

# Characterization of a Transthyretin (Prealbumin) Variant Associated with Familial Amyloidotic Polyneuropathy Type II (Indiana/Swiss)

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## Abstract

Amyloid fibrils were isolated from cardiac tissue of two brothers who died from familial amyloidotic polyneuropathy (FAP) type II. Sequence analysis on peptides derived from proteolytic cleavage with trypsin and fragmentation with cyanogen bromide reveal that the fibril subunit protein is derived from plasma transthyretin (prealbumin). About two-thirds of the fibril subunit protein was found to contain an amino acid substitution at position 84 where the normal isoleucine residue has been replaced by serine. Sequence analysis of the plasma transthyretin (prealbumin) from the two brothers as well as two clinically diagnosed FAP type II family members and two of four children of affected individuals showed the presence of serine at position 84. The presence of this substitution also correlates with low serum levels of retinol-binding protein and thus transthyretin (prealbumin) position 84 may be involved with the interaction of these two proteins.

## Introduction

In 1956 Rukavina et al. (1) reported a large kindred of Swiss descent with hereditary amyloidosis who had settled in northeastern Indiana. At the time the clinical features of this malady were distinctive from the familial amyloidotic polyneuropathy (FAP)<sup>1</sup> in Portuguese families that was described by Andrade (2). The Indiana/Swiss type of amyloidosis was characterized by early onset of the carpal tunnel syndrome followed by a more generalized peripheral neuropathy, blindness from vitreous deposits of amyloid, and cardiomyopathy. Unlike the Portuguese FAP, clinically significant renal involvement was absent. While a number of other kindreds with amyloidosis have been described in the last 30 years (Japanese, Swedish, Danish, Iowa, German, French) (3–8), the Indiana/Swiss syndrome has remained clinically distinct. Only the Maryland kindred described by Mahloudji, which also has a high incidence of carpal tunnel syndrome, clinically resembles the Indiana syndrome (7).

With the discovery that amyloid fibrils in the autosomal dominant amyloidoses were composed of transthyretin (prealbumin), it became obvious that these amyloidoses might be clas-

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1. *Abbreviations used in this paper:* FAP, familial amyloidotic polyneuropathy; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RBP, retinol-binding protein.

sified chemically (9). Pras et al. described a variant transthyretin (prealbumin) from the amyloid of a Jewish patient (10) and it is now known that at least one Swedish kindred (11, 12), two Japanese kindreds (13, 14), and the Portuguese patients with amyloidosis (15) all have a variant transthyretin (prealbumin) with a methionine substitution for valine at position 30 of the 127 amino acid residue transthyretin (prealbumin) molecule.

Recently we found that affected individuals in the Indiana/Swiss kindred have low serum levels of retinol-binding protein (RBP), whereas other kindreds do not (16). Since RBP binds to transthyretin (prealbumin) in the plasma, this led us to postulate that another transthyretin (prealbumin) variant is present in this condition. To investigate this we have isolated and structurally characterized the amyloid protein of the Indiana/Swiss type.

## Methods

Amyloid-laden tissue was obtained postmortem from two brothers who died of cardiac insufficiency and who had been diagnosed as having FAP type II. Amyloid fibrils were isolated from 30-g samples of heart tissue by the procedure of Pras et al. (17). The fibril subunit protein was then isolated from fibril concentrates by denaturation, reduction, alkylation, and size exclusion chromatography as previously described (12). The subunit protein (4 mg) was digested with trypsin and the peptides separated by reverse-phase high performance liquid chromatography (HPLC) on a column (1 × 25 cm) of Synchrom RP-8 resin. When necessary, peptides were repurified on Altex Ultrasphere C-18 or Waters bondapakphenyl columns. Polyacrylamide gel electrophoresis (PAGE) was done as previously described (11).

The amyloid subunit protein and plasma transthyretin (prealbumin) were cleaved with cyanogen bromide as reported previously (18). To 2 mg of amyloid subunit protein was added 0.5 ml of deoxygenated 70% formic acid and 2 mg of cyanogen bromide. The mixture was stirred in the dark at room temperature for 24 h and then dried under vacuum. The peptides were dissolved in 0.5 ml of 50% acetic acid and separated by reverse-phase high pressure liquid chromatography on a column (1 × 25 cm) of Synchrom RP-P resin using a 2-propanol gradient (0–56% during 90 min) to elute the peptides.

All protein and peptide samples were degraded in an 890C liquid-phase sequenator (Beckman Instruments, Inc., Fullerton, CA) using the 0.1 M Quadrol buffer program. 3 mg of polybrene was added to each sample to reduce extractive losses. Amino acid analyses were performed on a 119C instrument (Beckman Instruments, Inc.). All samples were hydrolyzed under vacuum for 24 h at 115°C using 5.7 N double-distilled HCl that contained 2 mg of phenol to reduce oxidation.

Plasma transthyretin (prealbumin) was isolated using a three-step procedure involving DEAE Sephadex ion exchange chromatography, Affigel Blue dye ligand affinity chromatography, and size exclusion chromatography as has been reported previously (18). After denaturation in 70% formic acid the protein was digested with trypsin and the peptides separated by reverse-phase HPLC as for the amyloid subunit protein (19).

## Results

Congo red staining of autopsy material from the two brothers revealed major amyloid deposits in their cardiac tissue. Both

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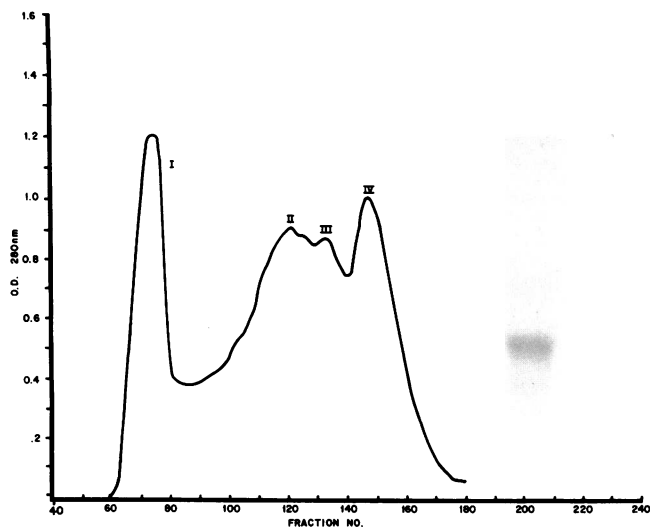
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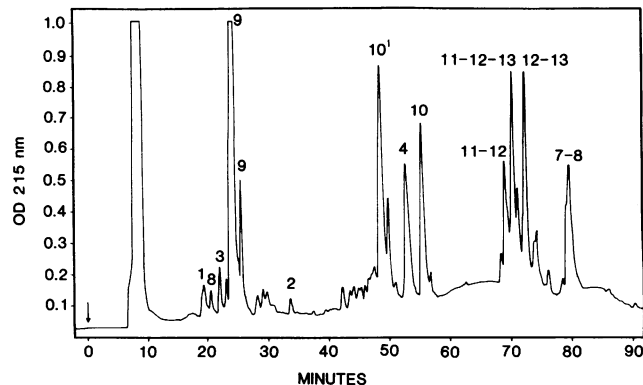
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individuals had enlarged hearts that weighed 900 and 750 g, respectively. From 30 g of starting tissue both samples yielded ~100 mg of water extract protein and 2 g of top layer protein. Both fractions were highly enriched for amyloid fibrils as demonstrated by Congo red staining. After denaturation, reduction, and alkylation, the fibril subunit protein was isolated by size exclusion chromatography (Fig. 1). On sodium dodecyl sulfate (SDS)-PAGE, peak IV was found to have molecular mass of ~14,000 D and to be size homogeneous. Reverse-phase HPLC also revealed a single major protein peak. The other fractions were found to be heterogeneous by the above criteria. From 200 mg of starting fibril material ~14 mg of peak IV was isolated, and it accounted for ~20% of the solubilized isolated proteins.

Sequence analysis of peak IV material revealed a heterogeneous structure from which the parent molecule could not easily be identified. The cyanogen bromide fragmented material gave an HPLC profile that was indiscernible from non-FAP type I transthyretins (prealbumins). Sequence analysis of the major cyanogen bromide peptide provided a sequence that was identical to that from positions 14–52 of normal plasma transthyretin (prealbumin). Tryptic digestion of peak IV material generated a peptide mixture that gave the reverse-phase HPLC profile seen in Fig. 2. In general, peptides were isolated in 90% or greater purity. Amino acid composition and sequence analysis revealed that these peptides were derived from plasma transthyretin (prealbumin): the only primary structure difference was that peptide T10' (residues 81–103) was found to have a serine instead of the normal isoleucine at position 84 (Table I). The amino terminal peptide started predominately from transthyretin (prealbumin) position 5, but longer and shorter peptides were also present. Finally, for a preparation from one of the brothers, peptide 7–8 (residues 49–76) appeared to have both a glycine and the normal threonine at position 49. The two small peaks after peptides 10' and 10 had amino acid compositions and sequence analysis consistent with their being derived from 10' and 10 with arginine 104 attached to the carboxyl terminal. Se-



**Figure 1.** Gel permeation chromatography of FAP type II amyloid subunit protein on a column (2.6 × 85 cm) of Sepharose CL6B. The buffer was 4.0 M guanidine hydrochloride, 0.05 M Tris HCl, pH 8.2, the flow rate was 30 ml/h, and the fraction size was 3.5 ml. The inset shows a 10% PAGE gel of peak IV. The single band migrated with the same mobility as isolated transthyretin (prealbumin).



**Figure 2.** Reverse-phase HPLC separation of peptides formed by tryptic digestion of 4 mg of amyloid subunit protein peak IV on a column (1 × 25 cm) of Synchrom RP-8 resin. The column was equilibrated in 0.1% trifluoroacetic acid and the peptides eluted with a linear gradient from 0 to 35% acetonitrile generated over 75 min. The flow rate was 2 ml/min and the column was maintained at room temperature. Peaks are numbered to correspond to the tryptic peptides as noted in Fig. 3.

quenator yield data for all major peptides are shown in Table II while the alignment of all peptides are shown in Fig. 3.

Tryptic peptide HPLC maps for FAP type II and normal plasma transthyretin (prealbumin) can be seen in Fig. 4. These profiles are identical except that the FAP type II protein has peak 10'. By sequence analysis this peak contains residues 81–103 of the transthyretin (prealbumin) molecule with a replacement at position 84 of a serine for the normal isoleucine.

## Discussion

While large amounts of amyloid fibrils were isolated from the cardiac tissue, there was a low recovery of fibrils in the water supernatant, which is comparable with that seen for FAP type I tissue samples (11). To obtain the best yields of the subunit protein, solubilization in 8 M guanidine required 48–72 h to dissociate the fibril subunit proteins from the matrix proteins. Sequence analysis of peak IV material revealed a multiple sequence. Review of these data after further studies on the tryptic peptides revealed that this fraction was composed of transthyretin (prealbumin) and that ~40% of the material being sequenced started at position 5 of the transthyretin (prealbumin) molecule with an additional 20–30% starting at positions 4 and 6.

The cyanogen bromide digest was done on two samples of the amyloid subunit protein. The first cleavage was performed using subunit protein obtained from the top-layer material. Sequence analysis of the major cyanogen bromide polypeptide gave the same amino acid residues as the starting material, which indicates that little or no cleavage had occurred. However, when the cyanogen bromide digest was performed on the subunit protein isolated from the water wash material, the major peptide isolated by HPLC gave a single sequence starting at position 14 of the transthyretin (prealbumin) molecule. This sequence was extended to position 52 with no differences from normal transthyretin (prealbumin) (Table II).

The tryptic digest of the subunit protein from one of the brothers gave the profile seen in Fig. 2. There are only a few differences in this profile from that of normal transthyretin (prealbumin). First, the amino terminal peptide (T1) in the sub-

Table I. Amino Acid Composition of Tryptic Peptides from FAP Type II Amyloid Subunit Protein\*†

Peptide	T1	T2	T3	T4	T7	T7-8	T8	T9	T10	T10 <sup>†</sup>	T11-12	T11-13	T12-13
Asp			1.1 (1)	1.1 (1)		1.3 (1)	1.0 (1)		2.1 (2)	1.8 (2)	1.2 (1)	0.7 (1)	0.9 (1)
Thr	0.6 (1)				2.5 (2.5)	3.4 (4)	0.9 (1)		0.7 (1)	0.8 (1)	3.6 (4)	3.5 (4)	3.6 (4)
Ser	0.7 (1)			0.8 (1)	2.1 (2)	2.3 (2)		0.8 (1)	1.8 (2)	2.7 (3)	3.1 (3)	3.1 (3)	3.2 (3)
Glu	1.2 (1)				6.2 (6)	6.6 (7)	1.0 (1)		2.0 (2)	2.3 (2)	2.2 (2)	1.1 (1)	0.8 (1)
Pro		0.6 (1)		0.8 (1)					2.2 (2)	2.1 (2)	2.2 (2)	2.2 (2)	2.1 (2)
Gly	1.1 (1)			1.1 (1)	3.4 (3.5)	3.4 (3)			1.8 (2)	2.0 (2)	3.3 (3)	3.3 (3)	3.1 (3)
Ala			1.0 (1)	2.1 (2)					3.1 (3)	2.8 (3)			
Cys		0.6 (1)							1.7 (2)	1.7 (2)	1.4 (2)	1.4 (2)	1.3 (2)
Val		1.2 (1)	1.9 (2)	2.7 (3)	1.0 (1)	2.0 (2)	1.1 (1)		1.1 (1)	1.1 (1)	1.2 (1)	0.8 (1)	1.0 (1)
Met		0.9 (1)							1.1 (1)	1.0 (1)	2.0 (2)	1.5 (2)	1.6 (2)
Ile				1.2 (1)	0.8 (1)	1.9 (2)	0.9 (1)		1.1 (1)	1.0 (1)	1.2 (1)	0.8 (1)	1.0 (1)
Leu		1.3 (1)	1.0 (1)		1.9 (2)	2.2 (2)			1.1 (1)	1.0 (1)	2.0 (2)	1.5 (2)	1.6 (2)
Tyr					0.9 (1)	1.3 (1)		0.9 (1)			2.6 (3)	2.7 (3)	3.2 (3)
Phe				1.2 (1)	1.1 (1)	1.0 (1)			1.9 (2)	2.1 (2)			
His				0.8 (1)	0.9 (1)	0.8 (1)			1.7 (2)	2.0 (2)			
Lys	1.2 (1)	1.2 (1)			1.2 (1)	1.9 (2)	1.1 (1)	1.2 (1)	1.1 (1)	0.8 (1)	0.8 (1)	1.1 (1)	1.2 (1)
Arg			1.0 (1)	1.3 (1)						0.8 (1)	0.9 (1)	1.2 (1)	
Trp								0.3 (1)					
Position	5-9	10-15	16-21	22-34	49-70	49-76	71-76	77-80	81-103	81-103	104-126	104-127	105-127
Yield (nmol)	41	14	45	37	5	17	12	57	14	30	7	22	20

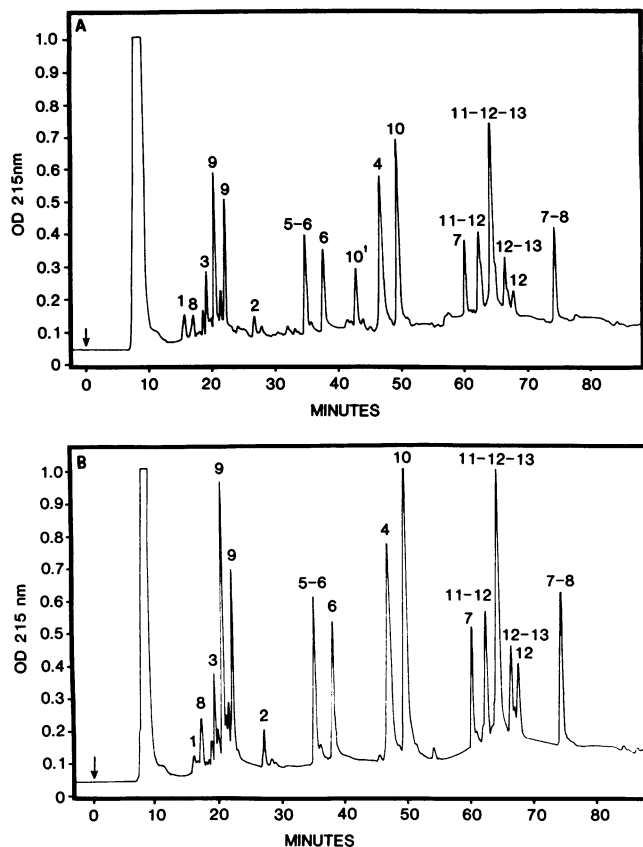
\* The numbers in parentheses represent integer residues found from sequence analysis. † Peptides 5-6 (35-48) and 6 (36-48) were recovered in yields too low to obtain reliable amino acid analysis.

Table II. Amino Acid Sequence Data for Peptides Used to Prove the Structure of FAP Type II Amyloid Subunit Protein\*

Cycle	T1	T2	T3	T4	T7	T8	T9	T10	T10'	T11-13	C2
1	5 T (3)	10 C (4)	16 V (19)	22 G (14)	49 T (3)	71 V (17)	77 S (20)	81 A (9)	81 A (20)	104 R (8)	14 V (17)
2	6 G (7)	11 P (4)	17 L (18)	23 S (12)	49 G (4)	72 E (16)	78 Y (42)	82 L (9)	82 L (22)	105 Y (16)	15 K (14)
3	7 E (5)	12 L (6)	18 D (10)	24 P (10)	50 S (5)	73 I (19)	79 W (26)	83 G (5)	83 G (15)	106 T (4)	16 V (12)
4	8 S (5)	13 M (4)	19 A (17)	25 A (16)	51 E (8)	74 D (7)	80 K (22)	84 I (6)	84 S (11)	107 I (11)	17 L (20)
5	9 K (4)	14 V (3)	20 V (14)	26 I (14)	52 S (4)	75 T (6)		85 S (3)	85 S (11)	108 A (13)	18 D (7)
6		15 K (2)	21 R (4)	27 N (10)	53 G (6)	76 K (9)		86 P (4)	86 P (8)	109 A (11)	19 A (12)
7				28 V (10)	54 E (6)			87 F (5)	87 F (16)	110 L (8)	20 V (17)
8				29 A (9)	55 L (9)			88 H (2)	88 H (7)	111 L (8)	21 R (10)
9				30 V (9)	56 H (5)			89 E (4)	89 E (10)	112 S (5)	22 G (16)
10				31 H (6)	57 G (4)			90 H (2)	90 H (5)	113 P (4)	23 S (7)
11				32 V (7)	58 L (8)			91 A (4)	91 A (11)	114 Y (10)	24 P (6)
12				33 F (5)	59 T (2)			92 E (4)	92 E (10)	115 S (4)	25 A (16)
13				34 R (1)	60 T (2)			93 V (4)	93 V (9)	116 Y (8)	26 I (13)
14					61 E (5)			94 V (4)	94 V (9)	117 S (4)	27 N (6)
15					62 E (5)			95 F (3)	95 F (9)	118 T (4)	28 V (8)
16					63 E (5)			96 T (2)	96 T (3)	119 T (3)	29 A (16)
17					64 F (3)			97 A (3)	97 A (9)	120 A (6)	30 V (11)
18					65 V (4)			98 N (2)	98 N (4)	121 V (6)	31 H (6)
19					66 E (3)			99 D (1)	99 D (3)	122 V (6)	32 V (16)
20					67 G (2)			100 S (1)	100 S (2)	123 T (1)	33 F (13)
21					68 I (3)			101 G (1)	101 G (3)	124 N (3)	34 R (3)
22					69 Y (3)			102 P (0.7)	102 P (2)	125 P (1)	35 K (10)
23					70 K (2)			103 R (0.5)	103 R (1)	126 K (2)	36 A (6)
24										127 E (1)	37 A (5)
25											38 D (3)
26											39 D (3)
27											40 T (2)
28											41 W (2)
29											42 E (2)
30											43 P (1)
31											44 F (3)
32											45 A (3)
33											46 S (1)
34											47 G (1)
35											48 K (2)
36											49 T (1)
37											50 S (1)
38											51 E (2)
39											52 S (1)

\* The single letter code is used to represent the amino acid identified at each cycle, while the number before the identified amino acid is the position number for that residue in the parent prealbumin molecule. The numbers in parentheses are the yield of that residue in nanomoles.





**Figure 4.** (A) Elution profile showing the separation of the peptides formed from trypsin digestion of plasma transthyretin (prealbumin) (3 mg) from an individual with FAP type II. Separation was obtained on a Synchrom RP-8 column (1 × 25 cm) using 0.1% TFA in water at a 2 ml/min flow rate as the initial buffer. A double gradient from 0 to 10% acetonitrile in 10 min followed by a gradient from 10 to 35% acetonitrile in 75 min was used to effect separation of the peptides. (B) Separation of the peptides derived from a trypsin digestion of normal plasma transthyretin (3 mg). Conditions were the same as above. All peaks are numbered as in Fig. 2. The two peaks labeled 9 gave identical composition and sequence analyses and may represent modification of the indole ring of Trp 79.

loss of cysteine, methionine, and tryptophan, this is consistent with the loss of these residues in the fibril subunit protein being due to tissue oxidation.

From these studies it would seem that the glycine that appears to be at position 49 is not part of the primary structure and that it is not attached to transthyretin (prealbumin) in the plasma. It is possible that the residue is attached after the fibrils are deposited into the tissue and that this modification may be tissue specific. This would explain the presence of the adduct in some amyloid-laden tissues but not in others as has been reported in the amyloid of Jewish origin (16, 19).

For the four presymptomatic individuals whose transthyretin (prealbumin) were studied, two of them had peptide T10' and two did not. Prior studies show that affected individuals in this kindred have low RBP levels, which was true for the two presymptomatic individuals who had peptide T10'. Therefore, it appears that the occurrence of low plasma RBP levels is associated with the position 84 substitution and may be used to identify carriers of the abnormal gene.

All the individuals with the gene coding for serine 84 showed

a consistent serum pattern. In all the people with active amyloid deposition the variant transthyretin (prealbumin) accounted for 20–25% of the total plasma concentration. However, in individuals not exhibiting active disease the variant concentration was between 30 and 35% of the total serum transthyretin (prealbumin) level. This is consistent with what has been seen in FAP type I individuals and probably represents the enriched deposition of the variant protein in the amyloid fibrils with the resultant depletion of the plasma (12).

From the x-ray structure of the transthyretin (prealbumin) molecule it has been determined that position 84 is located on an external loop on the outer surface of the protein between beta sheet strands E and F (20). This position is on one of the most exposed segments of the molecule, and how this substitution leads to the formation of fibrils is uncertain. However, certain features would appear to be important. Position 84 is at the end of a short alpha helical region (positions 78–83) and a mutation from isoleucine to serine will have effects on hydrophobicity and hydrogen bonding. Since low ionic strength is known to dissociate transthyretin (prealbumin) and RBP, it would appear that hydrophobic interactions may play an important role in the association of these proteins (21). Thus, the replacement of isoleucine at position 84 with a serine would lead to a major reduction of hydrophobicity in this region and may lead to a higher dissociation constant for these proteins. This reduced affinity for RBP could explain the low serum levels of RBP because unbound to transthyretin (prealbumin) it is quickly cleared by the kidneys (22). How the serine at position 84 will alter the protein structure will have to await x-ray structure analysis of the variant protein.

From the genetic code and transthyretin (prealbumin) complementary DNA (cDNA) sequence it is clear that the change from isoleucine to serine involves a single base substitution where the second codon is changed from a uracil (thymine) to a guanine (23–25). This replacement of a purine for a pyrimidine base should alter the DNA so that specific nucleases will give different patterns for the normal and variant genes. Thus, it should be possible to identify carriers of this trait by differences in restriction nuclease patterns as has already been shown for the methionine for valine substitution in FAP type I individuals (26) and for alanine for threonine substitution in FAP Appalachian (27).

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