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The amino acid substitutions of human α_1-antitrypsin M_3, X and Z

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α_1-Antitrypsin has been isolated from individuals with inherited genetic variants M_3, X and Z. A fragmentation and peptide mapping system is described which together with amino acid and sequence analyses revealed the substitutions in M_3 at 376 of Glu to Asp, in X at 204 of Glu to Lys and in the physiologically innocent Z a mutation at 213 of Val to Ala. The latter represents a second amino acid substitution in the Z protein.

α-$_1$-Antitrypsin; Genetic variant; Amino acid sequence; Peptide mapping

1. INTRODUCTION

The major human, extracellular serine protease inhibitor (Pi), α_1-antitrypsin (α_1-AT), has attracted biomedical interest owing to its genetic polymorphism [1], its unusually pronounced microheterogeneity [2], and because some of the genetic variants are accompanied by a slight to pronounced decrease in plasma α_1-AT content. A few of these mutations predispose for development of lung diseases [3], and one of them for liver diseases [4].

The complete amino acid sequence (394 residues) of baboon α_1-AT was deduced from cDNA studies [5], and established in humans for the most common PiM variant after direct amino acid sequence analysis [6]. A 24-residues long prepeptide has also been identified [7].

More than 50 alleles contribute to the genetic polymorphism of α_1-AT, which is preferably determined by means of isoelectric focusing of plasma after reduction of its mixed disulphides [8]. Single amino acid substitutions have been established where Glu in positions 264 and 342 has been replaced by Val and Lys, respectively, in the PiS and PiZ variants, both of which are accompanied by decreased concentration of α_1-AT in plasma [9,10]. Recent observations indicate that the altered plasma homeostasis for the PiZ variant is attributable to its postribosomal metabolism [11]. The entire nucleotide sequence of the Z gene has recently been completed [12]. In addition to the 342 Glu-Lys substitution, another point mutation (GTG to GCG) was found which predicts an amino acid substitution, at 213 of Val to Ala, without any apparent effect on the function or metabolism of the Z protein.

The reactive site of α-antitrypsin responsible for its inhibition of serine proteases, primarily elastase, is centered on the methionine residue 358, located 37 residues from the C-terminus of the molecule [13]. A mutation of this Met to Arg converts α_1-AT from an anti-elastase to an anti-thrombin, resulting in a bleeding tendency [14]. An α_1-AT mutant (Christchurch) with Lys-363 instead of Glu-363 close to the active center has a normal protease inhibitory activity [15].

A peptide mapping system for α_1-AT is reported here, and its usefulness is exemplified by the identification of the amino acid substitutions of the PiM_3, PiX and PiZ proteins.

2. MATERIALS AND METHODS

2.1. *Material*

Citrated blood plasma from subjects of various Pi types was obtained from the hospital blood center.

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2.2. *Reagents*

Most reagents and trifluoroacetic acid (Arista® grade) were reagents from BDH. Acetonitrile (HPLC grade) was obtained from Blackford, Wells Limited, England. Pharmalytes 4.2-4.9 were from Pharmacia (Uppsala, Sweden). Lysylendopeptidase was obtained from Wako (Osaka, Japan). Chemicals for sequence determination were from Applied Biosystems (Foster City, CA).

2.3. *Equipment*

A Pharmacia FPLC system, including two P-500 pumps, a liquid chromatographic controller (LCC-500), a 214 nm UV-detector with a HR-10 mm cell and a fraction collector with a RST-100 valve was used for all chromatographic separations.

2.4. *Purification of α_1-AT*

The genetic variants of α_1-AT were determined by isoelectric focusing, using a Pharmalyte 4.2-4.9 gradient in a polyacrylamide gel [8]. α_1-AT from individuals were purified with a SH-SS interchange chromatography system with minor modifications [16]. After an initial precipitation of low density lipoproteins (20 μl 10% dextran sulphate, 100 μl of 1 M $CaCl_2$ per ml plasma), the supernatant was slightly reduced with β-mercaptoethanol, at a final concentration of 0.02 M, for 0.5 h. Salt and reducing agent from 50 ml plasma was separated from proteins by gel filtration on a Sephadex G-25 column (5 × 30 cm with 0.05 M Tris buffer, pH 8). The plasma proteins with reactive SH-groups (α_1-AT, albumin, prealbumin) were retained in a column with activated thiol-Sepharose at pH 8. After washing and release of α_1-AT by reduction, contaminating albumin, prealbumin and some other contaminants were separated by passage through Blue Sepharose and anti-prealbumin Sepharose columns. The three columns had a volume of 50 ml each. After concentration of the proteins to some 3 ml, a final gel filtration on AcA-54 (1.6 × 80 cm in 0.05 M ammonium bicarbonate) gave 99% pure α_1-AT at a yield of about 50%.

2.5. *The peptide mapping system*

The purified α_1-AT was reduced and carboxymethylated. CNBr degradation (300 moles CNBr per mol Met) was performed in 70% (w/v) formic acid, at room temperature for 24 h for batches of 5 mg α_1-AT.

After lyophilization, the material was dissolved in 0.2 ml of 6 M guanidine-HCl and separated on two Superose 12 columns (Pharmacia) in tandem, equilibrated with 0.05 M ammonium bicarbonate in 30% acetonitrile. The flow rate was 0.8 ml/min and fractions were collected as marked on fig.1 and lyophilized.

Each of the three larger fragments (I–III) was dissolved in 0.1 M ammonium bicarbonate and digested with lysylendopeptidase at an enzyme to substrate ratio of 1:100 (w/w) for 3 h. The reaction was terminated by the addition of one drop of 6 M HCl. The lysylpeptides and the smaller CNBr fragments IV–IX were subjected to reversed-phase HPLC.

Peptides (0.25–0.5 mg) or CNBr fragments were directly injected into a Pharmacia HR 5/5 Pep RPC prepacked column (C_2/C_{18}, 5 μm particle size, 300 Å pore size). Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile/water (80:20). Elution was performed with an 80 min linear gradient from 0 to 60% B at a flow rate of 1.0 ml/min. The level sensor and delay functions in the fraction collector facilitated the collection of all major peaks.

2.6. *Amino acid and sequence determination*

Amino acid analyses of collected fraction peaks were performed after hydrolysis in 6 M HCl at 110°C for 24 h and using a Beckman 6300 amino acid analyzer. Automated Edman degradation was performed on a gas-phase sequencer (model 470 A) from Applied Biosystems equipped with an on-line detection system, Applied Biosystems model 120 A PTH-analyzer. The apparatuses were operated according to the manufacturer.

3. RESULTS

3.1. *The peptide mapping system*

After the CNBr degradation the lyophilized material had to be dissolved in guanidine-HCl and the column run in 30% acetonitrile to deaggregate the most hydrophobic peptides. Three larger (I–III) CNBr fragments were separated from a group of smaller ones by HPLC gel filtration on prepacked Superose 12 (fig.1). An increase in capacity from 1 to 5 mg material has recently been made possible by using a 1.6 × 80 cm Superose 12 column. The amino acid composition of the individual RP-HPLC peaks of lysylpeptides from fragments I–III was matched by the known sequence data. Position 220–221 represents a Met-Met sequence ending the largest CNBr fragment (II). The lysylendopeptidase gave more reproducible peptides than TPCK-trypsin, although some peptides became larger. The smaller CNBr fragments IV–IX were satisfactorily separated on the RP-HPLC system (fig.3). Met-358 appeared as homoserine in the C-terminal fragment VI (352–358) and as unreacted Met in the larger combined fragment VI–VII (352–374). It was evident

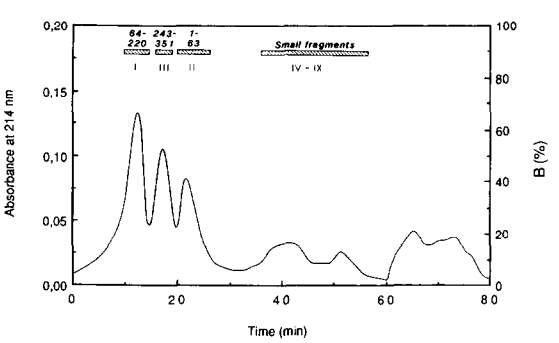

Fig.1. Gel filtration pattern of cyanogen bromide fragments of carboxymethylated α_1-antitrypsin. Two Superose 12 columns in tandem were eluted with 0.05 M NH_4HCO_3 in 30% acetonitrile. Parts of the effluent were collected as indicated by bars. Fraction collection was started after 40 min.

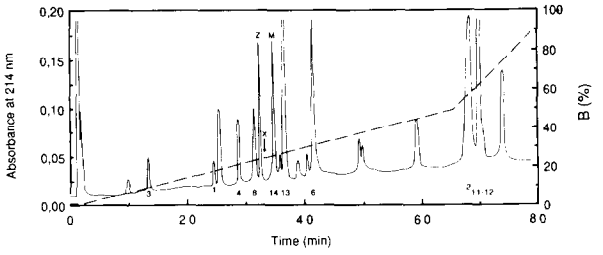

Fig.2. Separation of lysylpeptides from the largest CNBr fragment II on a C_2/C_{18} (Pep RPC 5/5) column. Peptide 14 (residues 202–217) represents the normal M position. The Z position reflects the 213 substitution (Val-Ala) in the Z protein. The X marks the position of peptide 14, devoid of the three first amino acids depending on a 204 Glu-Lys substitution in the X-protein.

from this chromatographic profile that Met-358 of the reactive site must have been partially oxidized to methionine sulphoxide, which does not react with CNBr.

3.2. *Peptide mapping of the genetic α_1-AT variants PiM$_3$, PiX and PiZ*

Both the HPLC gel filtration on Superose 12 and all RP-HPLC on C_2/C_{18} columns gave normal patterns of CNBr fragments and lysylpeptides from the M_3 protein. However, amino acid analysis of one of the purified smaller CNBr fragments gave evidence for a Glu-Asp replacement in fragment VIII. Sequence analysis of this fragment proved a substitution at 376 of Glu to Asp in M_3 protein. When separating the lysylpeptides from the CNBr fragment II of the X protein, an aberrant peak was obtained. The sequence of peptide 14 was found to be: Asp-Thr-Glu-Glu-Glu-Asp-Phe-His-Val-Asp-Glu-Val-Thr-Thr-Val-Lys. The first three amino acids in the aberrant peptide were missing as indicated both by the amino acid content and the sequence analysis. A new tripeptide, Asp-Thr-Lys, was found in the front peak indicating a Glu-Lys substitution for 204.

Z protein was run in the same peptide mapping system. The same peptide 14 now appeared to be less hydrophobic (see fig.2). Sequence analysis indicated a Val-Ala substitution for 213.

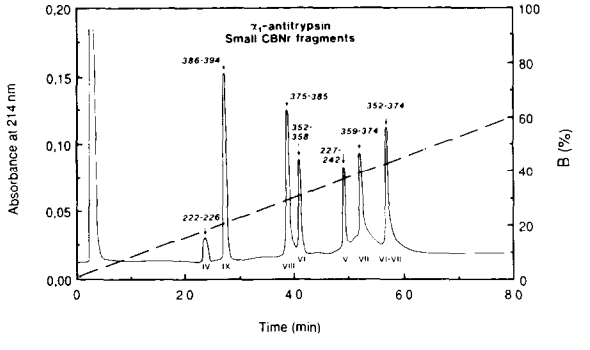

Fig.3. RP-HPLC of all smaller CNBr fragments IV–IX on C_2/C_{18} column. The actual residues are given at the top of the peaks. The methionine (358) in the active center is only partially cleaved by CNBr. Thus a combined VI + VII fragment is obtained besides the individual fragments VI and VII.

4. DISCUSSION

On electrofocusing, each phenotype of α_1-AT separates into five subfractions, two major, 4 and 6, and three minor, 2, 7 and 8. Subfractions 7 and 8 have the same variants of carbohydrate structure as the major subfractions 4 and 6, but have lost their first N-terminal amino acids: Glu-Asp-Pro-Glu-Gly [2]. This loss of three acid amino acids explains their higher p*I* values. The major subfractions 4 and 6 of the PiX protein which we have investigated have exactly the same p*I* values as the PiM 7 and 8, respectively. This is in good agreement with the finding of Glu-Lys substitution at 204 of PiX, adding two charge units. The same substitution pair was found already in 1976 in the Z protein [10]. Also, PiX and PiZ have similar positions on agarose electrophoresis, but show a small difference on electrofocusing. The second substitution in the Z protein at 213 (Val-Ala) as deduced from nucleotide sequence of the coding region of the PiZ gene [12], was verified with our peptide mapping system. In our earlier complete sequence of the M-protein we had obtained a clear Val in position 213 [6]. However, the cDNA work of Woo and co-workers [17] showed Ala, suggesting a polymorphism. This can now be explained by the findings that also 23% of the M1 haplotypes has the 213 Val-Ala substitution found in all Z proteins studied [12]. This 213 mutation is electrophoretically silent and located on the surface of the molecule.

The replacement of acid starch gel electrophoresis with electrofocusing for genetic Pi typing has revealed at least four additional M subtypes M_{2-5}. Unfortunately they have been named chronologically as they were detected, and

not with regard to differences in p*I* [1]. The PiM_3 occurs in 15% of the Scandinavian population. In contrast to the very small difference in p*I* values between M_1 and M_3 (less than 0.005 pH units), they separate on conventional agarose gel electrophoresis in barbital buffer at pH 8.6. M_3 protein migrates slightly faster than M_1 protein. The side chain of Glu-376 normally creates a hydrogen bond to His-350. The substituted amino acid Asp has a side-chain too short to establish this hydrogen bond at alkaline pH. Among five different DNA sequences published, one group [18] found a difference in one nucleotide sequence causing a single amino acid substitution in position 376, Glu-Asp. It is thus evident that this human liver cDNA library was isolated from a homozygous PiM_3 subtype.

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