Structural and functional characterization of the abnormal Z α_1-antitrypsin isolated from human liver

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α_1-Antitrypsin has been isolated from liver inclusion bodies of a subject with a homozygous Z deficiency. The inhibitor was recovered in a fully active form by extraction in high salt at either pH 2.0 or pH 8.0. Carbohydrate analysis indicated a protein in the 'high mannose' form, and this was collaborated by its sensitivity to endo-βN-glucosaminidase. These data suggest that the abnormal α_1-antitrypsin is blocked in the secretory pathway prior to its entrance into the Golgi, and that this blockage is not due to a gross misfolding of the polypeptide.

α_1-Antitrypsin *Human liver* *Inclusion body* *High mannose glycoprotein* *Proteinase inhibitor*

1. INTRODUCTION

α_1-Antitrypsin (also known as α_1-proteinase inhibitor) is the major proteinase inhibitor in human plasma. It inhibits serine proteinases, including pancreatic elastase, trypsin, and chymotrypsin, as well as leukocyte proteinases. It is a glycoprotein with a molecular mass 52 kDa whose structure, function and abnormalities have been recently reviewed [1]. A deficiency of this inhibitor commonly occurs in Europeans due to the presence of the Z variant (342 Glu⟶Lys). The homozygous state (ZZ) for this variant results in a plasma concentration of 15% of the normal MM genotype. This homozygous Z deficiency is associated with the development of pulmonary emphysema and hepatic cirrhosis [2]. Individuals of this phenotype, as well as some who carry a single Z allele, have hepatic inclusions of PAS-positive, diastase-resistant globules which react with α_1-antitrypsin antiserum [3,4]. Studies involving the material isolated from these inclusion bodies have demonstrated different carbohydrate structures from those observed with the secreted form [5].

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We have examined the oligosaccharide structure of the accumulated material to confirm that it is compatible with a blockage of processing prior to entry to the Golgi. The inhibitory activity of the accumulated material has also been measured to determine whether this blockage is due to a major alteration in polypeptide conformation.

2. MATERIALS AND METHODS

Human liver was obtained as in [6], stored at −80°C until used, and the PAS-positive globular inclusion bodies isolated by a modification of a previously described method [3]. Briefly, portions of liver were pulverised while still frozen and incubated for 60 min at 37°C, with agitation in 50 ml of Hank's buffer containing 25 mg collagenase (Sigma, Grade IV). The suspension was filtered through a nylon gauze and centrifuged at 3000 × g_{av} for 10 min at 4°C. The pellet was resuspended in 0.25 M sucrose containing 0.02% sodium azide at 4°C, and subjected to sonication for 30 s. It was then layered over a 10–40% sucrose gradient and centrifuged in a Beckman SW28.1 rotor for 2 h at 25000 × g_{av} and at 4°C.

The vesicles containing α_1-antitrypsin were pelleted by this procedure and were readily dis-

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solved in 0.125 M Tris (pH 6.8) containing 2% sodiumdodecylsulphate, and 3% β-mercaptoethanol. Protein content was determined using the Bradford assay [7], and α_1-antitrypsin protein levels were measured by electroimmunoassay [8].

Endo-β-*N*-glucosaminidase (Seikaghu Kogyo Co., Japan) was used to digest glycoprotein as in [6]. Protein molecular masses were determined by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) in a 10–15% gel containing 0.2% SDS [9]. Protein transfer from the polyacrylamide gel to nitrocellulose (Western blotting) was performed as in [10]. Antisera and immuno reagents were purchased from Dako-immunoglobins (Copenhagen, Denmark).

Carbohydrate analyses were performed on a Varian gas-liquid chromatograph as in [10], 1–3 μl of the final silylated material was injected onto a column (180 × 0.16 cm i.d.) of 3% SE 30 and the chromatograph temperature programmed from 125–225°C at 1°C/min. Neutral and amino sugars were detected using flame ionization.

The inhibitory activity of α_1-antitrypsin was followed by measuring the loss of elastase and trypsin esterase activity. Trypsin activity was measured using *N*-benzoyl-L-arginine ethyl ester [12] and elastase monitored with succinyl-tri-L-alanyl-*p*-nitroanilide [13].

3. RESULTS AND DISCUSSION

Vesicles containing α_1-antitrypsin were isolated from the liver of a patient previously phenotyped ZZ. The yield of vesicles was 240 ± 20 mg/10 g liver wet weight, with approximately 90% of the total protein present as α_1-antitrypsin when measured by: (i) scanning of a Coomassie blue stained PAGE; and (ii) electroimmuno analysis. The α_1-antitrypsin in these vesicles was readily soluble in the PAGE loading buffer containing 2% SDS and 3% β-mercaptoethanol, but sparingly soluble in 8 M urea or 6 M guanidine hydrochloride. When SDS–PAGE was performed on the internal Z α_1-antitrypsin as well as the purified serum forms of Z and M (fig.1a), the internal liver form was found to have an apparent molecular mass of 56 kDa, lower than either of the two serum forms. Treatment with endo-β-*N*-glucosaminidase resulted in a further reduction in molecular mass to 51 kDa, while the serum forms remained unaffected. This suggested that the band at 56 kDa was α_1-antitrypsin which had accumulated in the liver vesicles as the 'high mannose' form. This was confirmed by the technique of 'Western blotting', with only the most heavily staining band reacting with human α_1-antitrypsin antiserum (fig.1b).

Analysis of the carbohydrate content of the purified serum and internal liver forms of α_1-antitrypsin was performed using gas-liquid chromatography. Using a series of internal standards it was possible to both identify and quantitate the individual sugars present. Table 1 shows that an identical composition was obtained for both the M and Z serum forms of α_1-antitrypsin while the Z internal form was markedly different. There was a complete absence of sialic acid and *N*-acetylgalactose in the internal Z, and a large increase in the mannose content. When these results are considered together with the endoglycosidase sensitivity, the evidence indicates that protein accumulation must have occurred after core glycosylation in the endoplasmic reticulum, and prior to the high mannose 'pruning' which takes place in the Golgi. The limited number of glucose residues detected (table 1) suggests that some processing of the mannose-containing carbohydrate sidechains has occurred. The enzymes β-glucosidase I and II known to be present in the rough endoplasmic reticulum could account for this processing [14].

It has been reported [15] that the internal Z α_1-antitrypsin had no functional activity. However, both the protein synthesised in vitro, either in a wheatgerm cell-free system (D.M. Errington, personal communication) or in a reticulocyte lysate system using rat α_1-antitrypsin mRNA, and possessing the N-terminal, pre-protein extension [16], had readily detectable elastase inhibitory activity. To show that the material isolated from the liver vesicles was functionally active, we attempted to extract vesicles under non-denaturing conditions.

Two methods were employed to isolate active inhibitor, one involving extraction at neutral pH (50 mM Tris, pH 8.0, 0.5 M NaCl) and the second utilising acid conditions (50 mM acetate, pH 2.0, 0.5 M NaCl). Plasma α_1-antitrypsin has been shown to retain biological activity under either of these conditions [17]. After 48 h extraction, solubilised α_1-antitrypsin was quantitated using

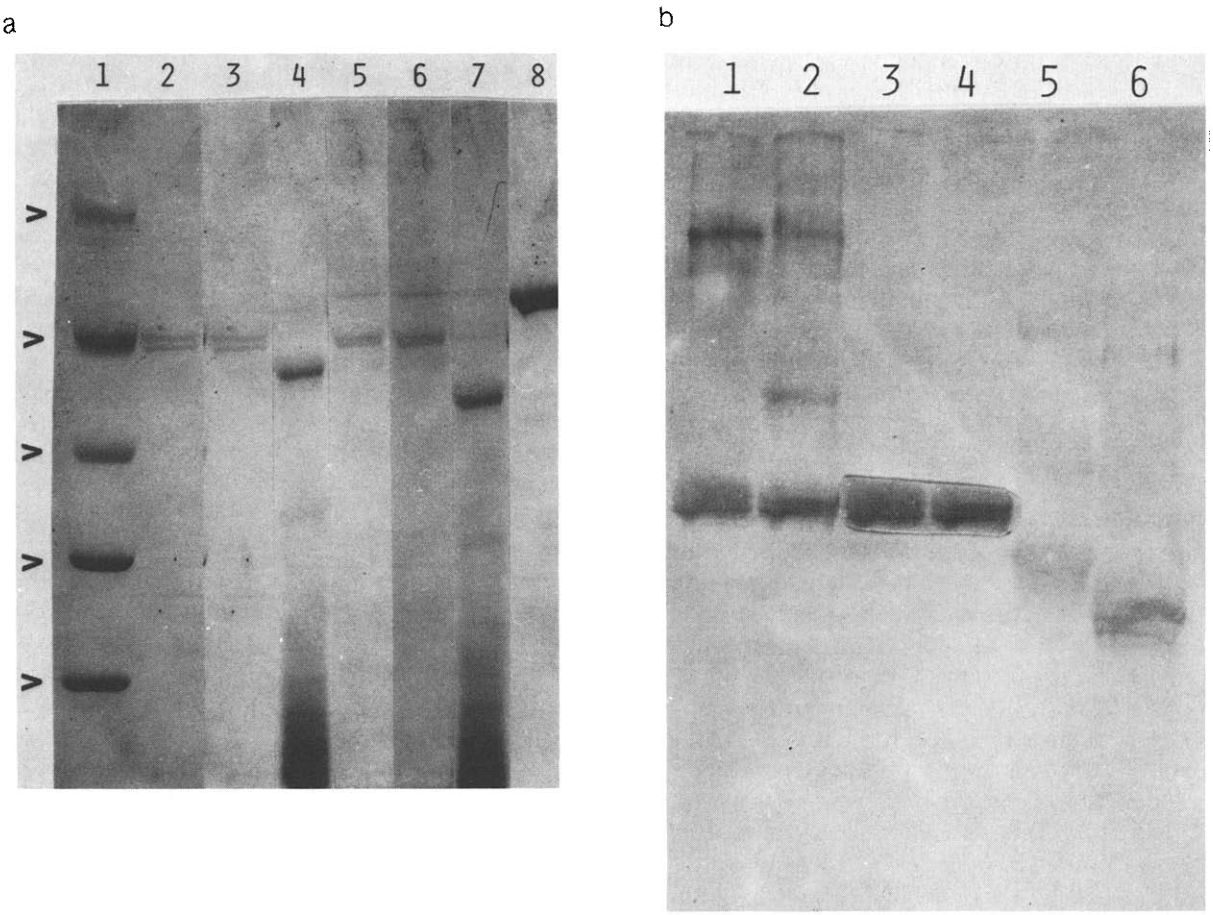

Fig.1. Molecular mass comparisons of human α_1-antitrypsin M and Z serum form and Z internal form and the effect of treatment with endo-β-*N*-glucosaminidase. (A) Coomassie blue stained 10–15% polyacrylamide gel electrophoresis. Lane 1, molecular mass markers, from the top of the gel, phosphorylase *B* (95 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.2 kDa); lane 2, M (serum form); lane 3, Z (serum form); lane 4, Z (internal form); lane 5, M (serum form) treated with endo-β-*N*-glucosaminidase; lane 6, Z (serum form) treated with endo-β-*N*-glucosaminidase; lane 7, Z (internal form) treated with endo-β-*N*-glucosaminidase; lane 8, overloading of endo-β-*N*-glucosaminidase. (B) Human α_1-antitrypsin immunologically detected after Western blotting from SDS–PAGE. Lane 1, M (serum form); lane 2, M (serum form) endo-β-*N*-glucosaminidase treated; lane 3, Z (serum form); lane 4, Z (serum form) endo-β-*N*-glucosaminidase treated; lane 5, Z (internal form); lane 6, Z (internal form) endo-β-*N*-glucosaminidase treated.

electroimmunoassay. The extraction at pH 2.0 yielded twice as much α_1-antitrypsin as that solubilised at pH 8.0.

As shown in table 2, both extracts inhibited trypsin and elastase, with the specific activity of each being identical and equivalent to the α_1-antitrypsin in normal serum. The specific activities for trypsin TIC (ΔA_{253}/s per mg α_1-antitrypsin) and elastase EIC (ΔA_{400}/s per mg α_1-antitrypsin) were: serum M (9.2 and 7.2, pH 2.0); Z (7.9 and 6.2, pH 8.0); and Z (10.8 and 8.7). This demonstrates that extracted material functions normally despite the immature carbohydrate sidechains. The fact that the TIC/EIC ratio is normal indicates that the internal Z form is not oxidized at the active site methionine.

Finally, it was possible to show that the Z internal liver form bound elastase by subjecting the

Table 1

Carbohydrate composition of human α_1-antitrypsin expressed as mole carbohydrate residue per mole protein assuming a molecular mass of 51 kDa and 12% carbohydrate for the serum form

	M (serum)	Z (serum)	Z (liver)
N-Acetyl-glucosamine	12.3	11.9	6.8
Mannose	11.4	10.6	22.1
Galactose	7.1	6.3	0
N-Acetyl-neuraminic acid	9.1	8.7	0
Glucose	0	0	2.0

complex to SDS–PAGE. [^{125}I]Elastase was mixed with excess α_1-antitrypsin, boiled in gel loading buffer, and immediately loaded onto PAGE. The molecular mass of the iodinated elastase shifted from 24 kDa in the free form to 78 kDa in the complex form (fig.2), thus demonstrating that the elastase inhibitory activity was due to α_1-antitrypsin and not a result of other contaminating proteins.

In summary, these results are compatible with a blockage in the transport of the protein prior to final processing in the Golgi vesicles. Our results support the observations that the Z internal liver form of α_1-antitrypsin accumulates in the region of the endoplasmic reticulum. Since normal inhibitory activity is retained in the high mannose

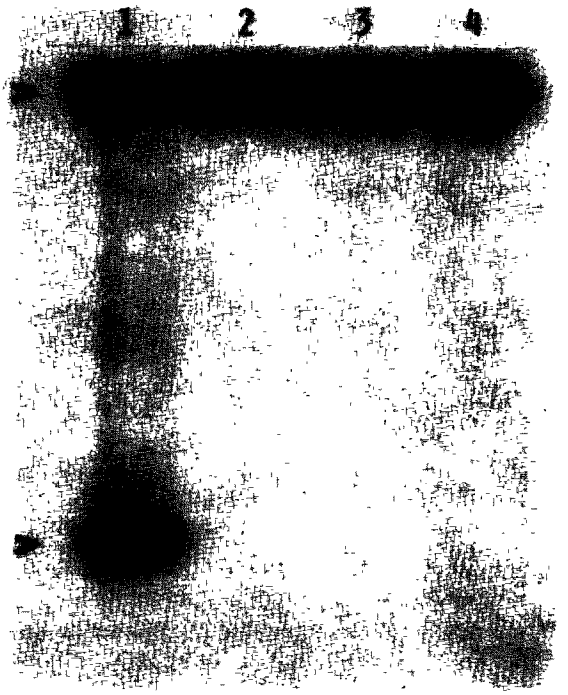

Fig.2. Autoradiograph of [^{125}I]elastase complexed to excess serum and internal forms of Z human α_1 antitrypsin. Lane 1, [^{125}I]elastase with 1/10 molar amount of M human α_1-antitrypsin. The upper band (78 kDa) represents the complex, the lower (24 kDa) unreacted elastase; lane 2, Z (serum form); lane 3, Z (internal form) extracted at pH 2.0; lane 4, Z (internal form) extracted at pH 8.0.

Table 2

Determination of the functional activity of human α_1-antitrypsin

	pH	TIC	EIC[a]	TIC/EIC
Internal α_1-antitrypsin Z (83 mg/l)	8.0	1.80	1.44	1.25
Internal α_1-antitrypsin Z (142 mg/l)	2.0	2.25	1.76	1.28
Serum α_1-antitrypsin M (132 mg/l)		2.42	1.89	1.28

[a] Trypsin inhibitory capacity (TIC) was measured as ΔA_{253}/2000 s and elastase inhibitory capacity (EIC) as ΔA_{400}/2000 s

form, the extracted α_1-antitrypsin must be in its native form with the reactive centre in the intact, strained conformation [18]. This, therefore, excludes the possibility that accumulation occurs due to a gross misfolding of the polypeptide chain. If the blockage at this step in secretion could be overcome, then the secreted α_1-antitrypsin would be a fully effective inhibitor [19].

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