M_r bands, the result of more extensive removal of carbohydrate sidechains (fig.2, lanes 7 and 8).

3.2. Mapping of CNBr fragments

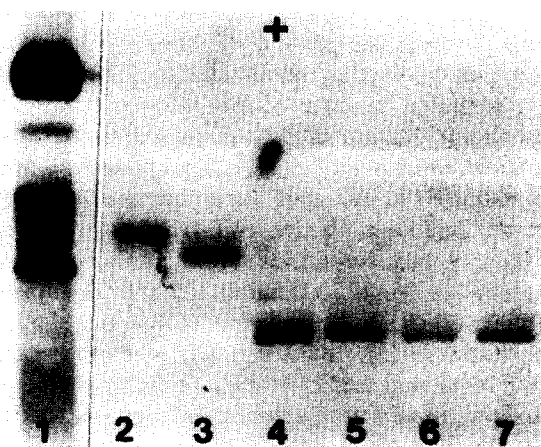CNBr digests of AT-IIIα showed the expected

Fig.1. Agarose gel electrophoresis pH 8.6 showing effect of sialidase on AT-III fractions. (1) Plasma control. (2) Purified AT-IIIα. (3) Purified AT-IIIβ. (4 and 5) AT-IIIα and AT-IIIβ, respectively, after 2 h incubation with neuraminidase (6 and 7). Antithrombins α and β, respectively, after 20 h incubation. Antithrombin (1 mg/ml) was incubated (20°C) with 5% w/w sialidase in 50 mM acetate buffer pH 5.5/5 mM $CaCl_2$.
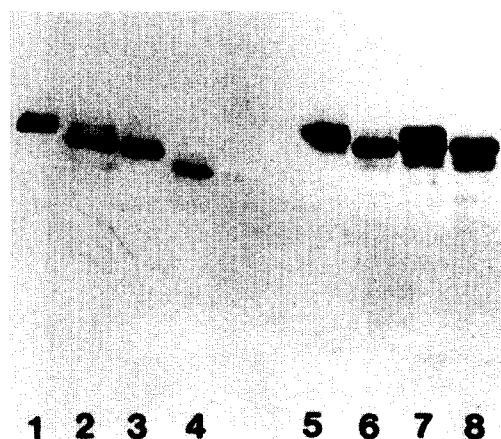
Fig.2. SDS-polyacrylamide gel (10%) electrophoresis showing effects of sialidase and endo-F (1 and 3). AT-IIIα before and after 2 h incubation with sialidase (2 and 4). AT-IIIβ before and after sialidase treatment (5 and 7). AT-IIIα before and after endo-F treatment (6 and 8). AT-IIIβ before and after endo-F treatment. Two μl of endo-F was added to 0.1 mg of antithrombin in 100 μl of 0.1 M phosphate buffer pH 6.1/50 mM EDTA/1% NP-40/0.1% SDS and incubated for 24 h at 20°C.

large 25 kDa fragment. This was missing from digests of AT-IIIβ and replaced by a smaller 22 500 kDa peptide (fig.3, lanes 4 and 5). These peptides, designated CNα 25 kDa and CNB 22.5 kDa, respectively, were purified by preparative SDS acrylamide gel electrophoresis (fig.3, lanes 8 and 9). Amino acid analysis showed that they had essentially the same composition and that they corresponded to the expected CNBr peptide encompassing residues 104–251. This was substantiated when both peptides were found to have the N-terminal sequence of Glu-Val-Phe-Lys-Phe-Asp-. The C-terminal tryptic peptide (below) of CNβ 22.5 kDa was also normal, with a composition of Ala, Asp, Gly, Glu, Ser, Cys, Ser, Ala, Ser, homoserine. This region of the molecule (104–251) contains three of the four expected oligosaccharide sidechains and the molecular mass difference between the peptides (2500) is accountable for by the loss of just one of these sidechains.

When peptides CNα 25 kDa and CNβ 22.5 kDa were digested with trypsin no significant differences were discernible on reverse phase HPLC mapping [11]. Two-dimensional mapping, however, showed that CNα 25 kDa contained a single
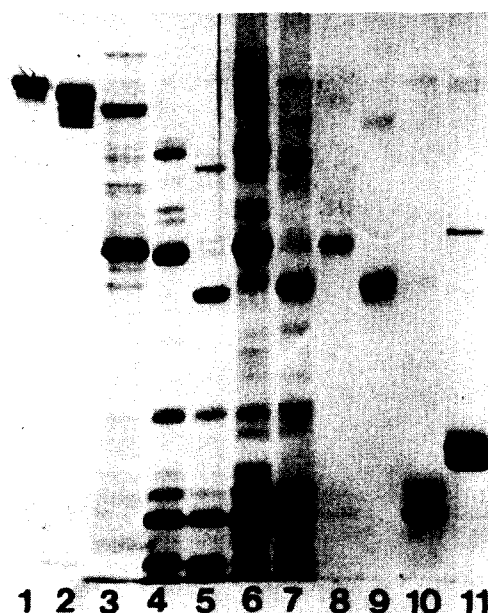

Fig.3. SDS-polyacrylamide gradient gel (10–20%) of CNBr fragments. (1) Native AT-IIIα. (2) Native AT-IIIβ. (3) Marker, partial acid hydrolysis of carboxyamidomethylated (CMn) human albumin, major bands at 25, 48 and 68 kDa. (4) CNBr digest of CMn AT-IIIα. (5) CNBr digest of CMn AT-IIIβ. (6 and 7) Acetone precipitates of 4 and 5, respectively. (8) Purified peptide CNα 25 kDa. (9) Purified abnormal peptide CNβ 22.5 kDa. (10) Purified CNBr peptide corresponding to antithrombin residues 339–423. (11) Red cell lysate containing markers of carbonic anhydrase (29 kDa) and α- (15.5 kDa) and β-globin (16 kDa).

neutral peptide (Trα0) that was missing from digests of CNβ 22.5 kDa (fig.4). PTH analysis of Trα0 gave a sequence of Lys-Ala-Asn*-Lys and established that it was the expected glycopeptide originating from cleavage at Arg 132 and Lys 136. Peptide Trα0 is confirmed as a glycopeptide by its observed neutral electrophoretic mobility, the negative charges of the two sialic acid residues being balanced by the positive charges on its two lysines. Fig.4 shows that this peptide is replaced by two basic peptides (Trβ1 and Trβ2) in digests of CNβ 22.5 kDa. Peptide Trβ1 had a composition of Lys, Ala, Asn, Lys and a pH 6.5 mobility of 0.85. This is in keeping with a predicted mobility of 0.84 for a peptide with a residue weight of 459 and a charge of +2 [13]. Peptide Trβ2 had a composi-

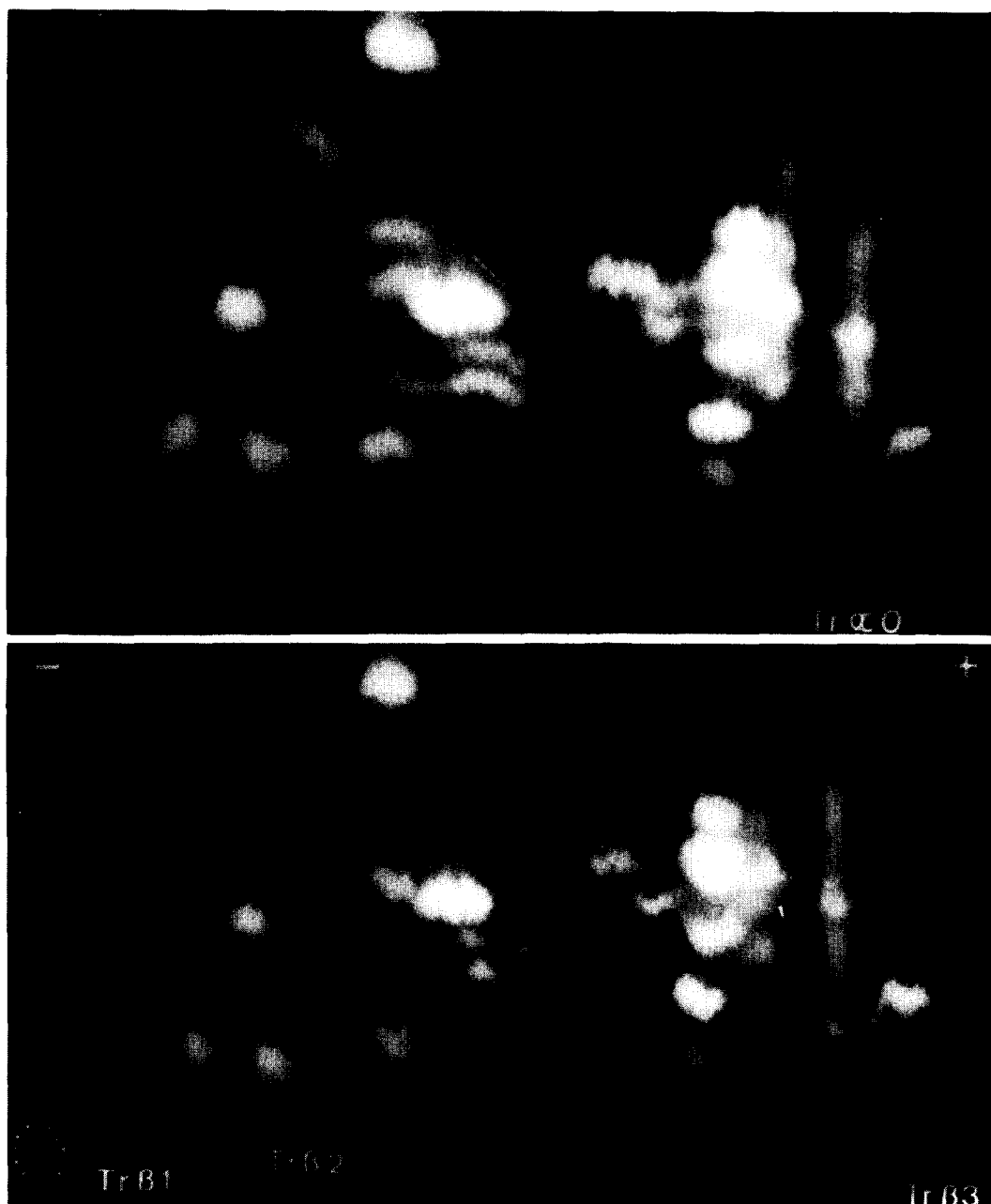433

Fig.4. Tryptic peptide maps of purified CNBr peptides. CNα 25 kDa (upper map) contains a neutral peptide Trα0, which is missing in digests of CNβ 22.5 kDa (lower map) and replaced by two new basic peptides Trβ1 and Trβ2. Electrophoresis pH 6.5.

tion of Ala, Asn, Lys and a predicted mobility of 0.50, the mobility of such a peptide (residue weight 331, charge + 1) being 0.54.

Amino acid analysis was performed on all major

tryptic peptides from CNβ 22.5 kDa and each corresponded to tryptic peptides expected to occur between methionines 103 and 251. In particular, peptides on the N- and C-terminal sides of Trβ1
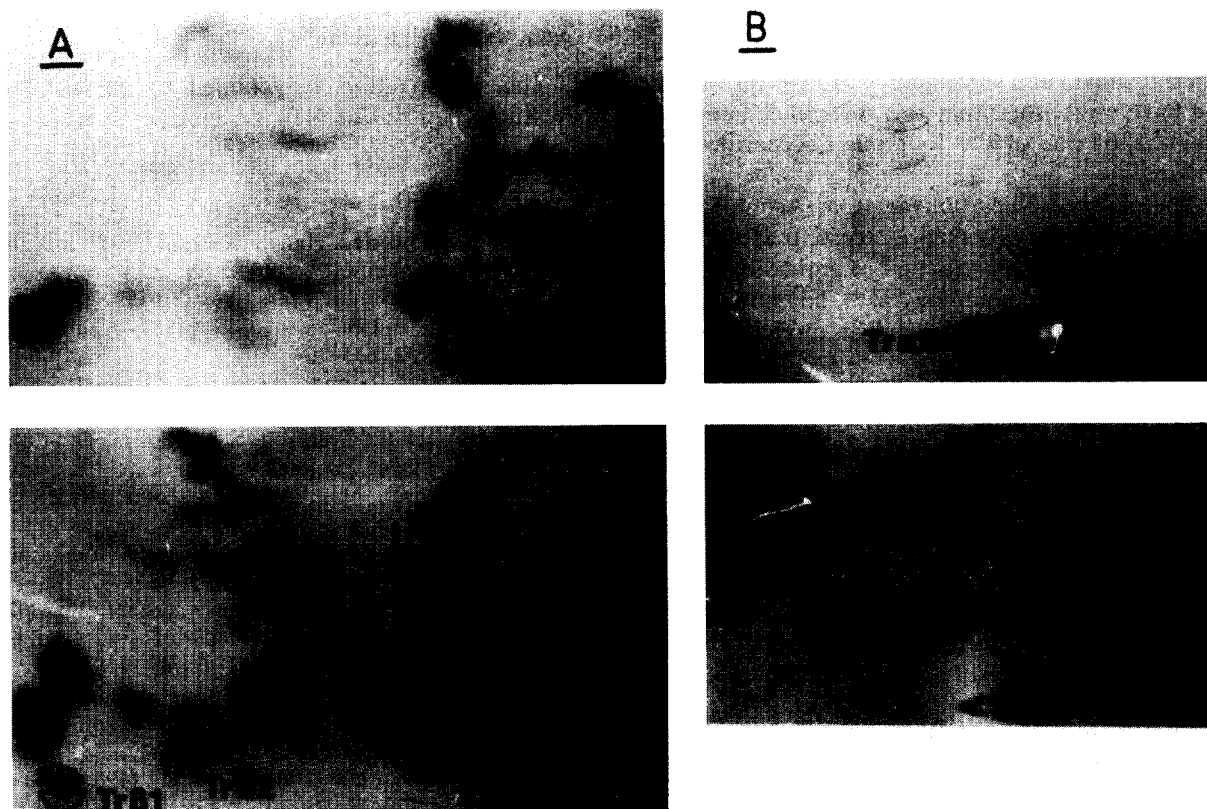
Fig.5. Tryptic peptide maps of peptides isolated by chromatography on concanaval in A-Sepharose. (A) Unbound fraction from AT-IIIα (upper) and AT-IIIβ (lower). (B) Bound peptides from AT-IIIα (upper) and AT-IIIβ (lower). Cathode at left.

were normal, with compositions of Leu, Tyr, Arg and Ser, Ser, Lys, respectively. Peptide Trβ3 was in the same position in both maps (fig.3) and its composition matched that of the expected tryptic glycopeptide, residues 151-169. Overstaining for tryptophan located the third glycopeptide, though it was not possible to analyse this peptide, it occupied the same position in both cases.

3.3. Isolation of glycopeptides

Tryptic digests of carboxyaminomethylated AT-IIIα and AT-IIIβ were chromatographed on concanavalin A-Sepharose. In each case, the unbound peptides were collected and mapped (fig.5A). In the case of AT-IIIβ, the two new basic peptides Trβ1, and Trβ2 were observed and, as before, these had compositions of Lys, Ala, Asn, Lys and Ala, Asn, Lys, respectively. Their electrophoretic mobilities and direct elution from concanavalin A

confirmed that they were not glycosylated. The glycopeptide fraction that bound to the concanavalin A was eluted with methyl α-glucoside. Peptide maps of this fraction (fig.5B) indicated that AT-IIIα possessed a single neutral glycopeptide Trα0 which was missing in the case of AT-IIIβ. The sequence of Trα0 was again established as Lys-Ala-Asn*-Lys.

This, therefore, confirms the earlier data and establishes that the differences between AT-IIIα and AT-IIIβ are due to the lack of an oligosaccharide sidechain on asparagine 135 in AT-IIIβ.

4. DISCUSSION

The initial observation in this investigation was that a minor component (some 5%) of circulating antithrombin possessed a higher heparin affinity. Peterson and Blackburn [3] showed that this, AT-

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IIIβ, had a lower molecular mass than AT-IIIα and that it contained 25–30% less hexosamine, neutral sugars and sialic acid. In this investigation we have established that this difference is due to the lack of one of the four oligosaccharide side-chains.

The absence of carbohydrate at Asn 135 is in keeping with the observed increase in affinity for heparin. The tertiary structure of antithrombin can be modelled from homology with the known structure of α_1-antitrypsin [16,17]. This model shows an area of positive charge formed by Lys 125, Arg 129 and Arg 132 on the D helix and Arg 47 on the A helix. This last residue is known to be essential for heparin binding [17,18], and the grouping of basic residues almost certainly forms an anionic site for the binding of the polysulphonated heparin molecule. The oligosaccharide at Asn 135 is immediately distal to the D helix in a site where its bulk presumably limits access to the adjacent heparin binding site. Thus absence of the oligosaccharide in antithrombin β results in a predictable increase in heparin affinity.

Plasminogen is another plasma glycoprotein that displays variation in its oligosaccharide content, with a concomitant alteration in its binding to an affinity resin (Sepharose-lysine) [19]. The high affinity form of plasminogen lacks a complex oligosaccharide at asparagine residue 238. The favoured explanation for its absence is that the oligosaccharide precursor was not transferred from the dolichol precursor to the plasminogen during synthesis, rather than removal of the oligosaccharide in the circulatory system. It is not clear which of these explanations applies to the AT-IIIβ; however, treatment of AT-IIIα with endo-F shows that AT-IIIα has one oligosaccharide that is readily removed. AT-IIIβ, on the other hand, is comparatively unaffected by this enzyme, the implication being that antithrombin possesses one labile oligosaccharide which could potentially be removed in circulation.

The physiological significance of the circulatory removal (or failure of attachment) of the oligosaccharide is not clear, but potentially, it provides another level of control on coagulation by altering the avidity of antithrombin for thrombin when heparin activation is suboptimal.

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