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Primary structure of an amyloid prealbumin and its plasma precursor in a heredofamilial polyneuropathy of Swedish origin

(amyloidosis/plasma protein/protein sequence/autosomal dominant)

FRANCIS E. DWULET*†‡ AND MERRILL D. BENSON*†§

*Rheumatology Section, Richard L. Roudebush Veterans Administration Medical Center and Departments of †Medicine and ‡Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46223

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ABSTRACT **Prealbumin from an individual with heredofamilial amyloid polyneuropathy of Swedish origin was isolated from plasma by using a three-step procedure involving ion exchange, Affi-gel Blue affinity chromatography, and gel filtration. This prealbumin and its associated amyloid fibril subunit protein were digested with trypsin and the resulting peptides were separated by high performance liquid chromatography. Comparison with normal prealbumin peptides showed that an amino acid substitution of a methionine for a valine had occurred at position 30. In the plasma prealbumin, the abnormal residue accounted for 1/3rd of the material while in the amyloid fibrils it accounted for 2/3rds. From this sequence information and the known three-dimensional structure of the prealbumin molecule, a mechanism for the amyloid formation is proposed. It involves formation of the amyloid fibrils by addition of prealbumin dimers or tetramers to the aggregate. Each dimer must contain at least one variant peptide chain while the tetramer must contain at least two abnormal chains. Either of these models can account for the observed amount of normal prealbumin in amyloid fibrils. No proteolytic processing of this molecule is required because the entire undegraded prealbumin molecule is found in the fibrils.**

The heredofamilial amyloidoses are a group of syndromes all having an autosomal dominant pattern of inheritance and most having polyneuropathy as a major manifestation of the disease. Since the first description of systemic amyloidosis in Portuguese familial amyloidotic polyneuropathy in 1952, similar syndromes have been recognized in a number of kindreds in several countries (1, 2). Patterns of organ involvement vary from one kindred to another, but all are characterized by extracellular deposition of fibrillar material having the physicochemical properties of amyloid (3).

In 1978 Costa *et al.* (4) showed that protein extracted from amyloid deposits from the tissues of patients with Portuguese familial polyneuropathy reacted with antiserum to plasma prealbumin. Subsequently, structural analyses of amyloid proteins from individuals in the United States (two separate kindreds of Swedish origin) and Israel have proven the presence of prealbumin or parts thereof in these types of amyloidosis (5–7). It has been hypothesized that a genetically determined structural defect in plasma prealbumin leads to amyloid fibril formation in these conditions. In the prealbumin amyloid from Israel, an amino acid change at position 49 from the amino terminus was noted, but no plasma prealbumin was available for study (7). Therefore, the question of whether an abnormal prealbumin was present in the serum could not be answered.

The finding of prealbumin in the tissue amyloid deposits of an American patient of Swedish origin with heredofamilial amyloidosis has been reported (5). We have now determined the entire primary structure of this amyloid protein and have found an amino acid substitution that is also present in the circulating plasma prealbumin.

EXPERIMENTAL PROCEDURES

Prealbumin was isolated from plasma by a modification of the procedure of Rask *et al.* (8). One hundred milliliters of plasma was equilibrated with 0.15 M NaCl/0.02 M Tris·HCl, pH 7.4. After removal of insoluble material, the sample was applied to a column (2.6 × 40 cm) of DEAE-Sephadex A-50 (Pharmacia) and the proteins were removed by elution with a linear gradient of sodium chloride from 0.15 M to 0.45 M (total vol, 2 liters). The prealbumin fraction was pooled, concentrated, reequilibrated with the starting buffer, and applied to a column (2.6 × 13 cm) of Affi-gel Blue (Bio-Rad). Final purification of the prealbumin was achieved by chromatography on a column (2.6 × 95 cm) of AcA 34 (LKB).

Amyloid fibrils were isolated from the kidney of patient GRO as described by Pras *et al.* (9). After reduction and alkylation, the amyloid subunit protein was isolated by gel filtration chromatography on Sephadex G-100 (Pharmacia) (5).

Prealbumin and amyloid fibrils were completely reduced in 6 M guanidine·HCl/0.5 M Tris·HCl/1 mM EDTA, pH 8.3. To each sample was added a weight of dithiothreitol equal to 1/2 the weight of the protein. After reduction for 14 hr, the samples were alkylated with iodoacetamide (1.2 mg/mg of protein) for 1/2 hour and any unreacted iodoacetamide was quenched with 100 μl of 2-mercaptoethanol. Glacial acetic acid was then added to lower the pH to 2.0, and the sample was dialyzed against 0.1% acetic acid and lyophilized.

Alkylated amyloid protein GRO, GRO plasma prealbumin, and prealbumin isolated from a normal individual (5 mg each) were suspended in 1.5 ml of water and enough 1 M ammonium hydroxide (usually 50–100 μl) was added to solubilize the proteins. Nitrogen gas was bubbled through the solutions to deoxygenate them and remove excess ammonia. The solutions were adjusted to 0.2 M NH_4HCO_3 (pH 8.2), heated in boiling water for 5 min, and quenched in an ice bath. Diphenylcarbamoyl chloride-treated trypsin (Sigma) was added to an enzyme/substrate ratio of 2:100 (wt/wt), the mixture was kept at 37°C for 8 hr, and then the reaction was terminated by lyophilization. Tryptic peptides were isolated by reversed-phase liquid chromatography on an Ultrasphere C-18 column (0.46 × 25 cm; Altex Scientific, Berkeley, CA) and impure pools were rechromatographed on an alkylphenyl μBondapak column (0.38 × 30 cm; Waters Associates). Peptides were separated using a gradient of 0–60% acetonitrile (Burdick and Jackson, Muskegon, MI) in 0.1% trifluoroacetic acid (pH 2.5).

Completely reduced and carboxamidomethylated GRO prealbumin and amyloid protein (5 mg each) were dissolved in 1 ml of deoxygenated 70% formic acid to which was added

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§To whom reprint requests should be addressed.

an equal weight of cyanogen bromide (Pierce). The mixture was kept in the dark at room temperature for 24 hr with constant stirring (10). The samples were then diluted with 20 vol of distilled water, lyophilized, and fractionated on a column (1.6 × 90 cm) of Sephadex G-75 (Pharmacia).

Peptides were hydrolyzed in 1.0 ml of double-distilled 5.7 M HCl to which had been added 50 μl of a 5% phenol solution to reduce tyrosine oxidation. After evacuation, the samples were sealed and heated at 110°C for 22 hr. Amino acid residues were identified and quantitated on a Beckman 119C automatic amino acid analyzer (Beckman). Automated sequence analyses were carried out on a Beckman 890C automatic sequenator using the 0.1 M quadrol program (121078). For peptides, 3 mg of Polybrene was added with each sample and one cycle in which phenyl isothiocyanate was not added was carried out to remove impurities. Protein samples were run either alone or with 3 mg of $NaDodSO_4$.

To the anilinothiazolinone derivatives obtained from the sequenator was added 15 nmol of norleucylphenylthiohydantoin as an internal standard. All samples were converted to the phenylthiohydantoin by heating at 80°C in 1 M HCl for 10 min. The phenylthiohydantoin amino acids were identified by chromatography on a Waters gradient HPLC system equipped with an Altex Ultrasphere C-18 column (0.46 × 25 cm) and an Eldex column heater. Peaks were identified by their absolute and relative (to the norleucine standard) retention times. Peak identification and quantitation were carried out on a Hewlett Packard 3390A reporting integrator. The chromatography conditions used were a minor modification of the procedure of Zimmerman *et al.* (11). Amino acid derivatives not clearly identified by this method were reconverted to the free amino acid by hydrolysis at reduced pressure in 5.7 M HCl/0.1% $SnCl_2$ at 150°C for 12 hr and identified on a Beckman 119C amino acid analyzer (12).

RESULTS AND DISCUSSION

The method outlined here provides a fast, simple, and easy technique for the isolation of human plasma prealbumin and gives at least as good yields as the more commonly used procedure of Rask *et al.* (8). Affi-gel Blue is important in this scheme, as shown in Fig. 1. It provides nearly pure prealbumin and separates retinol-binding protein from the prealbumin under nondenaturing conditions so that the material can be used for recombination studies. The prealbumin from the AcA 34 column is free of contaminating proteins to the limit of detection using immunodiffusion, immunoelectrophoresis, $NaDodSO_4$/polyacrylamide gel electrophoresis, and protein sequence analysis. Overall yield was 35–55% of theoretical.

Elution profiles for the tryptic digests of normal prealbumin, GRO prealbumin, and the GRO amyloid protein were similar (Fig. 2). The only difference between the two prealbumin profiles was the appearance of a new peak (12B) in the GRO prealbumin. Amino acid analysis of these fractions showed that peaks 12 and 12A had the same composition and that peak 12B was nearly identical but with the replacement of a methionine for a valine. All other corresponding peaks had identical compositions and could be matched to the reported sequence for prealbumin. Sequence analysis confirmed that peptides 12A and 12B were from position 22–31 with peak 12A having the normal sequence and peak 12B having the valine in position 30 replaced by a methionine. All other GRO prealbumin peptides gave the expected amino acid sequences.

The GRO amyloid fibril protein showed a pattern almost identical to that of the GRO prealbumin. The abnormal peptide (12B) was also present in the amyloid protein and the only other difference was that the amino-terminal peptide (peak 3) was eluted about 1 min later than the same peptide in the normal and GRO plasma prealbumin. The presence of a blocked amino terminus on this amyloid protein has already been reported, and our results concur that all of the α amino groups in this protein are blocked (5). In the normal and GRO prealbumin isolated from plasma, there are no peptide peaks with this elution time. It should be noted that peptides from the entire prealbumin molecule, including the carboxyl-terminal glutamic acid, were identified, proving that there was no proteolytic modification of the amyloid molecule either before or after its incorporation into the fibrils. At present, the nature of the amino-terminal blocking group is unknown. The structure of the GRO prealbumin and the peptides used to confirm the sequence are given in Fig. 3. Although not shown, all peaks in the elution profiles were identified as peptides originating from the prealbumin molecule. For the GRO amyloid, only peptide 12B was sequentially degraded to confirm the presence of methionine at position 30. All other peptides were identified by elution position and composition. Although the other two prealbumin proteins from heredofamilial amyloid that have been reported do not show a blocked amino terminus, it should be noted that senile cardiac amyloid, which is also derived from prealbumin, does have a blocked amino terminus (13). Further work may show how closely these molecules are related.

A number of chymotrypsin-like cleavages were observed and these are probably a result of the long digestion times required for fragmentation of these proteins. The logic for this conclusion is based on three observations. First, the prealbumins and amyloid gave single peaks of 14,600 daltons on $NaDodSO_4$/polyacrylamide gel electrophoresis. Second, the prealbumins gave single amino acid sequences and, lastly, the peptides derived from chymotrypsin-like cleavage were unblocked. Prealbumin is very resistant to proteolytic cleavage and, if the samples are not heat denatured immediately before enzyme addition, no fragmentation is observed. This is probably due to the tight β-sheet structure of the molecule, which makes it difficult for proteases to cleave the protein and thus make it more susceptible to further degrada-

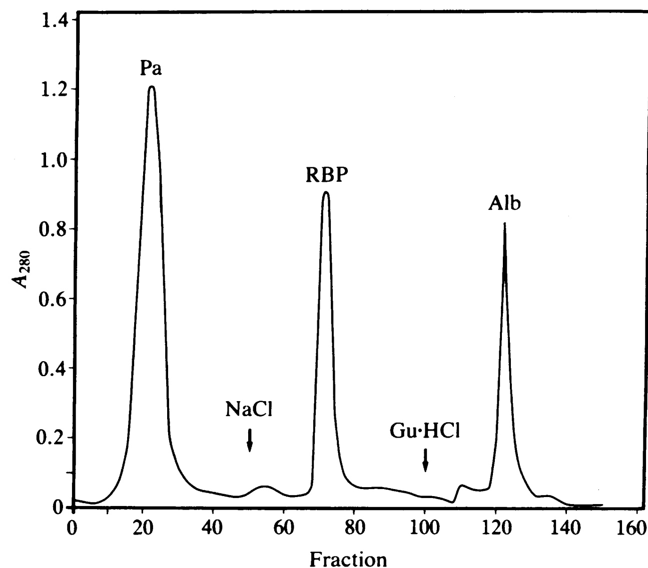

FIG. 1. Purification of plasma prealbumin on a column (2.6 × 13 cm) of Affi-gel Blue. The starting buffer was 0.02 M Tris·HCl/0.15 M NaCl, pH 7.4, the flow rate was 25 ml/hr, and the fraction size was 3 ml. At the first arrow, the buffer was changed to 0.02 M Tris·HCl/2.0 M NaCl, pH 7.4, and, at the second arrow, it was changed to 0.02 M Tris·HCl/4.0 M guanidine·hydrochloride, pH 7.4. The first peak (designated Pa) is composed primarily of plasma prealbumin, the second peak (designated RBP) contains retinol-binding protein, and the third peak (designated Alb) is composed predominately of serum albumin.

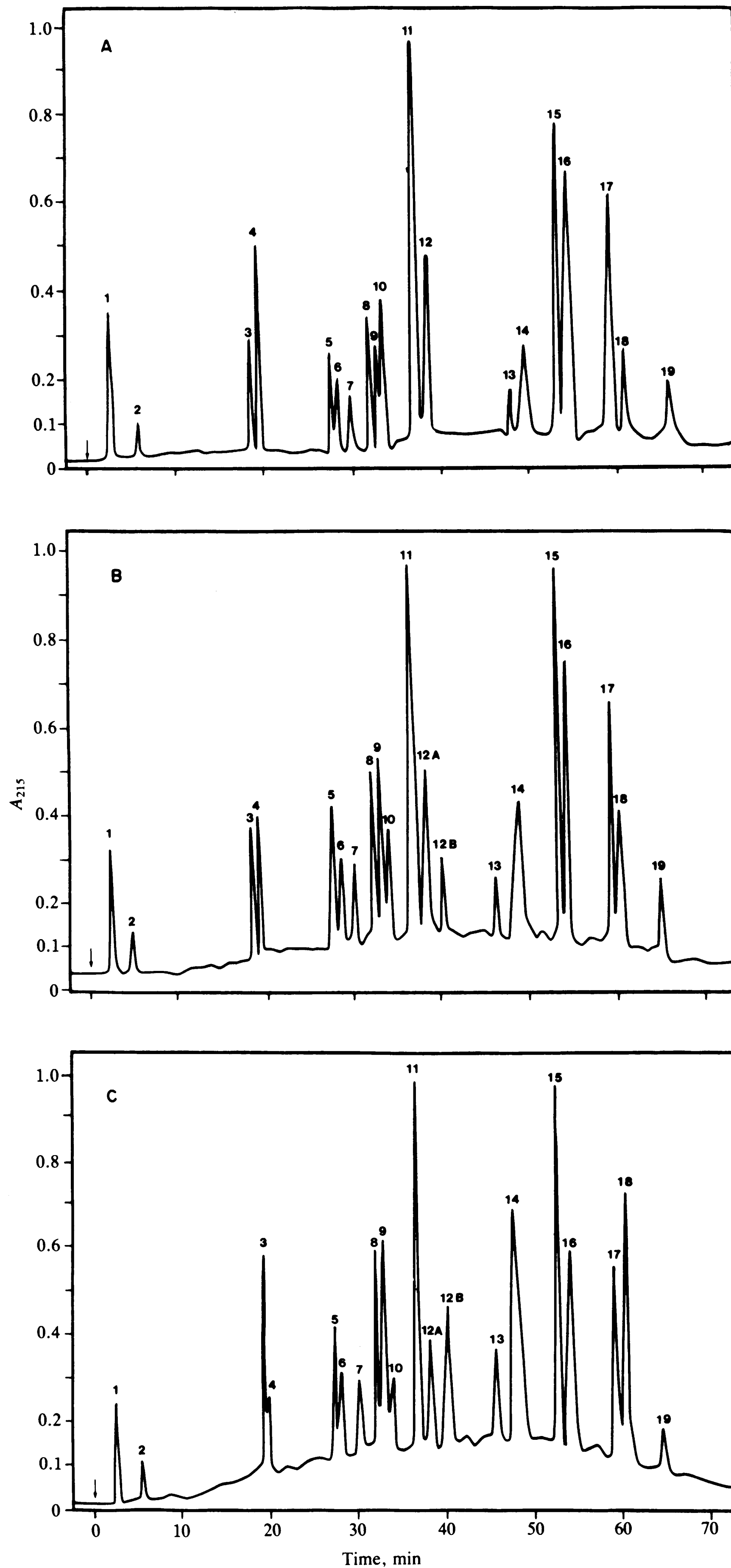

FIG. 2. HPLC separation of peptides formed by tryptic digestion of 1.0 mg of normal prealbumin (*A*), GRO prealbumin (*B*), and GRO amyloid (*C*). All columns were equilibrated with 0.1% trifluoroacetic acid and a linear gradient of 0–60% acetonitrile was generated over 90 min. The flow rate was 1.0 ml/min and the columns were maintained at room temperature (22°C).

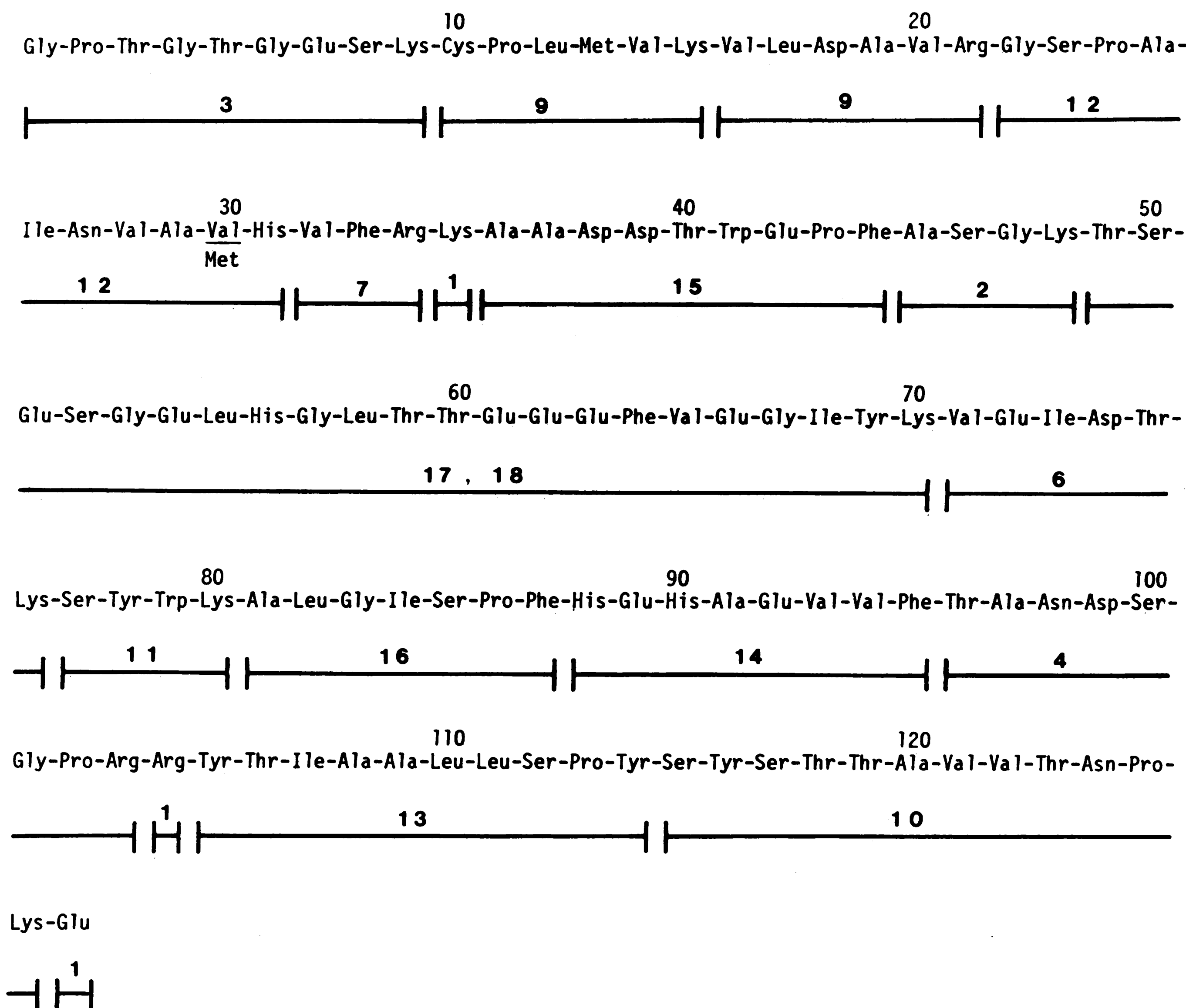

FIG. 3. Complete amino acid sequences of GRO prealbumin and amyloid proteins. The bars represent the isolated peptides and the numbers above them represent the pools from which they were isolated.

tion (14). Overall isolated yields of peptides were 5–25% of theoretical.

A most interesting observation is the relative amounts of normal and abnormal prealbumin molecules in GRO plasma and amyloid fibrils. The yield of isolated peptides 12A and 12B shows a 2:1 normal/variant prealbumin ratio in the plasma whereas, in the amyloid fibril protein, there is a normal/variant chain ratio of 1:2. To further confirm these observations, the GRO prealbumin and amyloid protein were fragmented with cyanogen bromide and the small peptides were removed by gel filtration. The retained high molecular weight material was then subjected to amino acid sequence analysis. The yields of the dual sequences from residues 14–127 and 31–127 were compared. The results of these sequenator runs confirm the peptide yield data from the tryptic peptides.

The difference in the concentration of the variant molecule in the plasma and the amyloid can be explained by either of two mechanisms. If it is assumed that the building block or subunit of this hereditary amyloid is a prealbumin dimer and that the dimer must contain at least one abnormal prealbumin chain, then statistics show that 2/3rds of the amyloid chains will be of the variant prealbumin. However, if the basic building block of the amyloid is the tetramer, then only those tetramers containing two or more abnormal subunits will be deposited in the amyloid. Under these conditions, 7/11ths of the amyloid chains will be of the variant prealbumin. Since the two models differ in the amount of abnormal chain by only about 3% and, since our results are not that precise, either can fit the observations. What is important is that both models explain the presence of normal prealbumin in the amyloid. The finding of normal prealbumin in the amyloid fibril protein suggests that fibril formation must occur at least at the dimer level.

Comparison of this amyloid prealbumin with the one reported by Pras *et al.* (7) shows that both proteins have single point mutations, which may account for their tendency toward amyloid formation. Pras *et al.*, however, did not have plasma available from patient SKO and therefore could not search for an abnormal prealbumin precursor. In the present studies, we have been able to show that the abnormal prealbumin is also found in the plasma. This lends credence to the hypothesis that the plasma prealbumin is the reservoir from which the amyloid fibrils form.

Analysis of the substitutions in the SKO and GRO proteins reveals a surprising pattern. From the x-ray crystallographic structure of human prealbumin, both changes are in close proximity (14). Also, both substitutions are near the ends of β-sheet strands. Position 30 (Val/Met) is the second residue on the amino terminus of β-strand B while position 49 (Thr/Gly) is the carboxyl-terminal residue of β-strand C. For the SKO mutation, the change from a threonine to a glycine will cause a major change in secondary structure. From the model of Chou and Fasman, threonine has a high β-sheet-forming potential while glycine has a low one (15). Thus, this end of strand C will be distorted and probably lose some of its β structure. The observed cleavage between residues 48 and 49 can be explained by the loosening of the β-strand C structure, which would make the lysine-48 residue more exposed to solvent and thus more susceptible to a trypsin-like (plasmin) cleavage. In the GRO protein, the valine has a high β-forming potential while methionine has a much lower one. Also, the methionine will occupy a greater volume than the valine, thus distorting this segment of the molecule. It would seem that distortion of this segment of the prealbumin molecule (the B–C strands) can lead to a protein that is susceptible to self-aggregation. This is not surprising because the B–C strands form the terminal segments of all four subunits and this is where the addition to the growing amyloid chain will take place. These results support the hypothesis that the formation of these hereditary amyloids from plasma prealbumin is due to the structure of the precursor molecule and not to a defective degradation mechanism.

Note should be made that there is one additional change in the protein sequences (positions 61–63) compared with the original prealbumin sequence of Kanda *et al.* (16). They reported the sequence of Glx-Glx-Gln while in this work we find the sequence to be Glu-Glu-Glu. Pras *et al.* (17) also reported protein SKO to have the Glu-Glu-Glu sequence, which we have identified not only in the GRO proteins but also in a normal prealbumin. Since our samples came from individual donors and Kanda *et al.* used pooled material, it is possible there is an additional variation at position 63 between glutamic acid and glutamine or that the glutamine at this position is very unstable. This is a possibility because the peptide eluted from the column as two peaks (fractions 17 and 18), both of which had the same composition and sequence. Thus, it is possible that during the isolation procedure some of the glutamine at position 63 was hydrolyzed to glutamic acid and that during the sequenator run the rest of the glutamine was hydrolyzed. These possibilities will have to be investigated by additional sequence studies on protein from a number of human sources.

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