Amyloid Fibril Protein in Familial Amyloidotic Polyneuropathy, Portuguese Type

Definition of Molecular Abnormality in Transthyretin (Prealbumin)

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Abstract. Amyloid fibril protein in patients with familial amyloidotic polyneuropathy is known to be chemically related to transthyretin (TTR), the plasma protein that is usually referred to as prealbumin. A genetically abnormal TTR may be involved in this disease. Studies were conducted on amyloid fibril protein (AF_p) isolated from tissues of two Portuguese patients who died with familial amyloidosis, and on TTR isolated from sera of patients with this disease. AF_p, purified by affinity chromatography on retinol-binding protein linked to Sepharose, resembled plasma TTR in forming a stable tetrameric structure, and in its binding affinities for both thyroxine and retinol-binding protein. The structural studies included: (*a*) comparative peptide mappings by reverse-phase high performance liquid chromatography (HPLC) after trypsin digestion; (*b*) cyanogen bromide cleavage studies; and (*c*) amino acid microsequence analysis of selected tryptic and CNBr peptides. On the basis of the known amino acid sequence of TTR, comparative tryptic peptide maps showed the presence of a single aberrant tryptic peptide (peptide 4, residues 22–34) in AF_p as compared with TTR. This aberrant peptide contained a methionine residue, not present in normal tryptic peptide 4. CNBr cleavage of AF_p produced two extra peptide fragments, which were demonstrated, respectively, by HPLC analysis and by sodium dodecyl sulfate-gel electrophoresis. Sequence analyses indicated the presence of a methionine-for-valine substitution at position 30 in AF_p as compared with TTR. Thus, the purified amyloid fibril protein comprised a TTR variant with a methionine-for-valine substitution at position 30. A single nucleotide change in a possible codon for valine 30 could explain the substitution. The variant TTR was also present in the TTR isolated from the pooled sera of amyloidoses patients, together with larger (four- to six-fold) amounts of the normal TTR. Thus, in these patients, the variant TTR was circulating in plasma, along with larger amounts of normal TTR. We suggest that the variant TTR represents the specific biochemical cause of the disease, and that this abnormal form of TTR selectively deposits in tissues as the amyloid characteristic of the disease.

Introduction

The heredofamilial amyloidoses represent a heterogeneous group of genetic syndromes that are defined by the presence of extracellular deposits of insoluble fibrillar protein. Most of these syndromes are characterized by the appearance of peripheral neuropathy, and are transmitted in an autosomal dominant manner (1).

In 1978, Costa et al. (2) presented evidence that the amyloid deposits in the Portuguese variety of familial amyloidotic polyneuropathy (FAP)[1] contain a protein related to transthyretin

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1. *Abbreviations used in this paper:* AF_p, amyloid fibril protein from Portuguese patients with FAP; C_a, C_b, extra CNBr fragments from AF_p: C_1, C_2, CNBr fragments from N-TTR; FAP, familial amyloidotic polyneuropathy; FAP-TTR, plasma TTR from patients with FAP; HPLC, high performance liquid chromatography; N-TTR, normal plasma TTR; o-PA, o-phthaldialdehyde; PAGE, polyacrylamide gel electrophoresis; RBP, retinol-binding protein; T*, aberrant TP-4 from AF_p; T_4, thyroxine; TFA, trifluoroacetic acid; TP-4, tryptic peptide 4 from N-TTR; TTR, transthyretin.

(TTR; usually referred to as prealbumin)[2] as a major constituent. Subsequently, TTR-related protein was found to be associated with amyloid deposits in patients with FAP from several countries and ethnic origins (3–7). It was suggested (2) that in FAP a genetic mutation in TTR may have occurred, leading to the production of an abnormal TTR, which is abnormally bound, degraded, and/or precipitated in tissues as amyloid fibrils.

To explore this hypothesis, we recently conducted studies designed to investigate whether Portuguese patients with FAP have an abnormal species of TTR circulating in their plasma (8). Human TTR is a protein of 54,980 M_r, composed of four identical subunits (9). TTR forms a stable complex with plasma retinol-binding protein (RBP), which is the specific transport protein for retinol (10), and is involved in the plasma transport of both thyroid hormone and retinol (vitamin A) (11). Significantly reduced levels of TTR were found in patients with FAP (8). TTR was isolated from serum of FAP patients and was examined in detail. FAP-TTR was indistinguishable from normal TTR with regard to a wide range of physical and chemical properties (8).

Since FAP-TTR and normal plasma TTR (N-TTR) were indistinguishable with regard to these physical-chemical characteristics, it became apparent that further progress towards the biochemical definition of the abnormality in FAP would require the study of the TTR-related amyloid protein from the tissues. Accordingly, the present study was undertaken with the goal of isolating purified amyloid fibril protein and then of conducting detailed studies of its chemical structure and physical properties, in comparison to those of TTR.

We now report studies on the primary structure of the purified amyloid fibril protein from Portuguese patients with FAP (AF$_p$),[3] compared with that of plasma TTR from patients with FAP and from normal persons. The amyloid fibril protein used in this work was purified by a novel procedure, and was characterized with regard to a number of physical-chemical properties. The present studies indicate that there is a methionine-for-valine substitution at position 30 in the AF$_p$ as compared with normal plasma TTR. The variant TTR is also present in plasma of FAP patients, together with a larger amount of normal TTR. The results of this work have been reported previously in abstract form (12).

Methods

Purified TTR and AF$_p$. TTR was isolated from pooled serum from 24 patients with FAP, and from normal plasma, as described in detail previously (8). The two TTR preparations used in the present work

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2. The name transtheyretin has been suggested recently by the Nomenclature Committee of the IUB and the IUPAC-IUB Joint Commission on Biochemical Nomenclature for the protein commonly called prealbumin (1981. *J. Biol. Chem.* 256:12–14).
3. According to the guidelines for nomenclature of amyloid proteins, the amyloid fibril protein of the familial disease of Portuguese origin is designed by AF$_p$ (see G. Glenner, P. P. Costa, and F. Freitas, editors. 1980. Amyloid and Amyloidosis. Experta Medica, Amsterdam. xi).

(FAP-TTR and N-TTR) were the same preparations of TTR previously used in the comparative study of some of the physical and chemical properties of FAP-TTR and N-TTR (8).

AF$_p$ was isolated from amyloid fibril concentrates that were obtained postmortem from the kidneys of two Portuguese patients with type I FAP. The first patient (case XII), a woman who died at age 41, had a typical family history, and a characteristic clinical picture (13), which began at age 27. Paresis of the extremities (particularly the lower ones) and gastrointestinal disturbances were prominent; these symptoms progressed over several years, and were followed by cachexia and death. Several members of the family of the second patient (case XIV), a man who died at age 43, were affected in four generations. At age 32 case XIV developed impaired thermal and pain sensation in the lower extremities. The diagnosis of FAP was confirmed by a positive nerve biopsy. The patient died after a progressive and typical evolution of his disease.

Amyloid fibrils were purified by repeated homogenization in phosphate-buffered saline, and then in distilled water, in a modification of the methods of Pras et al. (14) and Glenner et al. (15), as described previously (2). The lyophilized amyloid fibril concentrates were solubilized and reduced with dithiothreitol in 6 M guanidine-HCl, which was followed by alkylation with iodoacetic acid. These procedures were carried out as follows: First, 120 mg of lyophilized amyloid fibril concentrate was dispersed and solubilized in a solution of 6 M guanidine-HCl, 1 M Tris-HCl, 1 mM EDTA, and 20 mM dithiothreitol, pH 8.8, for 3 h at 37°C. Undissolved material was removed by centrifugation at 105,000 g for 1 h. To the clear supernatant, 36 mg iodoacetic acid was added, and alkylation was allowed to proceed for 15 min at room temperature in the dark at pH 6.9. The sample was then applied to a column of Bio-gel P-10 (Bio-Rad Laboratories, Richmond, CA) and equilibrated in a solution of 5 M guanidine-HCl, 0.1 M Tris, 4 mM EDTA, pH 8.6, to remove excess iodoacetic acid. The resulting solution was dialyzed against distilled water and lyophilized. Purified AF$_p$ was obtained from this solution by affinity chromatography on RBP linked to Sepharose.

Affinity chromatography. Human RBP covalently linked to Sepharose was prepared as an affinity support. The human RBP used was the same preparation of RBP previously used in studies of the amino acid sequence of RBP reported by Kanda and Goodman (16). The RBP had been isolated from the urine of Japanese patients with chronic cadmium poisoning and tubular proteinuria, and had been stored in the lyophilized state at −20°C. A sample (17 mg) of this RBP was saturated with retinol, by addition of an excess of all-*trans*-retinol in ethanol to an aqueous solution of RBP, followed by gel filtration to remove unbound retinol, as described previously (17). The resulting preparation, consisting mainly of holo-RBP (the retinol-RBP complex) was coupled to 3 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), according to the manufacturer's instructions and following established principles (18). The yield of coupled RBP was >90%.

The reduced and alkylated amyloid preparation (see above) was dissolved in 3 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, and was then applied to a column of RBP-Sepharose (1 × 10 cm), which was equilibrated with the same buffer. Elution with this buffer resulted in elution of material that did not bind to the gel. After such material was completely eluted, and no more protein appeared in the effluent, the buffer was replaced with distilled water adjusted with NH$_4$OH to pH 10.4, in order to elute protein bound to the column. It is known that the RBP-TTR complex dissociates in solutions of very low ionic strength and at alkaline pH (19, 20). The fractions containing eluted "bound" protein were pooled, dialyzed against distilled water, and lyophilized.

Thyroxine binding. Thyroxine (T_4) binding to purified AF_p was investigated by a gel filtration assay recently described for T_4 binding studies with TTR (21). L-[^{125}I]T_4 was purchased from New England Nuclear (Boston, MA) (1,250 μCi/μg sp act). The assay mixture (final volume 0.1 ml) contained 1.8×10^{-8} M AF_p (calculated as a tetramer of 55,000 mol wt) and 0.4×10^{-8} M L-[^{125}I]T_4 (0.04 μCi/assay) in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA. For each assay mixture, a paired assay mixture containing an excess (4×10^{-6} M) of unlabeled T_4 (Calbiochem-Behring Corp., La Jolla, CA) was also set up, in order to examine displacement of labeled T_4 from specific binding sites. After incubation for 30 min at room temperature, the assay tubes were chilled to 0°C, and protein-bound [^{125}I]T_4 was isolated by gel filtration on minicolumns of Sephadex G-25, which were equilibrated with Tris buffer. Fractions of 0.12 ml were collected. Free hormone (T_4) bound tightly to the gel matrix and was eluted later with 0.25 N NaOH (fractions of 1 ml each). Portions of all fractions were assayed for ^{125}I in a Packard model 5022 gamma counter (Packard Instrument Co., Inc., Downers Grove, IL). The radioactive fractions corresponding to the peak of protein-bound T_4 were subjected to polyacrylamide gel electrophoresis (PAGE). The resulting gels were sliced from top to bottom in 2-mm thick slices, and the radioactivity (^{125}I) in each slice was assayed in the same gamma counter.

Modification reactions. N-TRR and FAP-TTR were reduced and carboxymethylated with [^{3}H]iodoacetic acid (New England Nuclear) by a modification of the method of Crestfield as previously described (22). Unbound iodoacetic acid was removed by dialysis against 0.1 M ammonium acetate buffer, pH 6.8.

Proteolysis with trypsin. Digestions with trypsin (treated with N-tosyl-phenylalanine chloromethyl ketone) (Worthington Biochemical Corp., Freehold, NJ) were carried out at pH 8.4 in 1 M NH_4HCO_3, with an enzyme/substrate ratio (wt/wt) of 1:100. The reaction was stopped by the addition of an equal volume of 1 N HCl, followed by lyophilization. In most instances, the proteolytic reaction was carried out for 90 min, with trypsin being added at time 0 and after 45 min of digestion.

Cyanogen bromide cleavage. Cleavage with CNBr (Pierce Chemical Co., Rockford, IL) was effected in 70% formic acid, for 24 h at room temperature, with a protein concentration of 5 mg/ml, by using a 300-fold molar excess of reagent (23).

Separation of peptides by high performance liquid chromatography (HPLC). The tryptic and CNBr peptides were separated by reverse-phase HPLC on a μBondapack C18 column (0.4 × 30 cm; Waters Associates, Inc., Milford, MA), by using a Waters HPLC system that was equipped with the following components: model 6000A solvent delivery system, WISP 710B sample processor, model 720 gradient controller, and model 730 data module for automatic quantitation and documentation. Ultraviolet absorbance was monitored with both model 440 and model 450 absorbance detectors; for fluorescence measurements, a model 420AC fluorescence detector was used. All solvents were HPLC grade and were filtered through Millipore membranes and degassed before use.

Tryptic peptide mapping was carried out by separating the tryptic peptides by using two different solvent systems at different pHs. In most instances, the peptides were separated in a phosphoric acid/acetonitrile system (system 1), pH 2.0, and were detected by ultraviolet light absorbance. In other experiments, a trifluoroacetic acid (TFA)/propanol, pH 4.0, system, (system 2), with postcolumn fluorescence derivatization detection, was used (24).

Conditions for system 1 were as follows: Solvent A was 0.1% phosphoric acid and solvent B was the same in 50% acetonitrile. Absorbances at 220 and 280 nm were monitored at a flow rate of 1 ml/min. A gradient from 0 to 100% solvent B in 90 min was used as follows: 5 min isocratic

buffer A, a linear gradient from 0 to 60% B in 65 min, and 10 min isocratic elution with 60% B. Finally, after a 10-min linear gradient to 100% B, a 5-min isocratic elution in 100% B eluted undigested TTR. When necessary, the peptides were further separated by rechromatography on the same column, using 0.1% TFA, titrated to pH 4.0 with triethylamine (Pierce Chemical Co., sequanal grade), as solvent A and the same buffer in 50% 2-propanol, as solvent B. This system was called system 1a. Absorbance was monitored at 230 and 280 nm; a linear gradient from 30 to 60% solvent B in 40 min was used at a flow rate of 0.5 ml/min.

Conditions for system 2 were as follows: Solvent A was 0.1% TFA, titrated to pH 4.0 with pyridine, and solvent B was the same in 50% 2-propanol. A gradient for 50 min from 0 to 60% solvent B, at a flow rate of 0.6 ml/min was used. The peptides were detected by fluorometry, after reaction with o-phthaldialdehyde (o-PA) in the presence of mercaptoethanol (25). The reagent was prepared according to the instructions of the supplier (Pierce Chemical Co.). For preparative purposes, a stream-splitting system (24) was used.

Separation of the CNBr fragments was achieved with a solvent system consisting of 0.05% TFA as solvent A, and the same in 100% acetonitrile as solvent B. A gradient from 10 to 60% solvent B over 70 min, at a flow rate of 2 ml/min was used; absorbance was monitored at 210 nm. Gradient conditions were as follows: a linear gradient between 0 and 40% B for 35 min, followed by isocratic elution in 40% B for 25 min, and finally a 10-min linear gradient to 60% solvent B, which eluted uncleaved TTR.

Amino acid analysis. The amino acid compositions of the tryptic peptides and the CNBr fragments, as well as of the intact proteins, were determined by amino acid analysis on a 121 MB amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA) (26). The proteins and peptides were hydrolyzed in 6 N HCl in vacuo for 24 h at 110°C.

Amino acid analysis by HPLC, using automatic precolumn fluorescence derivatization (Waters Associates, Inc., Technical Bulletin, November 1982) was also used for the identification of individual amino acids, following Edman degradation or carboxypeptidase digestion (see below). After reaction with o-PA in the presence of mercaptoethanol, the fluorescent amino acid derivatives were separated by HPLC on a 5-μm reverse-phase C_{18} radial compressed column (Waters Associates) with gradient elution development. The separation of the standard amino acids (Pierce Chemical Co.) was achieved with conditions similar to those described by Hill et al. (27). Solvent A was methanol/tetrahydrofurane/0.08 M sodium acetate, pH 7.5 (2:2:96); solvent B was methanol/0.08 M sodium acetate, pH 7.5 (65:35). A linear gradient from 10% B to 100% B at a flow rate of 2.5 ml/min was developed.

Amino-terminal sequence analysis. A manual Edman microsequencing technique (28) was used to determine the NH_2-terminal sequences of selected tryptic and CNBr peptides. Individual amino acids were identified after "back" hydrolysis of the phenylthiohydantoin derivatives with 6 N HCl and 0.1% $SnCl_2$ for 4 h at 150°C. All reagents (from Pierce Chemical Co. and Beckman Instruments, Inc.) were sequanal grade and all glassware was pretreated by heating at 500°C for 4 h to minimize "background" levels of amino acids. For identification and quantification of the amino acids, blanks were run simultaneously for background subtraction. Criteria used for a positive identification were as follows: After subtraction of background (from a simultaneously treated blank sample), the amino acid observed had to be either the only one to increase, or the one with the greatest absolute and relative increase if more than one showed an increase.

Carboxypeptidase digestion. Diisopropylfluorophosphate-treated carboxypeptidases A and B (Worthington Biochemical Corp.) were used

for carboxy-terminal analysis (and sequence analysis) of some peptides (29, 30). The enzymes were purified by gel filtration on Sephadex G-25 in 0.2 M *N*-ethyl morpholine acetate buffer, pH 8.5, to remove autodigestion products. The peptides to be analyzed were dissolved in the same buffer and incubated at room temperature with carboxypeptidase A or a mixture of carboxypeptidases A and B; sample aliquots were withdrawn at various times and immediately injected on to the HPLC column. Quantitation was done after subtraction of the values obtained when using a blank sample that consisted of peptide and enzyme at time 0; when both carboxypeptidases A and B were used, blanks containing the two enzymes and incubated for the same periods, were introduced.

Other methods. Molecular weight estimates of the CNBr fragments and of the isolated proteins were made by vertical sodium dodecyl sulfate (SDS)-PAGE by the method of Laemmli (31) in 15% gels. BRL standards (Bethesda Research Laboratory, Gaithersburg, MD) were used as molecular weight protein markers. The proteins were detected both by Coomassie Blue and by silver staining (32). SDS-PAGE under non-dissociating conditions was performed according to the method of Weber and Osborn (33), as previously reported (8). The immunological relationship of the protein bands in the amyloid fibril preparation of TTR was explored by the immunoblotting technique, according to the method of Towbin et al. (34), by using rabbit anti-human TTR antisera prepared in this laboratory.

Results

Isolation of AF$_p$ by affinity chromatography on RBP-Sepharose. Previous work had shown that the amyloid fibrils from tissues of Portuguese patients with FAP contain a protein related to TTR that is not found in identically treated tissue preparations from normal humans (2). TTR is known to bind with high affinity to RBP (10, 11, 19, 20), and to form a stable TTR-RBP protein-protein complex. Moreover, our earlier studies showed that plasma TTR from patients with FAP bound to RBP with a capacity and affinity that were similar to that seen with normal TTR (8). Accordingly, pilot experiments were carried out to determine whether solubilized AF$_p$ would bind to RBP linked to Sepharose. These experiments indicated that some of the AF$_p$ did indeed bind to this affinity support. We therefore undertook to purify and isolate AF$_p$ by affinity chromatography on RBP-Sepharose of the entire solubilized amyloid fibril preparation.

After solubilization of the whole fibril preparation in 6 M guanidine, the preparation was alkylated with iodoacetic acid (see Methods). Previous studies have shown that reduction and alkylation of TTR do not affect its binding properties for RBP (35). The alkylated preparation was dialyzed to remove guanidine and was then lyophilized. The resulting preparation dissolved in the buffer used for affinity chromatography, in the absence of guanidine or of any detergent.

Fig. 1 shows the elution profile obtained after passage of the whole fibril preparation through a column of RBP linked to Sepharose. A major peak (peak I) of material that did not bind to the support was eluted with the void volume. When all unbound material had been completely eluted, the buffer was changed to distilled water at pH 10.4. This resulted in the elution
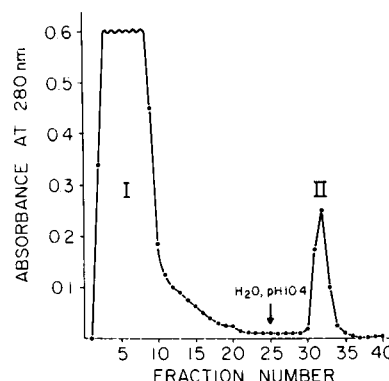

Figure 1. Affinity chromatography of a solubilized FAP amyloid fibril preparation on a column of human RBP linked to Sepharose 4B. Reduced and alkylated amyloid fibril material (120 mg) was solubilized in 3 ml 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, and applied to the affinity column. Fractions of 2 ml were collected at a rate of ~15 ml/h. At the position indicated in the figure by an arrow, the buffer was changed to distilled water at pH 10.4. Fractions 2 through 20 (referred to as peak I) and fractions 30 through 34 (designated peak II) were each combined, dialyzed against water, and lyophilized.

of a peak of protein (peak II) that had bound to the affinity support. About 20% of the total protein applied to the column was eluted in this peak. An identical affinity chromatography procedure has been used for the isolation of TTR from serum (36).

The peak II material migrated as a single protein band on PAGE. A single band of ~14,000 mol wt was also observed on SDS-PAGE, under dissociating conditions. Thus, peak II protein represented a purified AF$_p$.

Properties of the purified AF$_p$. To compare the mobilities of the AF$_p$ (the peak II protein) and normal plasma TTR (which had also been carboxymethylated with iodoacetic acid) a mixture of the two proteins was subjected to PAGE. Only a single band of protein was observed, which indicated that the electrophoretic mobility of the AF$_p$ was indistinguishable from that of TTR.

Table I shows the results of amino acid analyses of samples of the material in both peak I and in peak II, and of normal TTR, which were carried out together under the same conditions. The amino acid composition of peak II protein (AF$_p$) was very similar to that of normal TTR.

Since the AF$_p$ protein closely resembled human TTR in several ways (ability to bind to RBP, electrophoretic mobility), we set out to explore whether the AF$_p$ protein, like TTR, also formed a tetrameric structure. To this end, the AF$_p$ preparation was examined by SDS-PAGE under nondissociating conditions, where normal TTR (and plasma TTR from patients with FAP [8]) migrates as the undissociated tetramer. Under these conditions, AF$_p$ migrated as a band of protein of ~50,000 mol wt. Thus, the AF$_p$ protein appeared to form a stable tetramer under conditions where plasma TTR is normally found in tetrameric form.

Amino Acid	Peak I	Peak II	N-TTR	Kanda et al.*
Lys	8.0	7.4	8.4	8
His	3.6	4.1	4.1	4
Arg	7.2	4.2	4.0	4
Asp	11.5	9.1	8.0	8
Glu	15.8	13.9	11.9	12
Thr	8.6	10.6	11.3	12
Ser	9.4	10.6	10.4	12
Pro	7.9	7.5	8.7	8
Gly	12.9	10.6	10.5	10
Ala	10.8	11.5	12	12
Cys‡	2.2	1.2	1.6	1
Val	9.4	9.7	10.1	12
Met	2.2	1.6	0.7	1
Ile	5.7	4.7	4.8	5
Leu	10.8	7.4	7.1	7
Tyr	4.3	2.6	4.9	5
Phe	5.7	4.8	4.8	5

The values listed are the mean values obtained after duplicate analyses of each of the samples.
* Values taken from the amino acid sequence as reported (9).
‡ Cysteine was determined as carboxymethylcysteine from analysis of the reduced and carboxymethylated protein.

To explore further whether the AF_p protein forms a tetrameric structure similar to that of plasma TTR, the ability of the AF_p protein to bind thyroxine was examined. It has been shown that the TTR subunits are organized in the tetramer by extensive hydrogen bonding and hydrophobic contacts, giving rise to a molecule with a channel running through the center of its long axis (37). This central channel contains two identical binding sites for thyroid hormones. Thus, thyroxine binding depends upon the tetrameric structure of the molecule.

A gel filtration binding assay used recently in T_4-TTR binding studies (21) was used, in which the protein-bound and free hormone were separated by a rapid gel filtration step. Fig. 2 A shows the gel filtration elution profile obtained after incubation of AF_p with $[^{125}I]T_4$. Specific binding of $[^{125}I]T_4$ to AF_p protein, which could be fully displaced by excess unlabeled T_4, was observed. Thus, in the absence of added unlabeled T_4, radioactivity eluted with the peak of AF_p protein (fractions 4–7), followed by peaks of radioactive iodide and then of free hormone (eluted with NaOH). However, when the assay was carried out in the presence of excess unlabeled T_4, the protein-bound radioactivity was fully displaced (solid circles, Fig. 2 A). Parallel experiments (not shown) using the same conditions, but with plasma TTR (reduced and alkylated) as the protein, were performed. Very similar results were obtained.

To further document the binding of T_4 to AF_p, the labeled

material that eluted in fraction 5 (Fig. 2 A) was subjected to PAGE. The resulting gel was sliced from top to bottom and all slices were assayed for ^{125}I. The results are shown in Fig. 2 B. A sharp peak of radioactivity was observed in the gel, corresponding in location to the position of human TTR (reduced and carboxymethylated), which was treated by identical procedures. Thus, this experiment (Fig. 2) demonstrated that the AF_p protein has the capacity of forming tetramers which manifest binding properties for T_4 similar to those shown by normal TTR.

Studies were also conducted on the amyloid fibril protein that did not bind to the RBP affinity column. Amino acid analysis revealed that only approximately one fourth of the total mass of this material consisted of protein; the nature of the nonprotein component(s) is not known. The amino acid composition of the protein was, however, similar to that of TTR (see Table I). Analysis of this material by SDS-gel electrophoresis (see Appendix for details) showed several protein bands: major bands of molecular weights of ~15,000 and 32,000, and minor bands of ~42,000 and 54,000; higher weight bands were seen as well. Immunoblotting (see Appendix) demonstrated that all of these protein bands displayed TTR immunoreactivity. The results suggest that these protein bands all represented polymers of a TTR-related subunit, and that TTR-related protein may be the predominant, or almost the sole, protein component of the amyloid fibril.
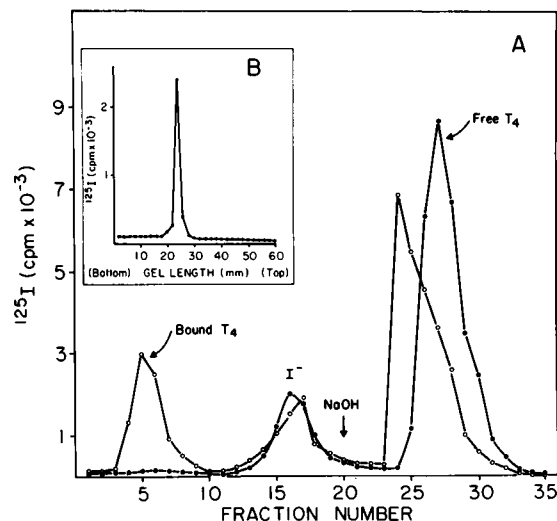

Figure 2. Binding of L-$[^{125}I]$thyroxine to AF_p. (A) Separation of the T_4-AF_p complex from unbound (free) T_4 and from free iodide (I^-) on Sephadex G-25 at 4°C. The assay mixture, 100 μl (see Methods for details) was incubated with (●) or without (○) unlabeled T_4. As indicated by the arrow, at fraction 20 the elution buffer was changed to 0.25 N NaOH to elute free T_4, tightly bound to the Sephadex matrix; 1-ml fractions were collected. (B) PAGE of $[^{125}I]T_4$ bound to AF_p. Fraction 5 (of A) was subjected to PAGE (24); the resulting gel was sliced from top to bottom in 2-mm thick slices and the slices were then assayed for ^{125}I.

Tryptic peptide map of normal TTR. On the basis of the known amino acid sequence of TTR (9), a tryptic peptide map was constructed for N-TTR, by digesting the reduced and carboxymethylated protein with trypsin, separating the fragments by HPLC, and identifying the peptide peaks by their amino acid composition (determined by amino acid analysis).

Fig. 3 shows the separation of all tryptic peptides on a C_{18} reverse-phase column, by using a phosphoric acid-acetonitrile solvent system (system 1). The nomenclature is the same as that of Kanda et al. (9), on the basis of the amino acid sequence of TTR. As shown in Fig. 3, all the tryptic peptides were separated by HPLC under these conditions. Peptides 4 and 10 eluted as a doublet peak; for preparative purposes, complete separation of these peptides could be achieved by rechromatography on the same column, in the TFA-propanol solvent system (system 1a) as shown in the inset in Fig. 3. Peptides 5 and 11 consist of Lys and Arg residues, respectively, and peptide 13 represents the COOH-terminal residue, which follows a Lys residue in the sequence. Several peptides were consistently found that represented combined tryptic peptides due to uncleaved Lys or Arg residues. These combined peptides included peptides 5 + 6, 7 + 8, 11 + 12 + 13, and 12 + 13.
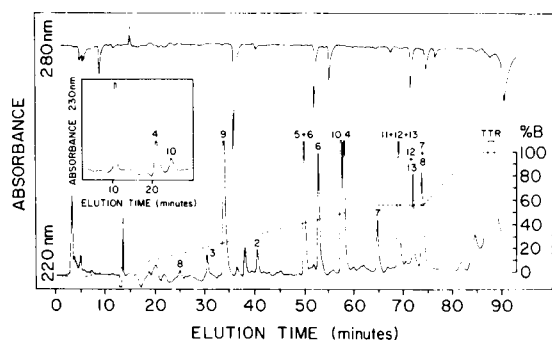

Figure 3. Tryptic peptide map of normal TTR. 1 mg of N-TTR was digested for 90 min with trypsin, and the resulting peptides were separated by HPLC on a reverse-phase C_{18} μBondapack (Waters Associates, Inc.) column, by using the phosphoric acid-acetonitrile solvent system (system 1) described in Methods. Peptides were detected by the 220 nm absorbance of the effluent, at a full scale of 0.2, which is represented by the lower tracing. Simultaneous detection at 280 nm was used at a full scale of 0.2 and is recorded in the upper tracing; the upper (280 nm) recording was 2.4 min out-of-phase with (i.e., later than) the lower recording. Gradient conditions as described in the text are represented by a dashed line. In this figure, the number above an individual peak identifies which tryptic peptide was eluted in this peak. Thus, the peak marked 9 represents trypic peptide 9. The nomenclature of the peptides is based on the known amino acid sequence of TTR (9). Several peptides representing combined tryptic peptides, due to uncleaved Lys or Arg residues, were found. These are labeled 5 + 6, 7 + 8, 11 + 12 + 13, and 12 + 13. (*Inset*) Rechromatography of a mixture of peptides 4 and 10 on the same column, using a TFA-2-propanol solvent system (system 1a). The peptides were detected at 230 nm with a full scale of 0.04. Gradient conditions are described in the text.
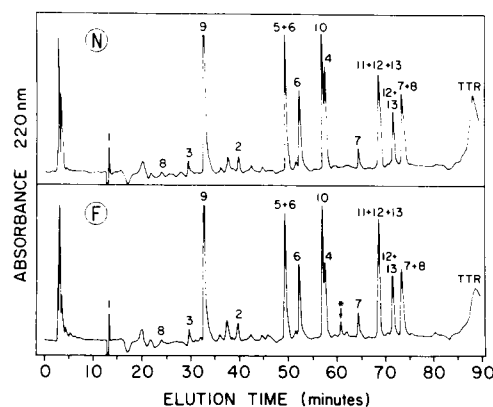

Figure 4. Comparative tryptic peptide maps of N-TTR (*upper, marked N*) and FAP-TTR (*lower, marked F*). 1 mg of each TTR was digested with trypsin for 60 min and one-tenth of the digest was applied to HPLC for separation of the tryptic peptides on a C_{18} μBondapack (Waters Associates, Inc.) column. The phosphoric acid-acetonitrile solvent system (system 1) described in Methods was used. Peptides were detected by the 220-nm absorbance of the effluent, at a full scale of 0.04. Identical HPLC profiles were obtained when the remaining part of the digest was applied to the same column, under the same conditions, for preparative purposes.

The tryptic peptide map shown in Fig. 3 was highly reproducible from experiment to experiment during the course of this work, provided that the digestion mixture was acidified before lyophilization and application to the HPLC column. We observed that without acidification, the reaction continued, which resulted in additional cleavages and different HPLC patterns.

Comparative tryptic peptide mapping. Samples of N-TTR, FAP-TTR, and AF_p were concurrently digested with trypsin. After an appropriate time interval, the reaction was stopped by acidification, and the peptide products were separated by HPLC, by using identical solvent and gradient conditions for the different proteins.

Fig. 4 shows the comparative tryptic peptide maps obtained with FAP-TTR (bottom panel) and with N-TTR (top panel). All the tryptic peptides were identified. The profiles for the two TTR preparations look nearly identical, except for a single subtle difference, namely a small peak, marked with an asterisk in Fig. 4 bottom panel, which eluted at ~61 min of the gradient in the FAP-TTR preparation. This tiny peak was consistently observed in each of four tryptic digests of FAP-TTR in experiments carried out during the course of this work. In contrast, it was never seen in five comparable digests of N-TTR, in both analytical and preparative-scale peptide maps.

Fig. 5 shows the tryptic peptide map obtained after the digestion of AF_p protein, in comparison to that of concurrently digested N-TTR. The AF_p peptide map showed a striking resemblance to that of N-TTR. There were, however, distinct differences. First, the AF_p peptide map showed the presence of a peptide peak not present in the N-TTR map. This "abnormal"
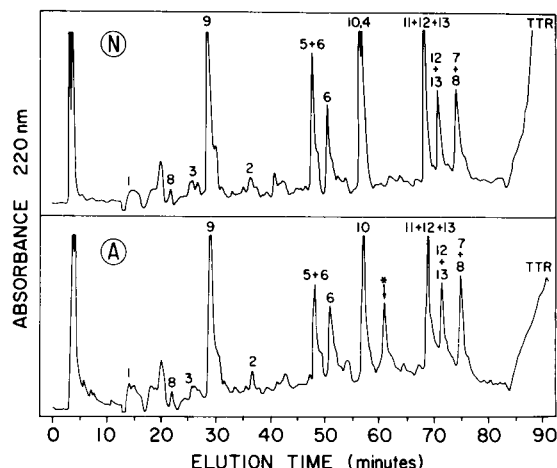
Figure 5. Comparative tryptic peptide maps of N-TRR (*upper, marked N*) and AF_p protein (*lower, marked A*). 1 mg of each preparation was digested with trypsin for 30 min and one-tenth of the digest was analyzed by HPLC using conditions identical to those used (and described) in Fig. 4.

peak, marked with an asterisk in Fig. 5 bottom panel, eluted at the same position as did the tiny "extra" peptide peak found previously in the FAP-TTR digest (also marked with an asterisk, in Fig. 4). This peak appeared in much larger concentrations in the AF_p digest in comparison to its counterpart in the FAP-TTR preparation. The second difference was that tryptic peptide 4 was absent from the AF_p preparation. This fact was firmly established by rechromatography, in buffer system 1a, of the fractions from the AF_p preparation that corresponded to the position of elution of tryptic peptide 4 of N-TTR. In this system (see inset, Fig. 3) peptide 4 was not detected with the AF_p preparation, in contrast with N-TTR. These two characteristic features of the AF_p tryptic peptide map compared with that of

N-TTR, namely the presence of an abnormal peptide and the absence of peptide 4, were consistently observed with every one of four tryptic digests of AF_p, and with AF_p preparations isolated from the two different patients.

The peptides obtained by digesting FAP-TTR and AF_p with trypsin were further compared with those obtained by tryptic digestion of N-TTR, by analyzing the tryptic digests by HPLC in a different solvent system (system 2, see Methods), with post-column fluorescence derivatization. With this system, only the lysine-containing peptides were detected, because of their higher reactivity with the o-PA reagent than the arginine peptides and because of the very small quantities of digests used. However, since only 3 of the 10 major tryptic peptides of TTR are arginine-containing peptides, system 2 gave useful information on the comparative chromatographic properties of the three preparations. Identical comparative tryptic peptide maps were observed for the lysine-containing peptides for the three different protein preparations in this second solvent system (data not shown). Thus, with two different solvent systems, the three proteins appeared to yield identical peptides on tryptic digestion, except for the specific differences noted in Figs. 4 and 5. These findings strongly suggest that the structures of the three proteins are largely identical, except for features responsible for the specific peptide differences seen.

Comparative kinetics of trypsin digestion. The structures of the three proteins (N-TTR, FAP-TTR, and AF_p) were compared further by comparing the rates at which different tryptic peptides appeared during digestion under identical conditions and at the same time. Samples of each of the three proteins were concurrently digested with trypsin, and portions of the digests were withdrawn after intervals varying from 15 to 90 min. The samples were analyzed by HPLC, and the relative amounts of the different tryptic peptides present in the digests were quantitated.

Fig. 6 shows the results of this experiment. Peptides 1, 2, and 8 were not detected with the quantities of material used
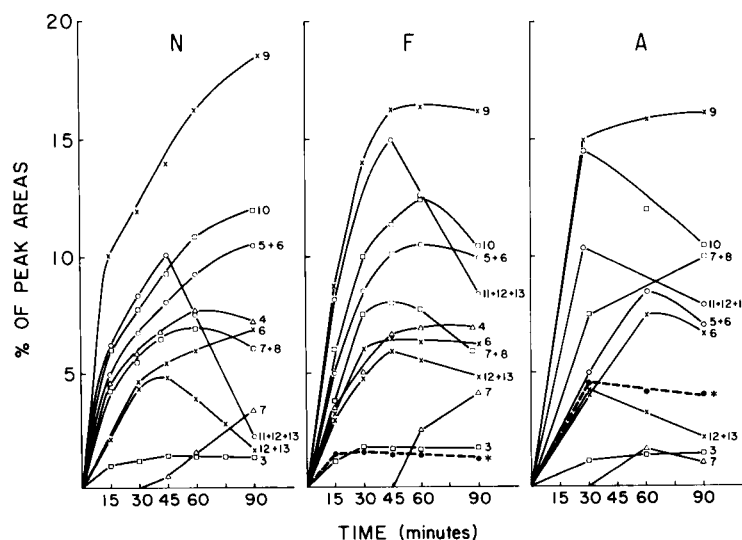

Figure 6. Kinetics of trypsin digestion of N-TTR, FAP-TTR, and AF_p protein. 1 mg of each protein was digested with trypsin and samples were withdrawn from the digest mixture at different times. The resulting peptides, from each timed interval, were subjected to HPLC analysis, under the conditions used for the comparative peptide maps (Figs. 4 and 5). On HPLC analysis, the area of each eluted peak (both for individual tryptic peptides and for undigested or partially digested TTR) was measured by an automatic integrator. For a given analysis, all peak areas were summed and the total considered to represent 100%. The area of each peptide peak was divided by the total sum of peak areas, to determine the percentage of peak area values plotted on the ordinate. In this figure, the number to the right of each plotted curve identifies which tryptic peptide is represented by the data points on the curved line.

for this study. As seen in Fig. 6, the patterns of appearance of the different individual tryptic peptides were generally similar for all three proteins. As discussed above, the "abnormal" peptide (asterisk) was readily observed in the AF_p digests, appeared in very small quantities in the FAP-TTR digests, and was not present in the N-TTR digests. Tryptic peptide 4 (TP-4) was absent from the AF_p digests, and present in the TTR digests.

The curves for peptides 12 + 13 and 11 + 12 + 13 decreased with time in a more pronounced manner than the curves obtained for the other peptides; this fact is related to chymotryptic cleavage that we observed to occur in tryptic peptide 12. These results further support the conclusion that was suggested from the comparative peptide mapping studies (Figs. 4 and 5), namely that the primary structures of the three proteins are probably identical except for differences connected with TP-4 and the "abnormal" peptide seen in the preparations from patients with FAP.

Analysis of tryptic peptide T*. Since the "abnormal" peptide (marked with an asterisk) eluted after peptide 4, which was absent from the AF_p tryptic digest, we suspected that the abnormal peptide might be closely related to TP-4. Accordingly, the individual tryptic peptides eluting from the HPLC column were collected and subjected to amino acid analysis.

Table II shows the amino acid compositions of TP-4 collected after HPLC separation of N-TTR and FAP-TTR digests, and of the asterisk-labeled peptide collected after HPLC separation of AF_p digests. The microscale level at which these latter peptides were analyzed (4–5 μg), together with possible small contamination with trypsin autodigestion products, probably contributes to the appearance of contaminating residues observed in all three proteins analyzed (Table II). The composition of the abnormal peptide (T*) closely resembled the composition of TP-4 from both N and FAP TTR preparations. A prominent difference was the presence of methionine, which was not seen in any of the TP-4 analyses.

The T* peptide was further examined by determining its carboxy-terminal sequence, in order to further document its close relationship to TP-4 (see Appendix for details). This sequencing study indicated that the carboxy-terminal sequence of the "abnormal" tryptic peptide T* is: . . . His-Val-Phe-Arg-COOH. This is also the COOH-terminal sequence for the normal peptide 4, documenting the close relationship between TP-4 and T*.

Cyanogen bromide cleavage. The apparent presence of methionine in peptide T* (Table II) was consistent with the finding that the overall amino acid composition of AF_p showed a higher content of methionine than did that of normal TTR (see Table I). Normal TTR possesses only one methionine residue, at position 13. CNBr treatment, therefore, cleaves the protein into two peptide fragments: a small peptide, residues 1–13 (called peptide C_1), and a large peptide, residues 14–127 (called peptide C_2). We reasoned that if another methionine residue was present in AF_p in the region of TP-4 (residues 22–34), CNBr treatment of AF_p would produce extra peptide fragments (as compared with TTR). Accordingly, samples of AF_p were subjected to CNBr

Table II. Amino Acid Composition of Tryptic Peptides TP-4 and T*

	Peptide: TP-4		T*
Protein:	N	F	A
Lys	0.2	0.3	0.3
His	1.0 (1)	0.9	1.0
Arg	0.8 (1)	1.2	1.0
Asp	1.1 (1)	1.3	1.9
Thr	0.4	0.4	0.6
Ser	0.9 (1)	1.3	1.6
Glu	0.8	0.9	1.0
Pro	1.1 (1)	1.0	0.9
Gly	1.2 (1)	1.7	2.2
Ala	1.9 (2)	1.9	2.3
Val	2.6 (3)	3.0	2.1
Met	—	—	0.3
Ile	0.8 (1)	0.9	1.2
Leu	0.3	0.6	0.8
Tyr	—	0.2	—
Phe	1.0 (1)	0.9	1.2
Cys	—	—	—

The symbols used for each protein are as follows: N, N-TTR, F, FAP-TTR; A, AF_p. The values listed are the relative amounts of each amino acid residue found on analysis of duplicate samples of each peptide. Values < 0.1 are not listed. The quantities of the peptides used for amino acid analysis are as follows: TP-4 12 μg (9 nmol) for N-TTR, 4.3 μg (3 nmol) for FAP-TTR. Peptide T* from AF_p: 5 μg (3.5 nmol). The numbers in parentheses represent the integer number of N-TTR taken from Kanda et al. (9), for residues 22–34 (TP-4).

treatment, followed by HPLC analysis and separation of the resulting peptide fragments. Samples of N-TTR and of FAP-TTR were treated and analyzed in an identical manner for comparative purposes.

Fig. 7 shows the comparative HPLC peptide maps obtained for each of the three proteins after CNBr cleavage. When intact TTR was analyzed under the same gradient conditions it eluted at the end of the gradient, mainly as a major peak at 66 min and a minor peak at 70 min.

N-TTR (Fig. 7, top panel) consistently showed an HPLC peptide pattern that consisted of a group of small peaks eluting between 6 and 15 min, and a group of larger, overlapping peaks eluting between 35 and 55 min. Distinct peaks were not observed between 15 and 30 min with normal TTR.

Normal TTR contains a cysteine residue at position 11; accordingly, since the N-TTR and FAP-TTR protein preparations had previously been alkylated with [³H]iodoacetic acid, fragment C_1 would be expected to be radioactive. The fractions eluted from the HPLC column were, therefore, collected and assayed for ³H-radioactivity. Radioactivity was detected at the beginning of the gradient, overlapping the early group of small peaks, with retention time of ~10–12 min. Radioactivity was also detected in late fractions corresponding to uncleaved TTR.
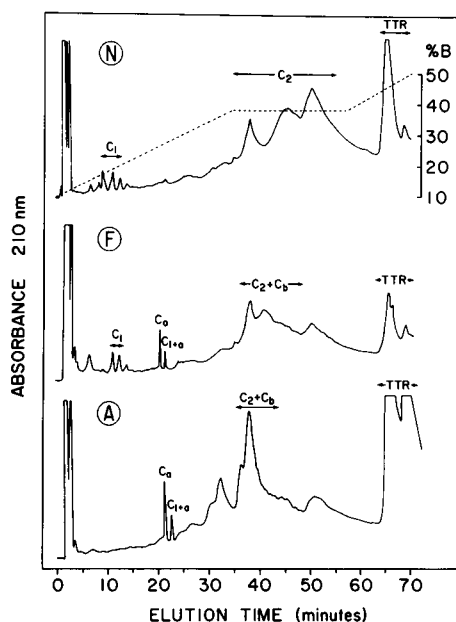
Figure 7. Comparative CNBr cleavage peptide maps of N-TTR (*N*), FAP-TTR (*F*), and AF_p (*A*). 1 mg of N-TTR and AF_p and 500 μg of FAP-TTR were concurrently digested with CNBr for 24 h. The resulting fragments were separated by HPLC by using a TFA-acetonitrile solvent system (See Methods). The peptides were detected by the 210 nm absorbance of the effluent at a full scale of 0.4. Gradient conditions as described in Methods are represented by a dashed line on the chromatogram shown at the top of the figure. The nomenclature of the peptides is discussed in the text.

More definitive identification of the CNBr fragments was achieved by amino acid analysis. Fragment C_1 was identified in the fractions overlapping the radioactive peak (eluting at ~10–13 min), both in the N-TTR and FAP-TTR preparations. In the AF_p profile shown in Fig. 7 (bottom tracing), this fragment (C_1) is not evident. However, in three other experiments carried out with AF_p over the course of this work, this peptide was evident on the chromatograms.

With N-TTR, the material eluting in the later, overlapping peaks at ~35–55 min was identified by amino acid analysis as fragment (peptide) C_2 (see Table III). This fragment (C_2) hence showed elution heterogeneity, probably due to amide differences and aggregation phenomena. The fractions corresponding to peptides C_1 and C_2 are so labeled in the top panel of Fig. 7.

In contrast to normal TTR, after CNBr cleavage of FAP-TTR or of AF_p, the HPLC peptide maps showed two sharp peptide peaks eluting at 21 and at 22 minutes (Fig. 7, middle and bottom panels, see peaks labeled C_a and C_{1+a}). These peaks were consistently found with FAP-TTR (five experiments) and with AF_p (four experiments), and were never seen with N-TTR. These peaks were collected and subjected to amino acid analysis. Homoserine lactone (Hsl in Table III) was found with both peaks, indicating that they both represented CNBr cleavage peptides with COOH-terminal methionine residues. The amino acid

composition of the first peak (C_a in Fig. 7), eluting at 21 min in the chromatogram, was very similar to that of a hypothetical peptide composed of residues 14–30, which would be generated by the presence of a methionine residue at position 30 (see Table III). The second extra peak, eluting at 22 min of the gradient, showed, on amino acid analysis, methionine and methionine sulfoxide (Met(o) in Table III) and carboxymethylcysteine; this peak also showed ^{3}H-radioactivity. The amino acid composition of this peptide (Table III) resembled that of a peptide composed of residues 1–30 with a methionine at position 30 and with an uncleaved methionine at position 13. This peak is, therefore, referred to as peptide C_{1+a}.

If AF_p (and in part FAP-TTR) contains a methionine residue at position 30, one would also expect to find a peptide fragment corresponding to residues 31–127 after CNBr cleavage. This "extra" CNBr fragment, which we refer to as peptide C_b, was not evident in the HPLC chromatograms (Fig. 7). We suspected, however, that this peptide was coeluting with uncleaved fragment C_2. Evidence for this conclusion came from the amino acid analysis of fractions from the AF_p preparation eluting in the normal position of the C_2 peptide (see Table III). These fractions from AF_p showed differences from the corresponding fractions from N-TTR, particularly decreases in valine and alanine content (which appear in relatively high amounts in peptide C_a), consistent with their being a mixture of C_2 and C_b.

To verify this hypothesis, SDS-PAGE was carried out on pooled fractions corresponding to the normal peptide C_2 (from N-TTR), and on pooled fractions from the AF_p preparation eluting at approximately the same position. The results are shown in Fig. 8. A single peptide band, of apparent molecular weight ~13,500–14,000 and representing fragment C_2, was seen in the normal TTR preparation. With the fractions from the AF_p preparation, however, two bands were observed: one corresponding to peptide C_2 and a band of apparent molecular weight ~12,000. This second band, never seen after CNBr treatment of normal TTR, corresponds to the predicted molecular weight of peptide C_b, and, therefore, represents the direct demonstration of the production of this "extra" CNBr fragment from AF_p. This experiment was repeated three times with identical results each time.

In an attempt to separate the CNBr fragments C_2 and C_b by HPLC and thus to isolate each individual fragment, we explored several types of columns, including reverse-phase, gel permeation, and ion-exchange supports, under various conditions. However, effective separation of these two fragments was not achieved, probably as a result of aggregation phenomena.

Two factors might account for the incomplete cleavages observed both for methionine 13 and for the putative methionine 30, namely, either the nature of the bond itself, or the oxidation state of methionine. It has been found (38) that CNBr cleavage of TTR is incomplete (~35%), even when the reaction is performed in the presence of guanidinium chloride and mercaptoethanol. The fact that methionine sulfoxide was seen in the chromatograms does not indicate that the methionine was originally present in an oxidized state. It has been shown recently

Table III. Amino Acid Composition of CNBr Peptides

Amino acid	Peptide C₂		N-TTR‡ 14–127	Peptide Cₐ		N-TTR‡ 14–30	Peptide C₁₊ₐ		N-TTR‡ 1–30
	N	A§		A	F		A	F	
Lys	7.2	6.3	7	1.0	1.0	1	1.9	1.4	2
His	4.0	3.5	4	0.1	0.2	—	0.3	0.2	—
Arg	4.0	3.5	4	1.0	0.8	1	1.0	1.2	1
Asp	8.4	7.7	8	1.9	2.1	2	3.1	2.0	2
Thr	10.0	9.4	10	0.2	—	—	1.8	1.5	2
Ser	8.8	8.3	10	1.1	1.3	1	2.6	1.5	2
Glu	11.6	11.6	11	0.4	0.6	—	2.7	1.1	1
Pro‖	4.2	4.8	7	0.7	(+)¶	1	2.4	1.2	3
Gly	8.8	7.6	7	1.3	1.9	1	4.6	3.6	4
Ala	11.6	8.8	12	2.5	2.8	3	3.2	2.4	3
Val	12.8	9.0	12	3.3	3.3	4	2.5	2.6	4
Met	—	0.2	—	—	—	—	—	(+)	1
Ile	4.8	4.3	5	0.8	0.8	1	1.1	0.9	1
Leu	6.4	6.3	6	1.0	1.1	1	2.0	1.3	2
Tyr	4.0	3.0	5	—	—	—	0.3	0.3	—
Phe	5.2	4.8	5	—	—	—	—	0.3	—
Cys	—	—	—	—	—	—	0.4	0.4	1
Hse**	—	—	—	0.8	0.2	—	0.2	0.1	—
Hsl**	—	—	—	0.8	0.8	1	0.8	0.6	1
Met(o)**	—	(+)	—	—	—	—	0.2	0.1	—
Amount‡‡ (µg)	17	13	—	2.7	1.3	—	2.7	4.6	—

* The symbols used for each protein are as follows: N, N-TTR; F, FAP-TTR; and A, AFₚ. The values listed are the relative amounts of each amino acid residue found on analysis of samples of each peptide. Values < 0.1 are not listed. ‡ The values listed for the three columns headed N-TTR represent the integer number of residues 14–127, 14–30, and 1–30, respectively, taken from the reported amino acid sequence of N-TTR (9). For the N-TTR 14–30 and 1–30 columns, the values listed are for hypothetical peptides 14–30 and 1–30 of TTR with a Met-for-Val substitution at position 30. § Peptide C₂ for the AFp preparation represents a mixture of peptide C₂ and the "extra" CNBr fragment of AFₚ referred to as Cᵦ, as demonstrated both by gel analysis (see Fig. 8) and NH₂-terminal amino acid analysis. This fact probably accounts for certain discrepancies in amino acid composition found for this fragment, relative to the C₂ fragment from the N-TTR preparation, namely its reduced valine and alanine contents. ‖ Low proline values were obtained in most of the samples, probably arising from destruction during acid hydrolysis. ¶ "+" means that the amino acid was definitely detected in the chromatogram, but in small amounts; under the integration parameters used, no numerical value was obtained. ** Hse, homoserine; Hsl, homoserine lactone; Met(o), methionine sulfoxide. ‡‡ The quantities of each peptide used for amino acid analysis, in micrograms, are listed in this row.

that the oxidation of methionine to methionine sulfoxide is a side reaction of the CNBr cleavage itself, and may, therefore, interfere with the reaction (39). The fact that both methionine and methionine sulfoxide were detected in fractions corresponding to peptide C₂ in the AFₚ preparation, however, in contrast with the normal composition of C₂, corroborated the hypothesis of the presence of an extra methionine in AFₚ.

The results obtained from these CNBr cleavage studies, therefore, supported the hypothesis that the AFₚ protein differed from normal TTR by having a methionine residue at position 30. Evidence in support of this conclusion was then obtained by microsequencing studies of both peptide Cₐ and of Cᵦ (see Appendix for details).

NH₂-terminal sequence analysis of peptide Cₐ, for five analyzed positions, indicated that its sequence corresponds to the NH₂-terminal sequence of normal peptide C₂, residues 14–18 of TTR (see Fig. 9). COOH-terminal sequence analysis of Cₐ gave the following results: . . . Ile-Asn-Val-Ala-Met-COOH. This sequence corresponds to that of residues 26–29 of normal TTR, with a methionine-for-value substitution at position 30. Thus, the NH₂- and COOH-terminal sequence analyses supported the hypothesis that Cₐ represents a peptide comprised of residues 14 to 30 of TTR, but with a methionine at its COOH-terminus (position 30).

To further confirm this conclusion, we carried out comparative NH₂-terminal sequencing with the normal CNBr peptide C₂ and with a mixture of AFₚ CNBr fragments Cᵦ and uncleaved C₂ (see Appendix). With the preparation from the AFₚ protein, in addition to the residues expected for the NH₂-terminal sequence of peptide C₂, valine was found in the second
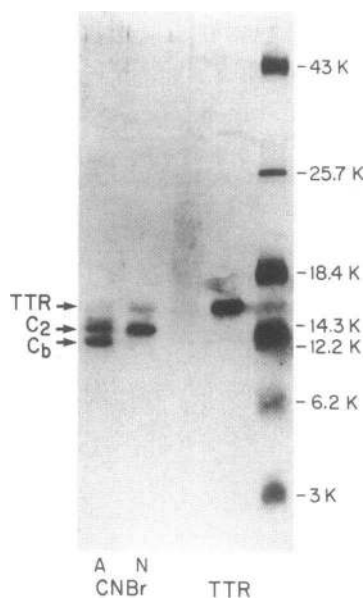
Figure 8. SDS-slab gel electrophoretic analysis of the CNBr fragments of N-TTR and AF$_p$ protein. The two proteins were cleaved with CNBr and the resulting fragments were separated by HPLC as shown in Fig. 7. Aliquots of pooled fractions corresponding to peptide C$_2$ from the N-TTR preparation (gel labeled *CNBr-N*) and from pooled fractions of the AF$_p$ preparation eluting at the same position (gel labeled *CNBr-A*) were subjected to SDS-PAGE, on 15% acrylamide gels, according to the methods of Laemmli (31). The bands were detected by silver staining (32). The gel labeled TTR represents an intact, untreated sample of N-TTR. The right hand gel shows molecular weight standards from BRL.

cycle and a large yield of phenylalanine was found in the third cycle. These residues presumably represent the second and the third NH$_2$-terminal residues of C$_b$, corresponding to Val-32 and Phe-33 of TTR (see Fig. 9). Thus, these data further support the conclusion that AF$_p$ has a Met at position 30, and that CNBr cleavage of AF$_p$ yields peptides C$_1$ (residues 1–13), C$_a$ (residues 14–30), and C$_b$ (residues 31–127).
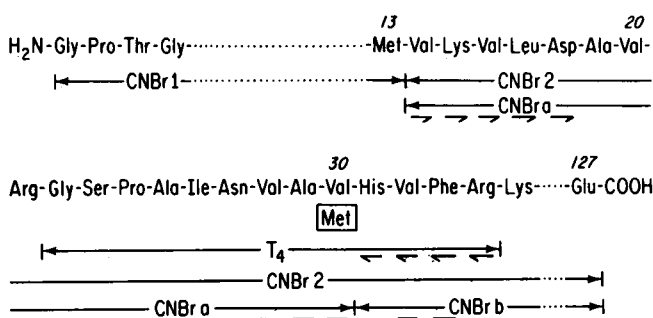

Figure 9. Partial amino acid sequence of the human TTR subunit taken from Kanda et al. (9). Symbols: Half arrows pointing to the right, sequence determined by manual Edman degradation (28); half arrows pointing to the left, sequence determined by the use of carboxypeptidases (29). The nomenclature of the peptides in this figure is as follows: T$_4$, tryptic peptide 4; CNBr 1 and 2, cyanogen bromide fragments of normal TTR; CNBr a and b, additional cyanogen bromide fragments of amyloid fibril protein AF$_p$. The Met residue (enclosed in a box) below Val 30 indicates the difference found between AF$_p$ and TTR, from the studies reported in this paper.

Discussion

The studies reported here are part of a project that has, as its long-term objective, the definition of the genetic and biochemical abnormalities involved in the etiology of the Portuguese type of FAP. The specific goals of the present studies were, first, to develop improved methods for the solubilization and isolation of pure and undenatured amyloid fibril protein, and then to characterize the amyloid fibril protein, and compare it with TTR, with regard to a variety of physical and chemical parameters. In particular, we aimed to compare the primary structure of the amyloid fibril protein with that of normal plasma TTR and of plasma TTR from FAP patients.

As reported here, a method was developed for the solubilization of the amyloid fibril preparation in a "physiological" buffer, and the purification of the AF$_p$ by affinity chromatography on RBP linked to Sepharose. Previous studies of amyloid fibril protein in FAP have all used amyloid protein isolated and solubilized under denaturing conditions. Although such preparations are suitable for studies of primary structure, they cannot be used to explore many physical-chemical and other structural properties of the amyloid fibril protein. The method reported here proved to be an effective and novel procedure for the isolation of undenatured AF$_p$ protein. Apart from demonstrating that the AF$_p$ protein shares with normal TTR its binding properties for RBP, the method provided us with a pure and intact protein suitable for in-depth investigation.

Studies of the physical-chemical properties of the purified AF$_p$ focused particularly on its ability to form a stable tetrameric structure, and on its binding properties for thyroxine and for RBP. SDS-PAGE under nondissociating conditions revealed that the purified AF$_p$ migrated almost entirely as a band with molecular weight corresponding to the TTR tetramer. The ability of the purified AF$_p$ to form tetramers was confirmed by studies with labeled thyroxine that demonstrated that AF$_p$ has binding affinity for thyroxine. The binding of iodothyronine molecules by TTR requires the tetrameric quaternary structure of the protein, since the tetrameric molecule has a channel running through the center of its long axis in which the two binding sites for thyroxine are located (37). Finally, the affinity chromatography procedure, on RBP linked to Sepharose, showed that AF$_p$ protein had binding properties for RBP very similar to those of normal TTR (19, 20). Thus, the AF$_p$ protein resembled plasma TTR in forming a stable tetrameric structure, and in its binding affinity for thyroxine and for RBP.

The structural studies reported here demonstrate that the amyloid fibril protein AF$_p$, purified from the tissues of two Portuguese patients who died with FAP, comprised a TTR variant with a methionine for valine substitution at position 30. The evidence for this conclusion included studies that involved: (*a*) comparative tryptic peptide mapping of AF$_p$ and plasma TTR by reverse-phase HPLC; (*b*) comparative peptide mapping after cyanogen bromide cleavage; and (*c*) amino acid microsequence analyses of aberrant CNBr and tryptic peptides.

In developing this work, the structural difference between

AF$_p$ and TTR was first localized to a single tryptic peptide (peptide TP-4) by comparative HPLC mapping of tryptic peptides. This method, comparative HPLC peptide mapping followed by sequence analysis of identified aberrant peptides, constitutes a relatively simple and powerful experimental approach to the study of variant forms of a given protein. For example, this approach was successfully used recently to identify the molecular abnormality in a mutant form of the human enzyme hypoxanthine-guanine phosphoribosyltransferase (40). The comparative tryptic peptide maps suggest that a second structural alteration in AF$_p$, as compared with normal TTR, is highly unlikely to be present, since all of the other tryptic peptides from AF$_p$ displayed exactly the same retention properties on the HPLC column as did the corresponding peptides from TTR. Moreover, the tryptic peptides were also screened by HPLC with a second solvent system, with different pH and elution properties; no additional peptide differences between AF$_p$ and TTR were observed. Further evidence that the structures of AF$_p$ and TTR are probably identical except for differences associated with TP-4 was obtained from the study comparing the kinetics of trypsin digestion of AF$_p$ with that of TTR (Fig. 6). The patterns of appearance of the different individual tryptic peptides were quite similar for both proteins.

Because of insufficient starting material, we were unable to isolate sufficient quantities of the abnormal tryptic peptide to permit direct Edman NH$_2$-terminal sequence determination through position 30. Despite this limitation, the sequence analysis studies conducted on peptide T* and on the CNBr fragments C$_a$ and C$_b$ provide strong evidence for a methionine-for-valine substitution at position 30 in AF$_p$ as compared with normal TTR. Nevertheless, it should be noted that the possibility of another mutation being present as well in the amyloid fibril protein, but not detected in this work, cannot be excluded. As discussed above, we consider this possibility to be unlikely for the purified AF$_p$ protein. No structural information was obtained for the peak I protein, which did not bind to the RBP affinity column. Although the peak I protein shows TTR immunoreactivity, it is conceivable that the TTR variant with a Met-for-Val substitution at position 30 might be preferentially selected for by study only of the peak II protein.

Amino acid substitutions in a protein may have drastic effects on its structure and function, or may cause little or no change in its properties. Since the isolated AF$_p$ protein resembled plasma TTR in forming a stable tetrameric structure, and in its binding affinity for both RBP and thyroxine, we can conclude that the methionine-for-valine substitution at position 30 in AF$_p$ does not substantially alter any of those aspects of the structure of TTR that are involved in formation of the tetrameric structure, or in the interactions of TTR with RBP or thyroxine.

The structure of the TTR subunit is dominated by eight β-strands, into which ~50% of the amino acids are organized, as two four-stranded β-sheets (37). The sheets are labeled A to H, according to their position in the sequence, and form two sheets, DAGH and CBEF, whose hydrogen bond interactions are predominantly antiparallel (37). The residues contained be-

tween the two sheets are almost exclusively hydrophobic and constitute the core of the subunit. The monomers are linked into stable dimers by further antiparallel β-sheet interactions, and the dimers are assembled into complete tetrameric molecules with equivalent β-sheets from each dimer opposed at the center of the molecule. This extensive β-sheet structure of TTR makes the molecule potentially amyloidogenic, since amyloid fibrillar proteins, from all forms of amyloidosis, are characterized by a β-pleated sheet configuration (41–43).

Valine 30 of TTR is located on β-strand B and is packed into the subunit core located between the two β-sheets. The substitution of a methionine for a valine at this position must cause some degree of structural perturbation in this part of the molecule. It is perhaps surprising that the structural distortions produced by this substitution did not appear to have a major effect on either the ability of the variant TTR (i.e., AF$_p$) subunit to form a stable tetramer, or on its interaction with either RBP or thyroxine. X-ray crystallographic studies of purified AF$_p$, in order to compare its full three-dimensional structure with that of TTR, would be of great interest.

Pras et al. (44) have reported recently that the amyloid fibril protein from tissues of a deceased Jewish patient with FAP consisted largely of a variant TTR subunit with a glycine-for-threonine substitution at position 49, along with variant TTR fragments 1–48 and 49–127. Threonine 49 in TTR is located on β-strand C of the core of the TTR subunit, in close proximity to valine 30. In fact, threonine 49 is one of the amino acids whose side chain is in contact with that of valine 30 in the three-dimensional structure of the protein (37). The close stereochemical relationship of residues 30 and 49, the two residues now found to be substituted in TTR-related amyloid proteins, raises the possibility that these residues are part of a structural domain which, if altered, is critically involved in the pathogenesis of FAP. The possibility that such a domain might be important in some as yet unknown aspect of TTR metabolism can also be considered. For example, it has been suggested that TTR may play some specific role in the peripheral nervous system (45). It has also been suggested that a circulating abnormal TTR molecule in FAP patients might be toxic to peripheral nerve tissues even before it is aggregated into sizable amyloid deposits (6). In this regard it should be noted that peripheral nerve fiber changes have been observed in some asymptomatic children of Portuguese patients with FAP (46).

No normal TP-4 was detected in the AF$_p$ preparation. Small amounts of this peptide, comprising <10% of the aberrant tryptic peptide T*, might well have gone undetected, however, with the methods used. Thus, we cannot exclude the possibility that a small proportion of normal TTR subunits might have been present, and not have been detected, along with the variant TTR subunits, in the amyloid fibril protein. In this regard it should be noted that Pras et al. (44) observed a 10–20% recovery of threonine at position 49, along with a much larger proportion of glycine, in their amyloid protein preparation.

Both normal TP-4 and the variant peptide T* were observed in the peptide maps of TTR from pooled sera of FAP patients.

In several peptide mapping studies carried out with FAP-TTR, during the course of this work, the normal TP-4 was approximately four to six times more abundant than the aberrant peptide T*. Thus, in these patients, two forms of TTR subunits (present as tetrameric TTR molecules) circulated in plasma, the variant TTR subunit with a methionine-for-valine substitution at position 30, and a larger amount (four to six times) of normal TTR. It is likely that these patients were genetically heterozygous with respect to TTR, and had two TTR alleles, one coding for the normal TTR subunit and the other for the variant TTR subunit. Very recently, after the initial presentation (12) of the present work, a brief communication appeared reporting the presence of a variant TTR in plasma with the same structural alteration (methionine for valine at position 30) as that described here, in a patient of Swedish origin with FAP (47). It is not yet known whether, in these Swedish patients, this variant TTR is also the amyloid fibril protein that deposits in their tissues; earlier studies on amyloid fibril protein from the same kindred (4) suggested that other differences in primary structure, compared with that of TTR, were present. In the present work, the reason for the relatively reduced amount of the variant TTR, compared with that of normal TTR, in FAP serum, has not been determined. One explanation might be a reduced rate of synthesis of the variant as compared with the normal TTR subunit (i.e., reduced gene expression of the allele for the variant TTR subunit). Alternatively, both TTR subunits might be produced at similar rates, with several forms of hybrid TTR tetramers circulating in plasma, but a particular species containing the variant TTR might be removed more rapidly than normal TTR from plasma, either because of more rapid degradation and/or its deposition in tissues. Any of these possibilities would be consistent with our previous finding (8) of a reduced plasma level of total TTR in Portuguese patients with FAP. Since the amyloid fibril protein AF_p was comprised almost entirely of the variant TTR, we suggest that more rapid removal of this species of TTR from plasma, with its selective deposition in tissues, was likely to have been present.

The detailed mechanisms whereby the alteration in the primary structure of TTR leads to the pathogenesis of FAP remain to be determined. The amino acid substitution reported here is, however, most probably the specific underlying biochemical cause of the disease. Thus, the disease is transmitted in a typical autosomal dominant manner, both forms of TTR (normal and variant) were found in plasma, and the abnormal (variant) form was found in the amyloid fibrils deposited in tissues. It is likely that the methionine-for-valine substitution represents a point mutation within the coding sequences of the TTR structural gene. Although the codon for valine 30 of human TTR has not been defined, one of the possible codons for valine is GUG, which could be converted to a codon for methionine by a single nucleotide change (GUG → AUG). Alternatively, as pointed out (44), human TTR may be genetically polymorphic; and the variant TTR may represent an isotype of the normal form. Polymorphism of TTR is known to occur in the Rhesus monkey (48, 49), in which it has been shown that the Rhesus TTR is

under the control of two autosomal codominant alleles. We believe that this latter possibility is unlikely for the human, particularly since two different variants of TTR (12, 44) (present work) have now been found specifically as components of amyloid fibril protein in FAP.

Although slightly different phenotypic clinical expressions exist among the different ethnic and geographic varieties of FAP, these syndromes all exhibit prominent peripheral neuropathy (1). Future studies are needed to determine whether or not these different varieties of FAP represent different mutations in the TTR molecule. That genetic heterogeneity at the TTR locus can occur in FAP of autosomal dominant inheritance, whereby different mutations on the same molecule produce a similar clinical picture, is indicated by the studies of the amyloid protein of a Jewish patient with FAP (44) along with the work on Portuguese patients reported here. Studies of amyloid proteins and serum TTR from FAP patients of other origins are needed in order to further define the extent of possible genetic heterogeneity. The methodology presented here could be applied effectively to this problem, by using comparative HPLC mapping of tryptic peptides to screen for possible alterations in the primary structure of TTR in the different varieties of FAP. In addition, these methods could be used to screen family members of FAP patients as to whether or not the variant TTR is present in their plasma. We are currently engaged in such studies on TTR from individual FAP patients and their asymptomatic children, in order to try to define whether or not the presence of the TTR variant can be used as a preclinical biochemical marker of the disease. Such a marker might ultimately be important in attempts at prevention or early treatment of this disease.

Appendix

This Appendix provides the detailed results of the studies carried out concerning the peak I protein from the solubilized amyloid fibrils, and of the microsequencing studies carried out on the AF_p (peak II) protein.

PAGE and immunological analyses of the solubilized amyloid fibril. Fig. 10 *A* (left-hand panel) shows the results of SDS-PAGE analysis of the material eluted in peaks I and II from the RBP-Sepharose affinity column. The conditions used were those in which normal TTR (reduced and carboxymethylated) is mostly dissociated into monomeric subunits, with a small amount of dimer being present. The peak II material (AF_p protein) migrated as a major band of molecular weight ~15,000, and a minor band of ~32,000 mol wt (Fig. 10 *A*, gel 2). Hence, as discussed above, AF_p protein showed electrophoretic properties that were virtually identical to those of TTR.

In contrast, peak I material showed several bands of protein on SDS-PAGE under the same conditions (Fig. 10 *A*, gel 1). Thus, major bands were seen at molecular weights of ~15,000 and 32,000, and minor bands at ~42,000 and 54,000; material of higher molecular weight was seen as well. Furthermore, an identical pattern was observed when PAGE was conducted under conditions which do not normally lead to dissociation of the TTR tetramer. Similar components (bands of varying molecular weight) have been observed in amyloid fibrils from type I FAP patients of Swedish and Japanese origin (3, 4).

It was noted that the apparent molecular weight values for the protein bands seen in the peak I material (Fig. 10 *A*, gel 1) were roughly multiples
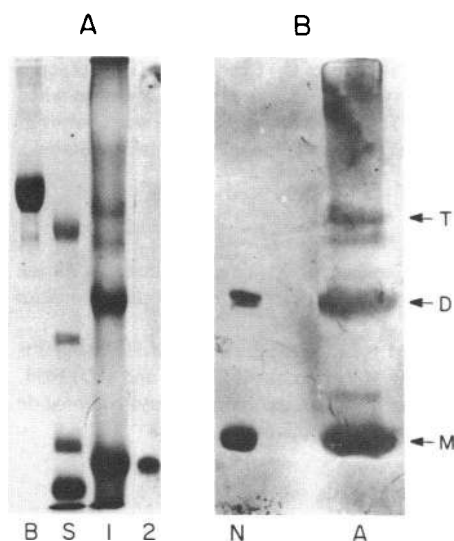
Figure 10. Gel analysis and immunoanalysis of amyloid fibril proteins: (A) SDS-PAGE in 10% slab gel (31), of material eluted from the RBP affinity column in peak I (gel 1) and in peak II (gel 2). Gels B and S show the simultaneous runs of molecular weight standards: gel B: BSA, 67,000; gel S: mixture consisting of (from top to bottom) ovalbumin, 45,000, chymotrypsinogen, 25,000, β-lactoglobulin, 18,000 and lysozyme 14,000. The lowest band in gels S and 1 represents the tracking dye, rather than a lower molecular weight protein band. (B) Immunoblotting study of peak I proteins (lane A) and of reduced and carboxymethylated TTR (lane N). The proteins were subjected to PAGE (A), and were than transferred to nitrocellulose paper, and stained specifically for human TTR. The arrows at the right hand side, labeled M, D, and T, represent the positions expected for TTR monomers, dimers, and tetramers, respectively.

of the monomeric TTR molecular weight. We therefore hypothesized that the several bands might all represent TTR-related amyloid fibril protein, but with the subunits aggregated to varying degrees. To investigate this hypothesis, the immunological relationship of the several bands to TTR was explored by the immunoblotting technique. Thus, the protein bands were transferred from the gel to nitrocellulose paper, and then specifically stained for immunoreactive TTR as described in Methods. The results are shown in Fig. 10 B. All of the observed bands in the peak I material, including the high molecular weight material, were TTR immunoreactive, suggesting that they all represented TTR-related amyloid protein, but aggregated to various degrees. These results suggest that a protein related to TTR may be the predominant, or almost the sole, protein component of the amyloid fibril.

Carboxy-terminal sequence of the abnormal tryptic peptide. COOH-terminal microsequence analysis was performed on peptide T* by the simultaneous use of carboxypeptidases A and B. A time course of digestion was performed, and the released amino acids were analyzed and quantitated by high sensitivity HPLC amino acid analysis, with precolumn derivatization. Fig. 11 shows the curve for the progressive release of the individual amino acids. These curves indicate that the carboxy-terminal sequence for the abnormal tryptic peptide T* is . . . His-Val-Phe-Arg-COOH.

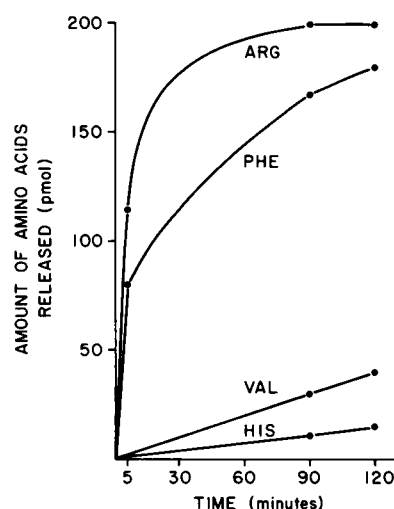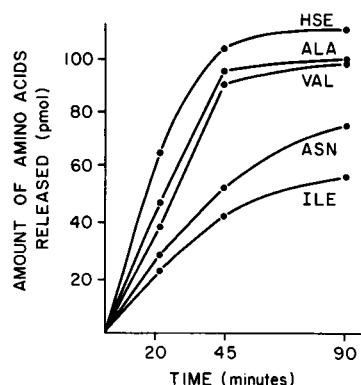*NH₂-terminal sequence of CNBr peptide Cₐ.* NH₂-terminal analysis

Figure 11. COOH-terminal sequence analysis with carboxypeptidases A and B of tryptic peptide T*. 2 nmol of peptide T* were incubated with carboxypeptidases A and B at an enzyme:substrate ratio of 2:1 for carboxypeptidase A and of 1:1 for carboxypeptidase B. At the indicated times, samples were withdrawn from the incubation mixture, and were immediately injected into the HPLC for detection and quantitation of the released amino acids, using precolumn fluorescence derivatization (see Methods) to achieve high sensitivity. After subtraction of the blank values (see Methods) the amounts of the released amino acids were plotted versus time of digestion.

of peptide Cₐ was performed by manual Edman microsequence analyses (see Methods). The phenylthiohydantoin-amino acid derivatives were hydrolyzed and the free amino acids quantitated by amino acid analysis using precolumn derivatization. Table IV shows the amount of the individual amino acids obtained after five cycles. These data indicate that the NH₂-terminal sequence of Cₐ is as follows: H₂N-Val-Lys-Val-Leu-Asp. . . .

Table IV. Identification of the NH₂-Terminal Amino Acid Sequence of CNBr Peptide Cₐ

Cycle	Val	Lys	Val	Leu	Asp	Amino Acid
1	370	—	—	—	—	Val
2	46	82	—	—	—	Lys
3	—	29	382	—	—	Val
4	—	—	—	103	—	Leu
5	—	—	—	13	56	Asp

2 nmol of peptide were used in this microsequence analysis. The phenylthiohydantoin-amino acid derivatives were hydrolyzed and the free amino acids were detected by amino acid analysis using precolumn fluorescence derivatization. Quantitation was performed as described in Methods and is expressed here in picomoles of amino acid detected. The underlined amino acid values represent the proposed NH₂-terminal sequence for peptide Cₐ.

Figure 12. COOH-terminal sequence analysis with carboxypeptidase A, of CNBr peptide C_a. Peptide C_a, resulting from the cleavage with CNBr of AF_p, was isolated by HPLC (see Fig. 7), lyophilized, and incubated with carboxypeptidase A; at the indicated times, samples were withdrawn from the incubation mixture, and were immediately injected into the HPLC for detection and quantitation of the released amino acids. (See legend to Fig. 11 for further details.)

COOH-terminal sequence of CNBr peptide C_a. End group analysis of C_a with carboxypeptidase A was performed in order to elucidate its COOH-terminal sequence. Fig. 12 shows the curves obtained for the progressive release of the individual amino acids, giving a COOH-terminal sequence as follows: . . . Ile-Asn-Val-Ala-Met-COOH. The same results were obtained with C_{1+a}.

NH_2-terminal sequence of CNBr peptide C_b. Comparative NH_2-terminal sequencing was performed with the normal CNBr peptide C_2 and with a mixture of AF_p CNBr fragments C_b and uncleaved C_2, by the manual Edman microsequence analysis. Table V shows the amounts of the individual amino acids obtained after three cycles of degradation. With the normal C_2 peptide, an NH_2-terminal sequence of H_2N-Val-Lys-Val . . . was obtained, corresponding to the expected sequence for residues 14, 15, and 16 of TTR (see Fig. 9). With the preparation from the AF_p protein, however, in addition to the residues expected for the NH_2-terminal sequence of peptide C_2, other residues were also found

Table V. Identification of the NH_2-Terminal Amino Acid Sequence of CNBr Peptide C_b

Cycle	Peptide‡	Amino acid residue*		
		Val	Lys	Phe
1	Nc	84	—	—
1	Ac	63	—	—
2	Nc	—	34.4	—
2	Ac	29	8.5	—
3	Nc	55	—	—
3	Ac	218	—	574

* Quantitation of the individual amino acids was performed as described in Methods section, and is expressed here in picomoles of amino acid detected.
‡ Fractions from the HPLC separations shown in Fig. 7 were used for these analyses. Peptide Nc was the purified C_2 peptide derived from pooled fractions of the N-TTR preparation, labeled C_2 in Fig. 7, top tracing. Peptide Ac was a mixture of peptides C_2 + C_b derived from the pooled fractions of the AF_p preparation labeled C_2 + C_b in Fig. 7, bottom tracing.

in the second and third cycles. Thus, in the second cycle valine was found in addition to lysine, consistent with it representing Val 32 of TTR (second NH_2-terminal residue of C_b) (see Fig. 9). The recoveries of this cycle were, however, very low, which is not uncommon when manual techniques are used. The results of the third cycle were, however, definitive, with a large yield of Phe, representing Phe 33, being obtained.

Acknowledgments

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