Aggregation of plasma Z type α_1-antitrypsin suggests basic defect for the deficiency

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The abnormal type of α_1-antitrypsin, PI (protease inhibitor) type Z, is associated with inclusion bodies in the liver, which contain non-secreted α_1-antitrypsin. Our studies show that Z protein has an inherent tendency to aggregate, even in plasma. Depending upon conditions, from 15 to 70% of the Z protein in plasma was in a high-M_r form, compared with 1.5% of M type α_1-antitrypsin. The high-M_r complex in plasma cannot be disaggregated using Triton X detergent or reducing conditions. This increased tendency to aggregate can be explained by the mutation affecting, tertiary structure and salt bridge formation in Z protein. We have observed this same tendency to aggregate for Mmalton α_1-antitrypsin, a rarer variant also associated with a plasma deficiency.

α_1-Antitrypsin deficiency *α_1-Antitrypsin aggregation* *Secretion defect*

1. INTRODUCTION

α_1-Antitrypsin (α1AT), or α-protease inhibitor, is a major plasma inhibitor of leukocyte and bacterial proteases, particularly elastase. A deficiency (usually PI type ZZ) occurring in about one in 2000–7700 Caucasians leads to an increased risk for chronic obstructive pulmonary disease in early adult life [1] or liver disease in early infancy [2]. A characteristic feature of the α1AT produced by the Z allele is lack of secretion from the liver, resulting in the formation of characteristic PAS-positive inclusion bodies [3] which do not readily dissociate [4,5]. The reason for this lack of secretion has not previously been explained. Two other types of α1AT deficiency alleles, PI Mmalton and PI Mduarte, occur at about 1/100 of the frequency of the PI Z allele [6], and similarly result in lack of secretion of α1AT from the liver [7,8]. We report here our studies which demonstrate that the plasma form of both the Z and Mmalton types of α1AT shows an unusual tendency to form high-M_r aggregates. This phenomenon can explain the liver retention of α1AT and the resulting plasma deficiency.

2. MATERIALS AND METHODS

PI (protease inhibitor) types in plasma or sera were identified by isoelectric focusing in polyacrylamide gels followed by immunofixation [9]. Samples from 5 PI type Z and 4 PI type M individuals were used. α1AT was partially purified from delipidated plasma or serum by binding to a thiol-Sepharose 4B column activated by 5′,5′dithiobis(2-nitrobenzoic acid) (DTNB) [10]. A single-step partial purification was used to maintain the α1AT in as natural a state as possible; some purification procedures could remove certain forms of α1AT. α1AT was selectively eluted as DTNB 1AT disulphide [10], from which DTNB was removed with 30 mM cysteine-HCl [11]. Monitoring of fractions was carried out by agarose electrophoresis [12]. Quantification of α1AT and other protein markers was carried out by electroimmuno assay [13]. Recovery after the thiol col-

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umn was 80–90%.

The M_r distribution of the thiol-purified α1AT, was assessed by Ultragel AcA 44 column chromatography (LKB, Broma) using one of several buffers (table 1). The effect of protein (albumin) on the elution pattern was assessed by removal of albumin from serum using a Blue Sepharose CL6B column [14] prior to thiol chromatography.

The molecular size of the high-M_r complex was determined on a Sephacryl S-300 column. Attempts were made to interconvert high- and low-M_r peaks, by changes in buffer ionic strengths, detergents and reduction, as outlined in section 3. Tests for the presence of IgA and other proteins in the high-M_r complex were carried out using cross-immunoelectrophoresis in agarose [15], and SDS-acrylamide gel electrophoresis [16].

Table 1

Percent of total α1AT in each peak collected from Ultragel column

type	Buffer[a]	Percent of total α1AT	
		Peak 1	Peak 2
M	A	2.5	97.5
	C	3.0[b]	97.0[b]
	D	1.5, 2.1[c]	98.5, 97.9[c]
	E	2.0	98.0
Z	A	32.6, 36.4	67.4, 63.6
	B	56.4	43.6
	C	18.7[b], 14.9[b]	81.3[b], 85.1[b]
	D	69.1, 23.1[c]	30.9, 76.9[c]
	E	64.2	35.8
Mmalton	D	55.9	44.1

[a] A: 0.1 M Tris-HCl, 1 M NaCl (pH 7.4); B: 0.1 M Tris-HCl, 0.3 M NaCl (pH 8.1); C: 0.05 M Tris-HCl, 0.05 M NaCl (pH 8.1); D: 0.05 M Tris-HCl, 0.05 M NaCl (pH 7.4); E: 0.05 M K_2HPO_4, 0.05 M KH_2PO_4, 0.008 M NaOH (pH 6.9)

[b] Albumin removed

[c] No thiol column

Replicate results are for samples from different individuals, except for Z buffer C. For Z: C, D (thiol) and E are from one individual

3. RESULTS

Marked differences in molecular size distribution by Ultragel chromatography were noted between PI types. PI Z and PI Mmalton eluted in two major peaks, shown as peaks I and II in fig.1. α1AT was quantified in each pooled peak. The α1AT in peak I, for PI Z and Mmalton α1AT, ranged from 15 to 70% of the total α1AT recovered (table 1). In marked contrast, only 1.5–3% of normal PI M α1AT eluted in peak I under identical conditions. With buffer A and 1 M NaCl, a mean of 34% of PI Z α1AT appeared in peak I, whereas with buffers B and D (with lower salt concentrations) 56 and 69%, respectively, of

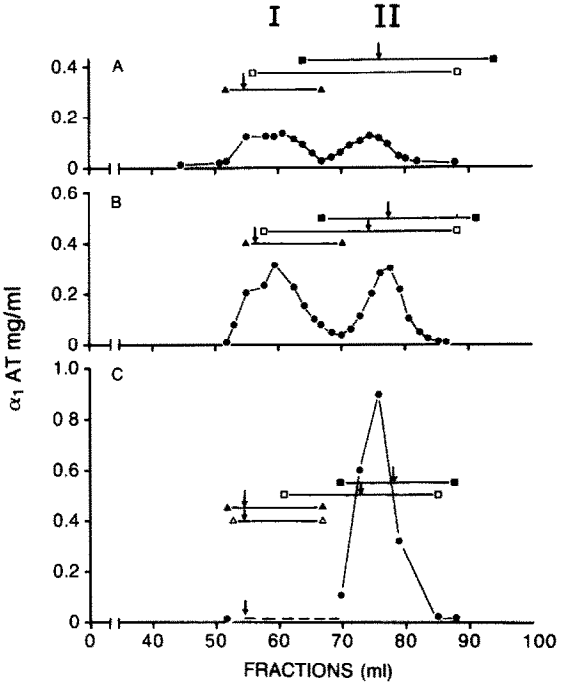

Fig.1. Gel filtration of thiol-purified α1AT PI type Mmalton (A), Z (B) and M (C) using 140 ml Ultragel AcA 44 column (90 × 1.75 cm), buffer D (table 1). Approx. 1.3 ml of sample was applied at a flow rate of 0.6 ml/min. 1.5 ml fractions were collected and quantitated for IgA (△), α_2-macroglobulin (▲), albumin (□), prealbumin (■) and α1AT (●). These plasma proteins were used as molecular mass markers: 160, 725, 66.3, 55 and 54 kDa, respectively. Fractions of α1AT eluted in two peaks, each of which were pooled (peaks I and II) and concentrated. Arrows indicate peaks and dashed line in C α1AT of <3 μg/ml.

PI Z α1AT was in peak I. Particularly noteworthy is the 64% of α1AT eluted in peak I with buffer E, chosen to represent as closely as possible the intracellular liver ion concentrations and pH.

Total protein (specifically albumin) appeared to influence the elution pattern of α1AT. When albumin was removed from one PI M and one PI Z serum (Blue Sepharose CL6B column), about 17% of total PI Z eluted into peak I, compared with 69% when albumin was not removed. Preferential removal of peak I on the Blue Sepharose column could not alone account for this difference: recoveries were 91 and 80%, respectively, for M and Z α1AT. The thiol purification of the plasma alone could not be responsible for the increased proportion of PI Z α1AT found in peak I. When defibrinated plasma was applied to the Ultragel column directly, we observed 23% of total Z type α1AT in peak I. The high-M_r component is apparently present even in circulating plasma. The thiol column may enhance the tendency to aggregate, or preferentially remove nonaggregated α1AT.

The aggregated Z α1AT in peak I showed a broad range of apparent M_r, from greater than that of α_2-macroglobulin (M_r 750000) to less than that of albumin (M_r 66000) (Sephacryl S300 column).

We were unable to demonstrate conversion of peak II (low-M_r) from types Z and Mmalton α1AT to peak I under several conditions. Peak II of M and Z α1AT was rechromatographed using buffer E and a higher ionic strength buffer (0.6 M K_2HPO_4, 0.6 M KH_2PO_4, pH 6.75). An increase in the amount of total protein load was also unsuccessful for converting Z α1AT peak II: when bovine serum albumin was added to peak II (in each of buffers C and E) to restore the protein concentration of the original plasma, no conversion to peak I was observed.

The high-M_r peak I of Z and Mmalton α1AT, once formed, was resistant to conversion into the lower-M_r component under a number of conditions. Peak I from PI Z plasma (in buffer B) was dialyzed and reapplied to the Ultragel column in a buffer of lower ionic strength (buffer C), with no resulting conversion of peak I to peak II. Peak I of each of Z and Mmalton α1AT was applied to the Ultragel column (in buffer D) following reduction with 5 mM dithiothreitol (DTT) and 10 mM iodoacetate with no effect on the fractionation pattern. Finally, an attempt was made to dissociate the aggregates by treatment with detergent (1% Triton X-100 overnight at 4°C) followed by Ultragel chromatography in buffer D with 1% Triton X-100, with no conversion of peak I to the lower-M_r form.

α1AT has previously been shown to bind to IgA [17]. IgA was not involved in the formation of the high-M_r component. There was no consistent relation between the IgA and α1AT concentrations in samples applied to and eluted from the Ultragel column and the resulting amount of complex found in peak I, suggesting that only a small fraction of IgA can bind α1AT, no matter how much α1AT is present. This was verified by electrophoresis of peak I of each of M and Z α1AT in agarose followed by crossed electrophoresis into anti-IgA or anti-1AT antibody (fig.2). A cathodal shoulder, seen with α1AT and migrating in the same position as IgA, was lost after reduction by DTT (fig.3) but this complex accounted for only a portion of peak I. A mixing experiment using peak I (M α1AT) and peak II (Z α1AT) was undertaken to determine whether IgA or other high-M_r proteins in peak I would bind to α1AT in peak II. There was no cathodal shoulder with crossed electrophoresis suggestive of binding to IgA, indicating that IgA present in peak I (M α1AT) was unable to bind further α1AT. The presence of other high-M_r proteins that bind to or cause aggregation of α1AT is unlikely: the major portion of α1AT in peak I migrated in the same position as nonaggregated α1AT (fig.3). Furthermore, SDS-acrylamide gel electrophoresis, which solubilizes aggregated α1AT from liver [5], converted the complexed α1AT to a component of $M_r \sim 55000$, with a small component of $M_r > 100000$ (as expected for IgA). There was no evidence of another major component in the complex. The aggregated form was not due to complexing of α1AT with the leukocyte protein elastase, as tested by Ouchterlony immunodiffusion using antileukocyte elastase antibody (kindly provided by Dr James Travis).

4. DISCUSSION

Our results have shown that Z α1AT in plasma, like that in liver, shows a pronounced tendency to aggregate into a high-M_r form, while PI M protein

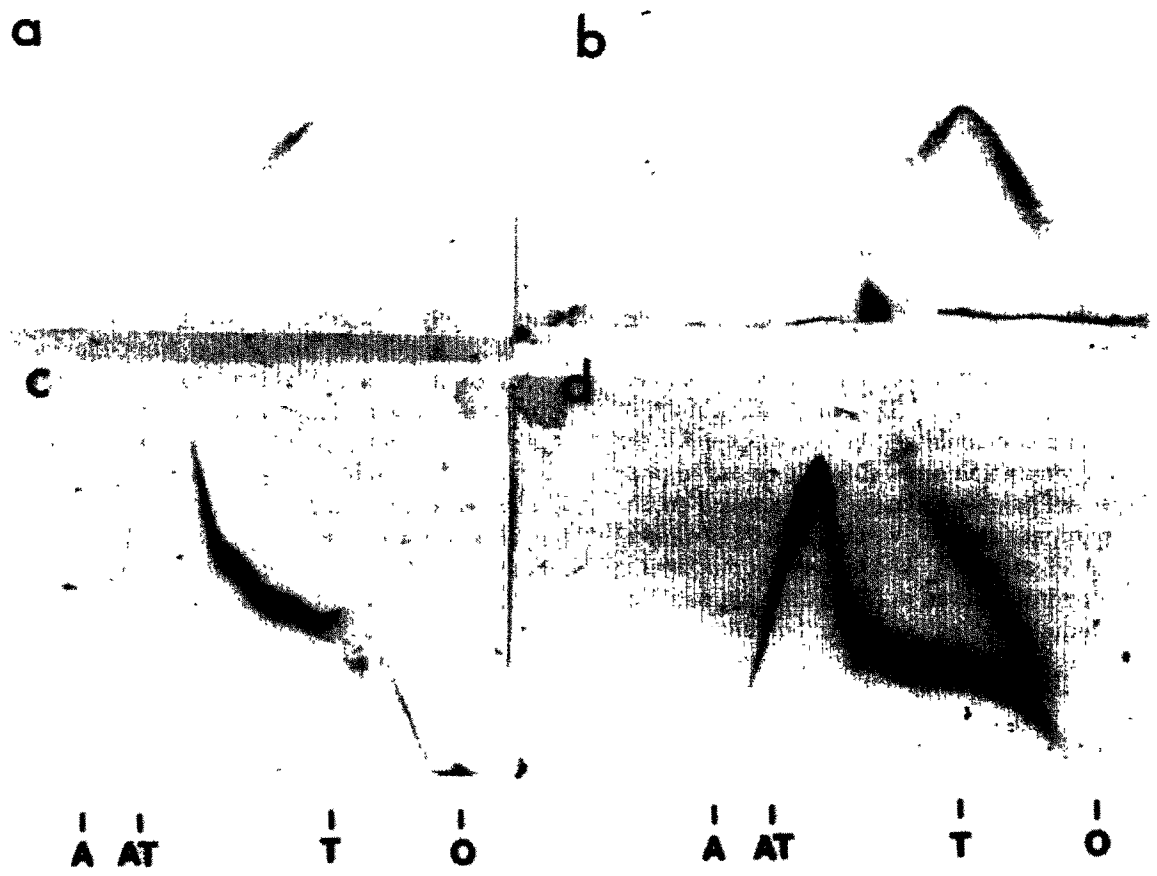

Fig.2. Crossed immunoelectrophoretic analysis of peak I, PI Z (b,d) and PI M (a,c) with anti-IgA (a,b) and anti-α1AT (c,d). Anode to the left in first dimension and to the top in the second dimension. Symbols indicate the mobility of plasma proteins: albumin (A), α1AT (AT), transferrin (T) and application (O).

does not. The amount of complex formed by Z α1AT appears to be influenced by phosphate ion, pH, salt concentration and total protein concentration. Aggregation occurs particularly, to a maximum of about 64%, in the presence of pH and salt concentrations typical of the hepatic intracellular fluid. This same tendency to aggregate is also observed for PI Mmalton. The high-M_r complex consists of a large range of M_r values, suggesting aggregates of from two to many α1AT molecules, which cannot be disrupted by a reducing agent or by treatment with Triton X-100 detergent, but can be dissociated by boiling in SDS. Apparently disulphide bonds are not involved in the complex formation and the bonds must be extremely strong.

The PI Z deficiency variant results in a plasma concentration of about 10–15% of that found for the normal PI M. According to the studies of the half-life of M and Z α1AT, the decrease is not due to increased removal from the plasma [18] and could therefore be due to decreased synthesis or decreased secretion. Synthesis of M and Z α1AT, in an in vitro system, is identical [19], pointing to a defect in secretion. The Z α1AT accumulating in the hepatocyte, in both rough and smooth endoplasmic reticulum [20] has a carbohydrate composition indicating lack of removal of the high-mannose core and lack of addition of the final oligosaccharide side chains [21]. However, incomplete processing appears to be secondary to the basic defect. Studies of microinjection of PI M and

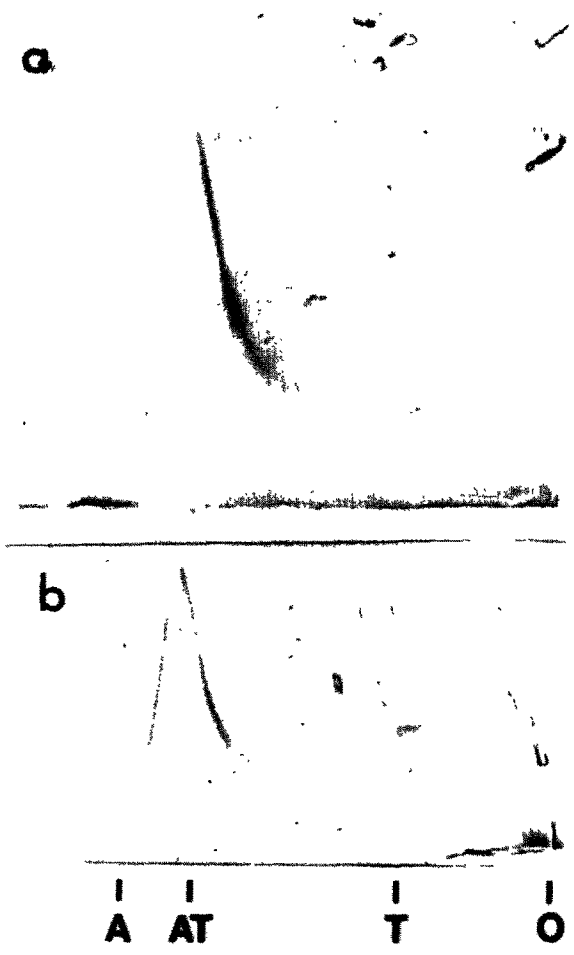

Fig.3. Crossed immunoelectrophoresis of peak I, PI M with anti-α1AT. Peak I (b) was treated with 5 mM DTT and 10 mM iodoacetate (a) for 30 min at room temperature.

Z mRNA into *Xenopus* oocytes demonstrated lack of secretion of the abnormal Z protein with accumulation of an intracellular form [22]. Studies reported here indicate that this lack of secretion is due to formation of very high-M_r aggregates. Glutamic acid residue 342 in the normal protein has been replaced by a lysine in the Z protein [23]. The three-dimensional structure of α1AT obtained from crystal analysis indicates that this substitution disrupts a salt bridge important for stabilization of the molecule and decreases the rate of folding. Our data suggest that the α1AT produced immediately begins to form aggregates of increasing size in the liver, because of the lack of the stabilization of the molecule by the salt bridge.

We have used the fully glycosylated plasma form of α1AT, rather than the incompletely processed form which would be present in the rough endoplasmic reticulum in the liver. However, in the studies of production of α1AT in oocytes [24], in the presence of tunicamycin, the nonglycosylated form of M α1AT was secreted normally into the medium, but the nonglycosylated Z protein was not. The tendency of α1AT to aggregate therefore is demonstrated with or without the presence of oligosaccharide chains on the molecule. The inherent tendency to marked aggregation of α1AT is, we propose, sufficient to produce the typical liver inclusions and subsequent plasma deficiency of PI Z or PI Mmalton α1AT.

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