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# Degradation and Deposition of Amyloid AA Fibrils Are Tissue Specific†

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ABSTRACT: The complete amino acid sequences of two related AA proteins ($M_r$ 9700 and 5300) derived from thyroid tissue from a patient, NOR, with the autosomal recessive disease familial Mediterranean fever were determined. Heterogeneity found at position 52 indicates these proteins are fragments of two allelic or isotypic SAA precursor molecules similarly degraded at unusual sites and deposited in the thyroid. Degradation appears to be tissue and/or enzyme(s) specific since the carboxy terminus of both fragments is Ala-Ala and is different from other AA amyloid fibrils extracted from various tissues in different patients. Electron micrographic studies reveal these fragments retain the characteristics of native amyloid fibrils under physiological conditions even after exposure to dissociating agents.

Amyloidosis comprises a heterogeneous group of diseases characterized by the systemic or localized extracellular deposition of proteinaceous fibrillar material in various tissues and organs. These fibrils can be isolated by flotation at low ionic strength and are composed of low molecular weight subunits which tend to aggregate. Fibrils have a $\beta$-pleated sheet conformation by X-ray crystallography and assume a green birefringence when stained with Congo red and viewed under a polarizing microscope.

There are four major types of systemic amyloidosis. Primary amyloidosis is seen in association with multiple myeloma and other plasma cell dyscrasias. The major fibrillar protein, AL, is the amino-terminal variable region or the intact light chain of immunoglobulin light chains (Glenner et al., 1970). Chronic infectious and inflammatory disease, as well as familial Mediterranean fever, may be associated with secondary amyloidosis. Here, the fibril subunit protein, AA (Levin et al., 1972; Sletten & Husby, 1974), is believed to form as a result of the enzymatic cleavage of an acute-phase reactant, serum AA protein. Systemic amyloid may be associated with some familial amyloidotic polyneuropathy (FAP) syndromes all of which have an autosomal-dominant mode of inheritance (Costa et al., 1978; Pras et al., 1981; Skinner & Cohen, 1981). FAP types 1 and 2 and senile cardiomyopathy (Westermark et al., 1977; Gorevic et al., 1980a) have been shown to be due to fibrils composed of prealbumin.

Recently, a novel form of amyloidosis has been described in patients on long-term hemodialysis (Gejyo et al., 1985; Gorevic et al., 1985). Fibrils consist of polymers of normal intact $\beta_2$-microglobulin, clinically presenting as tumoral masses in bone or causing peripheral nerve entrapment. Other tissues, such as skin and blood vessels, may also be affected, indicating the systemic nature of the disease. Localized forms of amyloid restricted to the central nervous system include hereditary cerebral hemorrhage with amyloidosis (HCHWA) due to a variant of cystatin C (or $\gamma$ trace) (Cohen et al., 1983; Ghiso et al., 1986) and the $\beta$-protein in cerebrovascular and senile plaque core amyloid in Alzheimer's disease and Down's syndrome with dementia (Glenner & Wong, 1984; Masters et al., 1985).

Amyloid A protein (AA) is a heterogeneous proteolytic cleavage fragment of a larger precursor. The complete sequences and the molecular weight determinations of a number of partially sequenced AA proteins showed that most of them are 76 residues long although proteins of different lengths [5–13.4 kilodaltons (kDa)] have been reported (Ein et al., 1972; Levin et al., 1972; Sletten et al., 1976; Isobe et al., 1977, 1980; Husby & Sletten, 1980; Lian et al., 1980; Moyner et al., 1980; Van Rijswijk, 1981). Whether this diversity reflects fragmentation during isolation or different sites of proteolytic cleavage of the precursor, SAA, is still unresolved. SAA is part of $HDL_3$ and circulates as a complex of approximately 180 000 daltons with $\alpha$ 1–2 electrophoretic mobility (Benditt et al., 1979). Upon denaturation a 12 000-dalton protein component with AA cross-reactivity is derived. SAA is the presumptive precursor of AA amyloid (Anders et al., 1975; Linke et al., 1975; Rosenthal et al., 1976).

There are two major and four minor isotypes of human SAA (Eriksen & Benditt, 1980; Bausserman et al., 1980). The amino acid sequence of SAA1 (Parmelee et al., 1982) has two allelic forms, $\alpha$ and $\beta$, with a double substitution of alanine for valine and valine for alanine at positions 52 and 57, respectively. Partial sequence analysis of SAA2 shows that it

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lacks the N-terminal arginine; however, the following 30 residues are identical with SAA1 (Bausserman et al., 1980). Four human cDNA clones have been identified, and three of these have been completely sequenced. The deduced amino acid sequence of pA1 (Sipe et al., 1985) is identical with SAA1$\alpha$; SAAg9 (Woo, 1986) is homologous to the first familial Mediterranean fever amyloid A protein sequenced (Levin et al., 1972), and pSAA82 (Kluve-Beckerman et al., 1986) is not expressed thus far.

Although two major isotypes of SAA have been identified in several inbred strains of mice, murine amyloid A fibrils contain fragments only of the SAA2 isotype (Hoffman et al., 1984). Recently, the cDNA sequences for these two isotypes were reported: SAA1 and SAA2 cDNAs encode proteins of 103 amino acids with 91% protein sequence homology (Yamamoto & Migita, 1985; Lowell et al., 1986). A third SAA3 gene and cDNA encoding an SAA isotype which is 70% homologous to the SAA1 and SAA2 isotypes and 76% homologous to human SAA was reported by Stearman et al. 1986). Although comparative studies of SAA and AA proteins in humans and many animals, as well as the sequence of three murine serum amyloid A genes, reveal polymorphism and heterogeneity, the SAA structure is largely conserved phylogenetically (Hermodson et al., 1972; Skinner et al., 1974; Eriksen et al., 1976; Anders et al., 1977; Gorevic et al., 1977; Waalen et al., 1980; Benson et al., 1985; DiBartola et al., 1985), implying that the protein has an important biological function. The amino-terminal region is the site of greatest diversity, followed by a highly conserved region, residues 32–54, that is present in all vertebrates studies.

In this paper, we present the primary structure of two related proteins, derived from tissue obtained at the time of thyroidectomy, of patient NOR with familial Mediterranean fever. Study of the NOR proteins provides direct evidence in man that more than one SAA molecule may deposit in tissue to form amyloid fibrils. These fibrils in turn consist of fragments of different length that copolymerize. The implications of these findings in the setting of a genetically determined reactive amyloidosis are discussed.

## Materials and Methods

*Protein Isolation and Purification.* Amyloid fibrils were isolated by the method of Pras et al. (1968) from thyroid tissue. Fibrils were solubilized in 3 mL of 6 M guanidine hydrochloride/0.1 M tris(hydroxymethyl)aminomethane (Tris)/0.17 M dithiothreitol, pH 10.2, and stirred for 48 h at room temperature. Then 1 mL of 2 M guanidine hydrochloride/4 M acetic acid was added, and the solution was applied to a column consisting of a 1:1 mixture of Sephadex G-75 and Sephadex G-100, 2.5 × 180 cm, equilibrated with 5 M guanidine hydrochloride/1 M acetic acid. Individual peaks were pooled and dialyzed exhaustively against distilled water before lyophilization.

Purity and molecular weight of fractions were determined on 17% polyacrylamide gels containing 0.1 sodium dodecyl sulfate (Laemmli, 1970). For Western blots after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the proteins were transferred to a nitrocellulose membrane in a Bio-Rad Trans-Blot cell at 4 °C for 3 h (150 V, 550 mA) using 25 mM Tris/192 mM glycine buffer (pH 8.3)/20% (v/v) methanol (Towbin et al., 1979). Unbound sites were blocked with 0.2% gelatin in phosphate-buffered saline, pH 7.0. Immunoreactive bands were visualized by color development with 3,3′-diaminobenzidine and hydrogen peroxide. Rabbit anti-SAA or anti-AA antisera were used as a first antibody, and peroxidase-labeled goat anti-rabbit antiserum was used as the second antibody.

*Enzymatic Digestion.* Peaks III and IV were dissolved (1 mg/mL) in 0.2 M ammonium bicarbonate, pH 8.2, and incubated (enzyme to substrate ratio 1:50 w/w) for 4 h at 37 °C with 1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin (Worthington); proteolysis was terminated by rapid freezing followed by lyophilization.

*High-Performance Liquid Chromatography (HPLC).* Tryptic peptides were isolated by HPLC with the use of a reverse-phase support medium ($\mu$Bondapak $C_{18}$ Waters) on a 0.78 × 30 cm column with a 0–66% linear gradient of acetonitrile (Burdick and Jackson) in 0.1% (v/v) trifluoroacetic acid (Pierce). The column eluents were monitored at 210 nm.

*Amino Acid Analysis.* An automated analyzer (Durrum D-500) was used to determined the amino acid composition of peptides hydrolyzed for 24 h in vacuo at 110 °C with 6 M HCl. Forty microliters of a 1% aqueous solution of phenol was added to prevent degradation of tyrosine. The presence of tryptophan was determined by amino acid sequencing.

*Sequence Analysis.* Automated sequence analyses by Edman degradation were performed with a Beckman 890C sequencer and the 0.1 M Quadrol program (Beckman); for small peptides, polybrene was added to the cup. Thiazolinone–amino acids were converted to phenylthiohydantoin (PTH)–amino acids in an autoconverter (Sequemat P-6) by using methanol/HCl, 7:1 (v/v) at 65 °C. The PTH–amino acids were identified by HPLC (Waters Model ALC/GPC-204) with the use of 4.5 × 150 mM IBM octadecyl, 5-$\mu$m column developed with a methanol/water gradient.

*Electron Microscopy.* To characterize the structure of the isolated amyloid fibrils, freshly extracted fibrils, peaks III and IV were suspended in saline for 24 h followed by centrifugation. For electron microscopy, 0.5 $mm^3$ blocks of thyroid tissue and amyloid fibril precipitates were fixed by immersion in 2.5% glutaraldehyde in 0.2 M buffer, pH 7.4, postfixed in 1% osmium cacodylate, stained enbloc in uranyl acetate, dehydrated, and embedded in LX 112 resin. Thin sections were double stained with uranyl acetate and lead citrate and examined and photographed in a Zeiss EM 10A microscope (Carl Zeiss Inc., New York, NY).

For negative staining, a drop of amyloid suspension was allowed to dry on a carbon-coated Formvar-covered grid after which it was contrasted with 1% uranyl acetate.

## Results

Gel filtration of amyloid fibrils extracted from thyroid yielded four peaks (Figure 1). Peak I (void volume) consists of heterogeneous, mucopolysaccharide material present in all amyloid fibrils (Gorevic et al., 1980b). Peak II contains P-component, a serum $\alpha$-globulin that constitutes 5–10% of amyloid deposits (Pepys et al., 1977; Skinner et al., 1980). After rechromatography on a Sephadex G-50 column equilibrated in 5 M guanidine hydrochloride in 1 M acetic acid, the purity and molecular weights of peaks III and IV, $M_r$ 9700 and 5300, respectively, were determined in a SDS–17% polyacrylamide gel. Western blot analysis of peaks III and IV using rabbit antisera to human SAA or AA proteins revealed that both proteins were immunoreactive.

Automated Edman degradation of peak IV gave the sequence of 44 residues from the amino terminus, starting with Ser (residue 2 of human SAA1), with equivocal results at positions 34, 37, 39, 41, 42, and 43. Six peptides were obtained by HPLC after trypsin digestion of peak IV. Peptides T4 and T5 coeluted and were not purified further. Limited trypsin hydrolysis yielded peptide T4.5 without a lysyl–tyrosyl cleavage at positions 34–35. The amino acid compositions are shown in Table I. Only peptides T4.5 and T6 were subjected to automated Edman degradation and determined the sequence

Table I: Amino Acid Composition of Tryptic (T) Peptides of NOR Peak IV (Residues per Mole)

| amino acid | T-1 2–15 | T-2 16–19 | T-3 20–25 | T-4.5 26–39 | T-6 40–45 |
|---|---|---|---|---|---|
| Asp | 1.0 (1) | 1.0 (1) | 1.0 (1) | 2.2 (2) | 2.1 (2) |
| Thr | | | | | |
| Ser | 1.6 (2) | | 0.9 (1) | 0.9 (1) | |
| Glu | 1.0 (1) | | | 1.1 (1) | |
| Pro | | | | | |
| Gly | 2.0 (2) | | | 1.1 (1) | 1.1 (1) |
| Ala | 2.0 (2) | | 1.0 (1) | 2.1 (2) | 2.1 (2) |
| Val | | | | | |
| Met | | 0.9 (1) | 0.9 (1) | | |
| Ile | | | | 1.0 (1) | |
| Leu | 1.0 (1) | | | | |
| Tyr | | | 1.0 (1) | 1.6 (2) | 0.9 (1) |
| Phe | 3.6 (4) | | | 0.9 (1) | |
| His | | | | 1.0 (1) | |
| Lys | | | | 0.9 (1) | |
| Arg | 1.1 (1) | 1.06 (1) | 1.1 (1) | 1.2 (1) | |
| Trp | | *a* (1) | | | |

[a] Detected by amino acid sequencing.

of peak IV to position 45 of SAA (not shown).

The complete amino acid sequence of peak III is shown in Figure 2. Automated Edman degradation yielded the sequence of 30 residues from the amino terminus also starting with position 2. Trypsin digestion yielded 10 peptides after HPLC separation (Figure 3). The amino acid compositions are shown in Table II. Limited tryptic digestion resulted in partial hydrolysis at positions 34–35 and 47–48, yielding peptides T4.5 (positions 26–39) and T7.8 (positions 47–62). Peptides T4.5, 6, 7.8, 9, and 10 were subjected to automated Edman degradation and placed by homology to known SAA proteins to establish the sequence of peak III to position 82 of human SAA. Although position 52 was heterogeneous, yielding both valine and alanine, 90% and 10%, respectively, position 57 was only alanine. In the corresponding region of sequence (residues 52–57), amyloid A proteins from patients with various disorders have been observed with each form and with mixtures of both (Sletten & Husby, 1974; Moyner et al., 1980; Parmelee et al., 1982).

Sections of the thyroid tissue and freshly extracted, unfractionated amyloid fibrils were processed and embedded for electron microscopy and stained with uranyl acetate and lead citrate. The tissue is richly laden with amyloid fibrils (Figure 4A), and the extracted fibrils appear to have some twisted fibrils. Peaks III and IV were suspended in distilled water or phosphate-buffered saline for 1 day. Negative-stain micrographs reveal twisted amyloid fibrils (Figure 4B), 10 nM wide

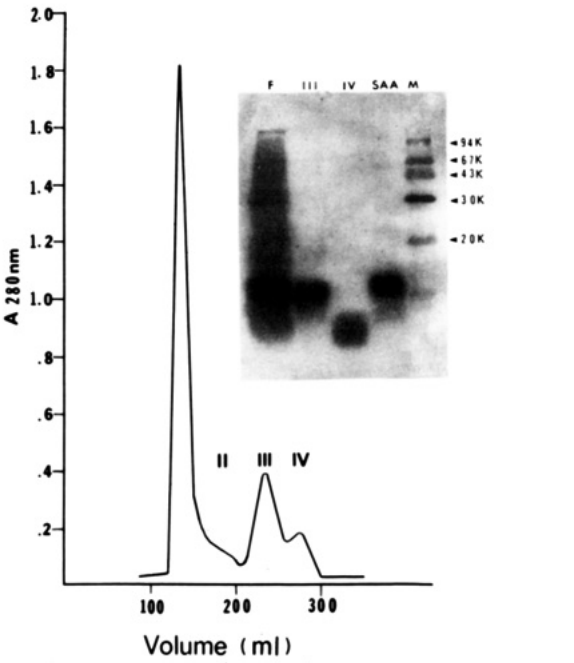


FIGURE 1: Fractionation of NOR amyloid fibrils from thyroid on 2.6 × 100 cm Sephadex G-75/Sephadex G-100 columns equilibrated in 5 M guanidine hydrochloride/1 M acetic acid. Inset: SDS–17% PAGE gel of amyloid fibrils extracted from thyroid (F) and purified components (peaks III and IV). Lane 4 is SAA. M, markers: phosphorylase *b* (94K); bovine serum albumin (67K); ovalbumin (43K); carbonic anhydrase (30K); soybean trypsin inhibitor (20K).

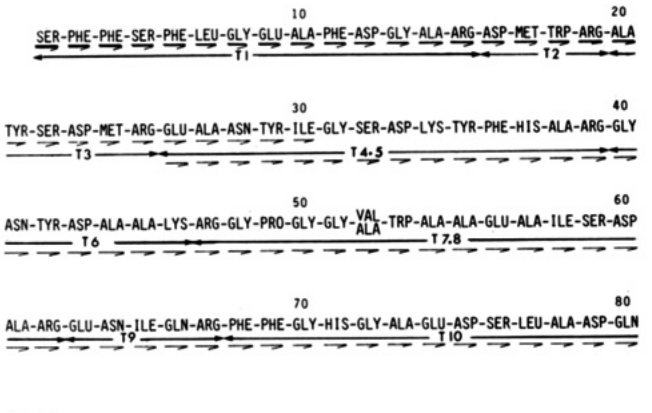


FIGURE 2: Amino acid sequence of NOR (peak III) amyloid protein. (→) Positions determined by sequence analysis. Numbering according to SAA1.

Table II: Amino Acid Composition of Tryptic (T) Peptides of NOR Peak III (Residues per Mole)

| amino acid | T-1 2–15 | T-2 16–19 | T-3 20–25 | T-4.5 26–39 | T-6 40–46 | T-7.8 47–62 | T-8 48–62 | T-9 63–67 | T-10 68–82 |
|---|---|---|---|---|---|---|---|---|---|
| Asp | 1.0 (1) | 1.0 (1) | 1.0 (1) | 2.2 (2) | 2.0 (2) | 1.5 (1) | 1.1 (1) | 1.0 (1) | 2.4 (2) |
| Thr | | | | | | | | | |
| Ser | 1.6 (2) | | 1.0 (1) | 0.9 (1) | | 0.9 (1) | 0.8 (1) | 1.9 (2) | 0.8 (1) |
| Glu | 1.0 (1) | | | 0.9 (1) | | 1.5 (1) | 1.1 (1) | | 2.4 (2) |
| Pro | | | | | | 0.9 (1) | 0.9 (1) | | |
| Gly | 1.9 (2) | | | 1.8 (1) | 1.0 (1) | 3.2 (3) | 2.3 (3) | | 2.4 (2) |
| Ala | 2.1 (2) | | 1.1 (1) | 3.2 (2) | 2.1 (2) | 4.2 (4) | 2.8 (3) | | 3.4 (4) |
| Val | | | | | | 0.8 (1) | 0.4 (1) | | |
| Met | | 0.8 (1) | 0.9 (1) | | | | | | |
| Ile | | | | 1.0 (1) | | 0.8 (1) | 0.5 (1) | 0.9 (1) | |
| Leu | 1.0 (1) | | | | | | | | 0.8 (1) |
| Tyr | | | 0.8 (1) | 1.0 (2) | 0.9 (1) | | | | |
| Phe | 3.5 (4) | | | 1.1 (1) | | | | | 2.0 (2) |
| His | | | | 0.9 (1) | | | | | 0.8 (1) |
| Lys | | | | 0.7 (1) | 0.9 (1) | | | | |
| Arg | 1.0 (1) | 1.0 (1) | 1.1 (1) | 0.9 (1) | | 1.7 (2) | 1.3 (1) | 1.1 (1) | |
| Trp | | *a* (1) | | | | *a* (1) | | | |

[a] Detected by amino acid sequencing.

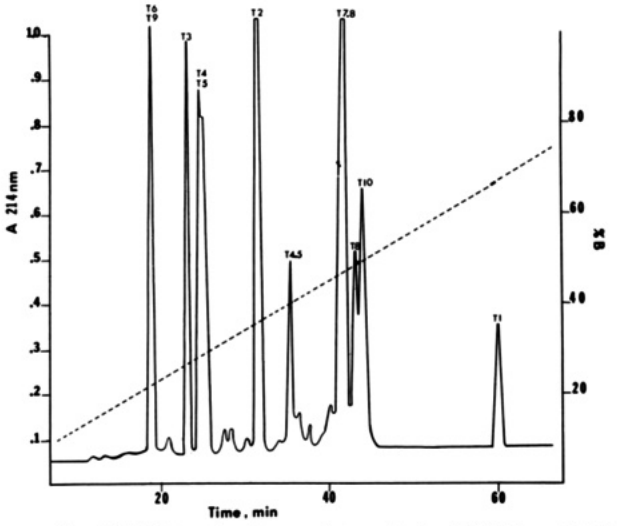


FIGURE 3: HPLC tryptic fingerprint analysis of NOR peak III. T1–T10 tryptic peptide (Table II).

with a periodicity of 100 nM in both preparations; however, there was significantly less fibrillar material in peak III.

## Discussion

Amyloidosis is heterogeneous with regard to both clinical expression and the composition and distribution of amyloid fibrils. In our study, the amyloid fibrils isolated from thyroid tissue of a patient with familial Mediterranean fever were found to be composed of two fragments of SAA, each starting with Ser (position 2). One was found to be larger (81 residues) and the other smaller (44 residues) than the 76-residue AA that has been isolated from most cases of secondary amyloidosis studied to date. Both had identical sequences through position 45, and the larger fragment (peak III) displayed heterogeneity at position 52 (valine/alanine), which was previously found in amyloid proteins obtained from reactive and inherited AA types. Thus, it is not unique to patient NOR and represents normal expression of two isotypic genes. Moreover, the prototypal familial Mediterranean fever amyloid A protein sequence previously reported by our laboratory (Levin et al., 1972) is a distinct SAA isotype from the two presented by patient NOR. This would imply that in the hereditary amyloidosis of familial Mediterranean fever, as well as in reactive amyloidosis, multiple SAA isotypes may undergo processing to form amyloid fibrils and that the composition of amyloid deposits may reflect more the organ or tissue in which it forms than the nature of the precursor.

The deposition and/or degradation appears to be tissue specific and may proceed sequentially. These AA molecules lack the N-terminal arginine residue as does SAA2, and their relative recoveries suggest that the COOH-terminus of both SAA molecules is degraded to yield a major peak III followed by further degradation to yield a minor peak IV. Alternatively, the latter may represent the preferential stepwise degradation of only one precursor SAA molecule. Although we do not have amyloidotic tissue from other organs to prove tissue-specific degradation with certainty, a second case (unpublished observations) where amyloid fibrils extracted from thyroid tissue obtained at thyroidectomy yielded similar molecular weight

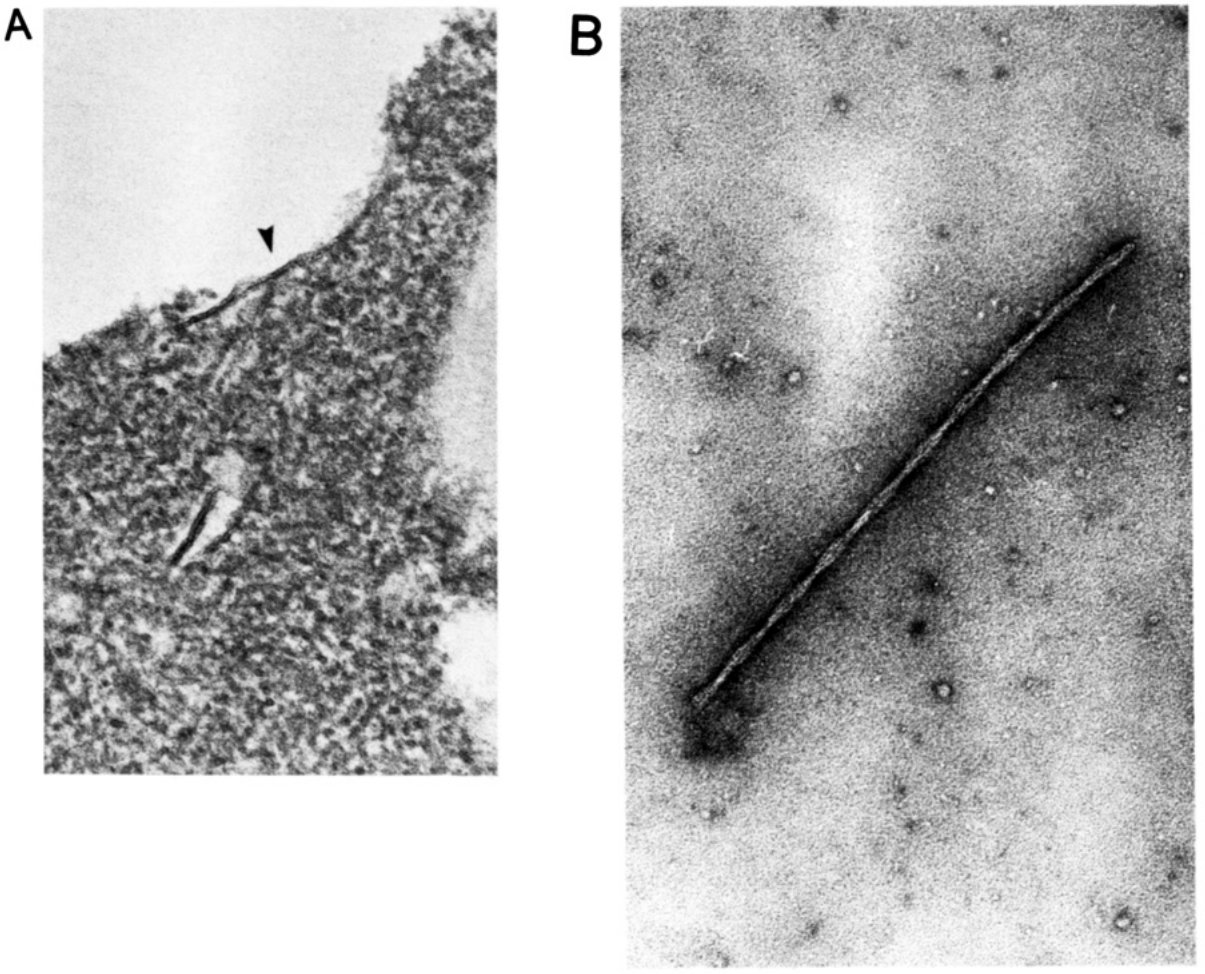


FIGURE 4: Electron micrographs of NOR amyloid fibrils. (A) Water-extracted fibrils embedded in LX112 resin and stained with uranyl acetate and lead citrate. Arrow points at a twisted fibril consisting of two filaments. Magnification 145000×. (B) Peak III fibrils negatively stained with 1% uranyl acetate. Magnification 105000×.

fragments of amyloid A protein tends to support this point of view. Moreover, tissue specificity has been observed in hereditary cerebral hemorrhage with amyloidosis (HCHWA), an autosomal-dominant form of amyloidosis that is restricted to the cerebral vasculature, although the precursor, cystatin C (or $\gamma$ trace), is present in many body fluids.

The completely sequenced AA proteins reported range from 45 to 83 amino acids. There are two reports of large AA proteins isolated from thyroid tissue, with molecular weights of 12000 (Van Rijswijk, 1981) and 13000 (Isobe et al., 1980), respectively. These singular AA proteins as well as our characterization of two NOR proteins of diverse and unusual size isolated from the same may signify that different tissues are potentially able to cleave the precursor of AA at different sites due to the presence of a distinct set of enzymes and their inhibitors. Degradation of the NOR SAA precursor molecules appears to be rather specific since the carboxy terminus of both fragments is Ala-Ala and is compatible with elastase specificity (Geneste & Bender, 1969). It has been demonstrated that human monocytes are able to degrade SAA in a two-step process which is inhibited by diisopropyl fluorophosphate, indicating neutral serine proteinases are involved in this degradation (Lavie et al., 1978, 1980). A similar degradation of SAA by polymorphonuclear leukocytes is inhibited by both serine esterase inhibitors and the acute-phase protein $\alpha_1$-antitrypsin (Silverman et al., 1980). These observations suggest the possible significance of tissue enzyme diversity and serum enzyme inhibitors in the pathogenesis of AA amyloidosis. Although many amyloid fibrils are composed of proteolytic cleavage products of precursor molecules, such as immunoglobulin light chains and serum amyloid protein A, the finding of amyloid deposits composed of apparently intact precursor molecules alone (Terry et al., 1973; Block et al., 1976; Lian et al., 1977; Costa et al., 1978; Gejyo et al., 1985; Gorevic et al., 1985) or in combination with fragments (Pras et al., 1981) suggests that proteolytic cleavage of a precursor in some instances may not be a prerequisite for amyloid formation.

Our electron microscopy studies revealed that the majority of amyloid fibrils were twisted. Twisted fibrils have been previously described in AA amyloid (Shirahama & Cohen, 1967) and in Alzheimer's disease (PHF) although the periodicity is different (Kidd, 1963; Gorevic et al., 1986). Although NOR amyloid fibrils were subjected to denaturing agents during isolation and purification procedures, they were capable of forming twisted fibrils with the same characteristics of the native amyloid fibril. Of interest was the fact that the shorter subunit fragment (2–45) appeared more fibrillar than the larger one (2–82).

Our data showed the following: we determined (i) the first complete characterization of two AA proteins from thyroid tissue of a patient, resultants of sequential degradation of the SAA precursors. (ii) No amino acid substitutions were found as reported in FAP or HCHWA. (iii) Heterogeneity at position 52 revealed normal expression of two allelic or isotypic genes whose products were similarly degraded and deposited in the thyroid; hence, in humans, at least two SAA proteins are potentially amyloidogenic. (iv) Although cleavage is not necessary for amyloidogenicity, when cleavage is present, the sites may be tissue and/or enzyme specific. (v) Under certain conditions, AA proteins, treated with dissociating agents, are capable of characteristic fibrillar re-formation.

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**Registry No.** Amyloid (human fibril NOR degradation product), 111209-28-0; amyloid (human fibril NOR valine isoform), 111209-30-4; amyloid (human fibril NOR alanine isoform), 111209-29-1.

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