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POLYMORPHISM OF HUMAN PLASMA THYROXINE BINDING PREALBUMIN

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Amyloid fibrils from an individual with heredofamilial amyloidosis were found to be composed of plasma prealbumin. To study this protein a three step procedure to isolate prealbumin from plasma was developed. It entailed ion exchange chromatography on DEAE Sephadex, affinity chromatography on Affi-gel Blue and gel filtration on AcA-34. Trypsin Digests of prealbumin were separated by reverse phase HPLC and the pattern compared to that from the normal protein. Only one unexpected peptide was found and it represented the substitution of a methionine for a valine at position 30 in the molecule. This substitution accounts for about 1/3 of the isolated molecules and it represents the first point mutation identified in human plasma prealbumin.

Amyloidosis is the extracellular accumulation of insoluble protein complexes having certain physico-chemical properties (1). These complexes are composed mainly of a single protein which has organized itself into long strands of stacked and interacting subunits which form fibrils of indeterminate length having an internal ß pleated sheet structure. There are several classes of amyloid which are identified by the subunit protein from which the fibrils are assembled. Primary or AL amyloid is composed of monoclonal immunoglobulin light chains or segments thereof. These segments are always derived from the variable region of the light chain and can be as small as half the variable region or as large as the entire light chain. Secondary or reactive amyloid is composed of a 76 residue polypeptide (AA) which is derived from an acute phase reactant protein (SAA). This protein which is 104 amino acids long is found associated with high density lipoprotein particles in diseases characterized by chronic inflammation or infection (2). A third type of systemic amyloidosis is inherited as an autosomal dominant trait. Families having this disorder have been identified

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as originating in Sweden, Portugal, Switzerland and Japan (3). In all cases where the subunit protein has been identified it has been shown to be related to plasma prealbumin.

During the course of studying one of the heredofamilial amyloids of Swedish origin it was shown that the amyloid fibril protein was related to prealbumin (4). Antibodies to prealbumin showed complete cross reactivity to the amyloid subunit, while antibodies to the amyloid indicated the presence of determinants not found in normal prealbumin. In addition the amyloid subunit had a faster migration on immunoelectrophoresis than did normal prealbumin. Taken together this indicates that the disorder may involve a mutation in the gene coding for prealbumin. This work was undertaken to determine any amino acid substitutions in this plasma prealbumin.

MATERIALS AND METHODS

Materials. Plasma from patient GRO was obtained by plasmaphoresis and stored at -20°C. Normal plasma was obtained from individuals with no signs of amyloidosis or liver disorder. DEAE Sephadex A-50 and G-75 were obtained from Pharmacia, Affi-gel Blue from Bio-Rad and AcA-34 from LKB. Antiserum to human prealbumin was obtained from Atlantic Antibodies (Westborough, Maine) and diluted 1:2 with 0.15M NaCl before use. Cyanogen bromide and trifluoroacetic acid were obtained from Pierce Chemical. Sequenator chemicals were obtained from Beckman-Spinco.

Prealbumin Purification. All columns used for prealbumin purification had the same initial buffer which consisted of 0.15M NaCl/0.02M Tris, pH 7.4. One hundred ml of normal or GRO plasma were dialyzed against starting buffer and, after removal of insoluble material, applied to a column of DEAE Sephadex A-50 (2.6 x 40 cm). The bound proteins were eluted with a 2 liter linear gradient of sodium chloride from 0.15M to 0.50M. The prealbumin fraction, identified by immunodiffusion, was concentrated, re-equilibrated with starting buffer and applied to a column of Affi-gel Blue (2.6 x 13 cm). The prealbumin which eluted in the unretained peak was again concentrated and applied to an AcA-34 column (2.6 x 95 cm).

Prealbumin was completely reduced in 6M guanidine HCl/0.5M Tris/1 mM EDTA/pH 8.3. To 5 mg of normal or GRO protein was added 5 mg of dithiothreitol and the samples were allowed to reduce for 4 hours at 37°. The free sulfhydryls were then alkylated with 12 mg of iodoacetamide and after 1/2 hour the reaction was quenched with 100 ul of 2-mercaptoethanol, dialyzed and lyophilized.

Peptide Isolation. Five mg each of alkylated GRO and normal prealbumin were suspended in 1.5 ml of water and 50-100 ul of 1.0N ammonium hydroxide was added to solubilize the proteins. Excess ammonia and traces of oxygen were removed with a stream of nitrogen gas. The solutions were then made 0.2M in ammonium bicarbonate at pH 8.2 and the proteins denatured by heating in boiling water for 5 minutes. After cooling, 100 ug of DPCC treated trypsin (Sigma) was added to each sample. The digests were kept at 37° for 12 hours and then lyophilized. One mg aliquots of trypsin digested prealbumin were fractionated by reverse phase liquid chromatography on an Altex Ultrasphere

C-18 column (0.46 x 25 cm) and impure pools were rechromatographed on a Waters alkylphenyl u-bondapak column (0.38 x 30 cm). For both columns the peptide mixtures were separated with a 0-60% gradient of acetonitrile in 0.1% trifluroacetic acid (pH 2.5). Normal and GRO prealbumins were also fragmented with cyanogen bromide. Five mg of protein was dissolved in 1 ml of 70% formic acid and, after deoxygenation with nitrogen gas, an equal weight of cyanogen bromide was added. The samples were stirred in the dark for 24 hours and diluted with 10 ml of water and lyophilized. the peptides were dissolved in 1 ml of 10% formic acid and applied to a column of G-75 (1.6 x 90 cm) in the same buffer.

Peptide identification and sequence analysis. Peptides were hydrolyzed in 1 ml of 5.7N HCl containing 50 ul of a 5% phenol solution at 110° for 20 hours. After drying the amino acids were identified and quantitated on a Beckman 119C amino acid analyzer. Protein and peptide samples were degraded in a Beckman 890C sequenator using the 0.1M quadrol program (121078). Peptide samples were run using 3 mg of polybrene as a carrier while protein samples were run with 3 mg of sodium dodecylsulfate. The anilinothiazolinone derivatives were converted to phenylthiohydantoins by heatin at 80° for 10 minutes in 1N NCl and were mixed with 15 nmoles of norleucine phenylthiohydantoin as an internal standard. The phenylthiohydantoin amino acid derivatives were identified by high performance liquid chromatography using a minor modification of the procedure of Zimmerman et al. (5).

RESULTS AND DISCUSSION

Since only a few hundred ml of GRO plasma were available, an isolation procedure was needed which gave good yields of the protein in a highly purified form. The procedure outlined here achieved these goals. The use of Affi-gel Blue was essential and not only provided a major purification of prealbumin but also separated the retinol binding protein without using denaturing conditions. Yields of 40-60% of theoretical were obtained.

Heat denaturation was absolutely necessary because, even after reduction and alkylation in 6M guanidine, no digestion occurred with trypsin on unheated prealbumin. The peptide elution patterns for the two prealbumin samples (Figure 1) are identical except in the GRO protein peak 12A is reduced and a new peak (12B) is generated. Amino acid analyses of these two peaks indicate that these peptides are from residues 22-31 and that in peak 12B a methionine has been substituted for a valine (6). Sequence analysis of both of these peptides confirms this and positions the methionine at residue 30.

In Figure 2 the entire sequence of the prealbumin molecule is presented and the positions of all the isolated peptides are given. It is obvious from this figure that there were a number of chymotrypsin like cleavages. This is probably due to the long digestion time needed to obtain a satisfactory degree

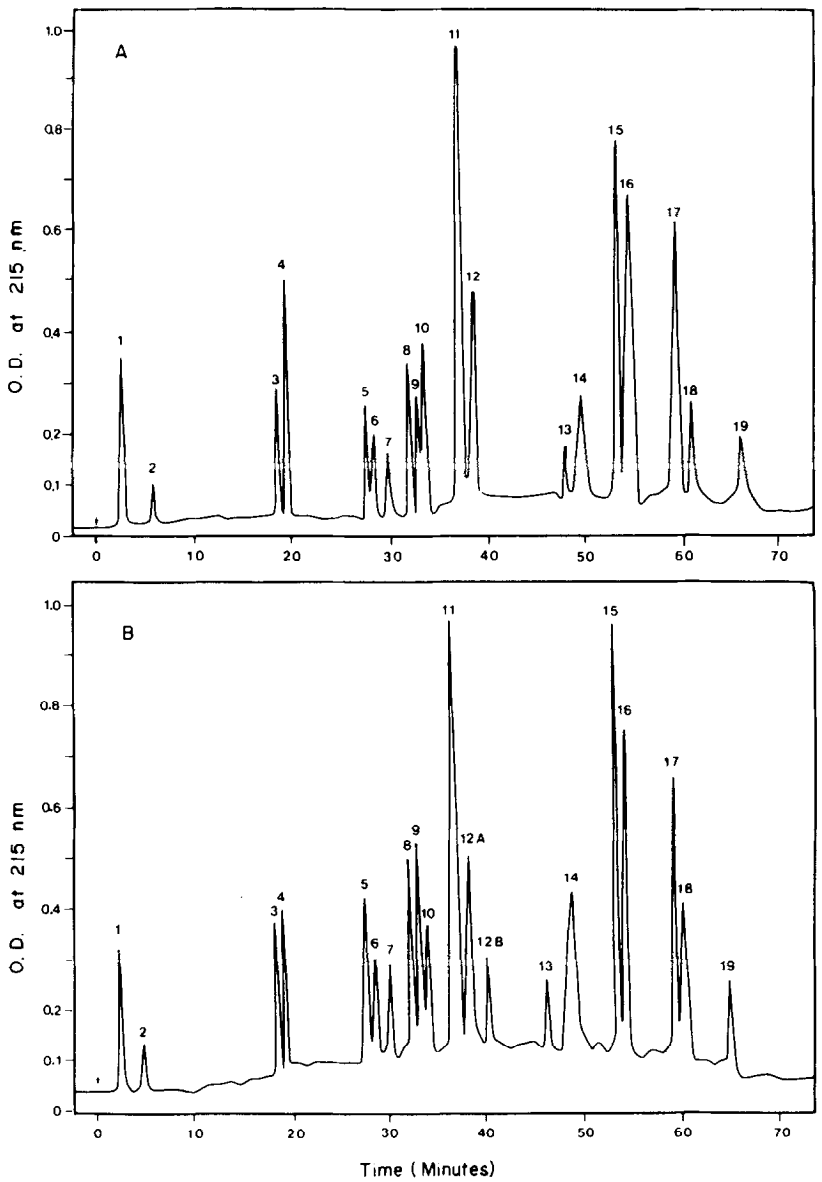

Figure 1. Reverse phase high performance liquid chromatographic separations of the tryptic peptides obtained from reduced and carboxamidomethylated prealbumin. Profile A shows the peptide pattern obtained from prealbumin of a normal individual and profile B shows the peptides obtained from GRO prealbumin. Conditions are as reported in the text with a gradient run time of 90 minutes and a flowrate of 1 ml/min.

of fragmentation. The overall isolation yields for the peptides is between 5 and 30% and, although these yields are somewhat low, they are consistent from digest to digest.

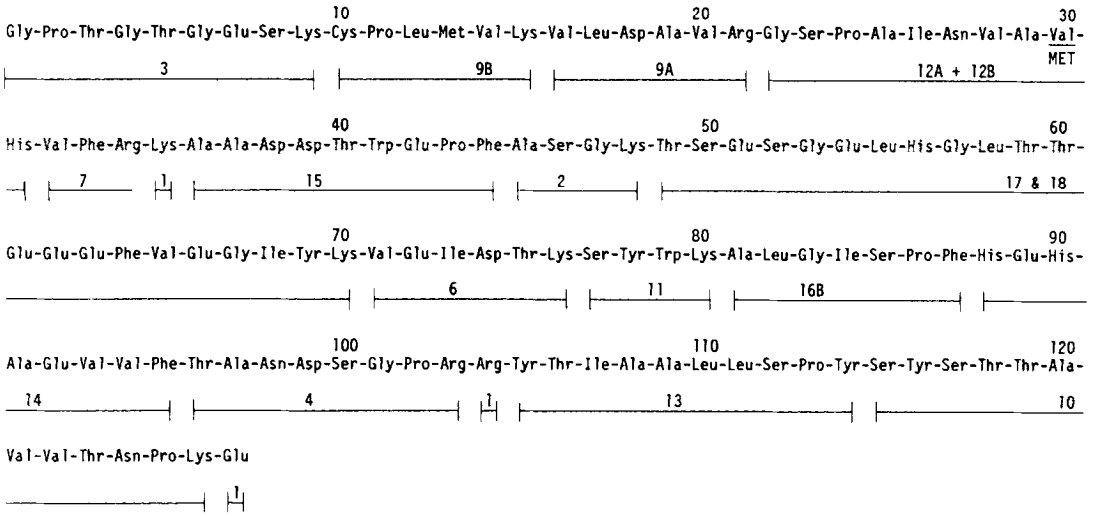

Figure 2. The complete amino acid sequence of GRO prealbumin. The bars beneath the sequence designate the isolated peptides and the number above the bars are the HPLC pools from which the respective peptides are isolated.

This is the first report of a mutation in the human plasma prealbumin molecule; although heterogeneity in the Rhesus monkey prealbumin has been identified (7,8). In the GRO plasma prealbumin the abnormal peptide was found to account for 1/3 of the sample. To reconfirm this ratio the GRO protein was cleaved with cyanogen bromide and the small peptides removed by gel filtration on G-75 Sephadex. The high molecular weight peptides were then applied to the sequenator and the ratio of the yields from positions 14-127 and 31-127 was calculated. Again it was found that the abnormal methionine was present in 1/3 of the molecules. If the inheritance of prealbumin structure represents the expression of codominant alleles, the reason for the lower level of abnormal prealbumin in the plasma is uncertain. It could be that the variant molecule is preferentially deposited as amyloid, has a reduced rate of biosynthesis, or an increased rate of degradation.

Attempts to determine if the variant prealbumin is the cause of this hereditary amyloidosis have been delayed by the small size of the family. The affected mother of patient GRO died before initiation of this study and no tissue or blood samples were available. Patient GRO had one brother who also had hereditary amyloid. Structural studies revealed that he also had the

abnormal plasma prealbumin. Both brothers each had two children but they all are too young for symptoms of amyloidosis to appear. Therefore, at the present time, concordance between inheritance of amyloidosis and the variant prealbumin in this family can not be determined.

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