The Amino Acid Sequence of a Major Nonimmunoglobulin Component of Some Amyloid Fibrils

MARK LEVIN, EDWARD C. FRANKLIN, BLAS FRANGIONE, and MORDECHAI PRAS

From the Department of Medicine, Rheumatic Diseases Study Group, New York University Medical Center, New York 10016

ABSTRACT The complete amino acid sequence of a protein, acid soluble fraction, (ASF) which constitutes up to 50% of amyloid fibrils from a patient with familial Mediterranean fever has been obtained. Partial amino acid sequences of three other proteins from patients with secondary amyloidosis were identical in the regions studied except for an alanine-valine interchange in one. The ASF contains no cysteine, does not resemble any known immunoglobulin, and has not been detected as yet in myeloma-associated amyloid.

INTRODUCTION

Amyloid is a fibrillar protein which infiltrates a variety of tissues and is associated with many different disease states. On the basis of organ distribution and associated diseases, it has been classified into four main types: primary, secondary, multiple myeloma associated, and a large number of different familial types (1). The major fibrillar protein component of all types of amyloid has a similar ultrastructural appearance. Nevertheless, there are a number of differences among them in their ability to bind Congo red and certain metachromatic dyes. These binding differences have raised the possibility that there may be differences among different types of amyloid (2). This possibility has been supported by the recent discovery of chemical differences between the major protein components of different types of amyloid fibrils. On the one hand, the variable region of homogeneous light chains is the major subunit of certain amyloid fibrils derived primarily from patients with the primary and multiple myeloma-associated types (3). In contrast a component which is not related to any known immunoglobulin has been shown to constitute up to 50% of the protein in amyloid fibrils from patients with secondary amyloidosis and one type of familial amyloidosis, i.e., familial Mediterranean fever (FMF).[1] This component, which has been termed acid soluble fraction (ASF) (4, 5) is a homogeneous protein with a mol wt of 8,500 daltons and corresponds to the A protein of Benditt and Eriksen (6). Amino acid analyses of several preparations (4, 5) showed the absence of cysteine and the presence of two residues of methionine. The partial amino acid sequence of four of these proteins has been presented (5, 7, 8).

The present report describes the complete amino acid sequence of this protein from a patient with FMF and the partial amino acid sequence of three other ASF proteins. It also presents preliminary evidence for the concomitant existence of heterogeneous components related to light chains in these amyloid fibrils.

METHODS

Preparation of amyloid fibrils and ASF. Amyloid fibrils were obtained from the livers and spleens of patients with FMF, tuberculosis, Hodgkin's disease, and bronchiectasis by extraction with distilled water (2). The purified amyloid fibrils were green birefringent when viewed with the polarizing microscope after having been stained with alkaline Congo red (9), and showed the characteristic fibrils on electron microscopy. ASF was extracted according to the method of Pras and Reshef (4). Fibrils were suspended in normal saline made 0.02 M with HCl, and homogenized with a VirTis 45 homogenizer (VirTis Co., Gardiner, N. Y.) for 5 min, heated to 60°C for 1 hr, and centrifuged at 50,000 rpm for 2 hr in a No. 50 rotor in a Spinco Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The supernatant, containing more than 50% of the starting material by dry weight, was dialyzed against normal saline and then against distilled water. The precipitate that formed was purified further by redissolving it in acid and

This work was presented in part at the Annual Meeting of the Society for Clinical Investigation, 1 May 1972.

Received for publication 12 June 1972 and in revised form 25 July 1972.

[1] *Abbreviations used in this paper:* ASF, acid soluble fraction; FMF familial Mediterranean fever.

1 5 10 15
Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Glu-Ala-Phe-Asp-Gly-Ala-Arg-Asp-Met-Trp-Arg

20 25 30 35
Ala-Tyr-Ser-Asp-Met-Arg-Glu-Ala-Asn-Tyr-Ile-Gly-Ser-Asp-Lys-Tyr-Phe-His-Ala

40 45 50 Trp* 55
Arg-Gly-Asn-Tyr-Asp-Ala-Ala-Lys-Arg-Gly-Pro-Gly-Gly-Ala-Arg-Ala-Ala-Glu-Val

60 65 70 76
Ile-Ser-Asn-Ala-Arg-Glu-Asn-Ile-Gln-Arg-Leu-Thr-Gly-Arg-Gly-Ala-Glu-Asp-Ser

FIGURE 1 The complete amino acid sequence of ASF from a patient with FMF. * The automatic sequencer yielded tryptophan in position 53 while the corresponding tryptic peptide contained arginine in this position. This discrepancy could not be resolved due to lack of material.

subjecting it to vacuum dialysis. ASF was recovered in the dialysate and lyophilized. The residual material was discarded.

Cyanogen bromide (CNBr) and proteolytic cleavage of ASF and separation of peptides. ASF extracted from the patient with FMF as described above was dissolved in 70% formic acid at a concentration of 20 mg/ml. CNBr at a concentration of 150 mg/ml was added and allowed to react at 25°C for 24 hr. The reaction mixture was suspended in 5% formic acid and centrifuged at 1500 *g* for 15 min. The precipitate which formed was washed with 5% formic acid and subjected to amino acid analysis. The supernatant was chromatographed on a column of Sephadex G-25 (3 × 130 cm) (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) in 5% formic acid.

One CNBr fragment was digested with TPCK trypsin (Worthington Biochemical Corp., Freehold, N. J.) in 0.2 M ammonium bicarbonate (pH 8.3) for 5 hr at 37°C enzyme-substrate ratio 1:50 (w/w) and then purified by high voltage paper electrophoresis on Whatman No. 3 MM paper at pH 6.5, pH 3.5, and pH 2.1, consecutively. Mobilities of the tryptic peptides were determined at pH 6.5 and expressed as fractions of the distance between ε-DNP-lysine and aspartic acid (10).

Amino acid analysis, amino acid sequence determination, and peptide map analysis. Peptides were hydrolyzed at 110°C for 21 hr with 6 N HCl containing 0.1% phenol in sealed tubes. Quantitative amino acid analyses were performed on a Beckman Model 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.)

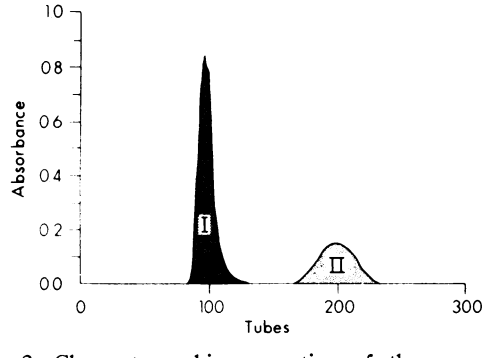

FIGURE 2 Chromatographic separation of the supernatant after CNBr cleavage of the ASF protein on a Sephadex G-25 column in 5% formic acid. Peak I represents the carboxy-terminal fragment (residues 25–76) and peak II represents the fragment from residue 18–24. Absorbance was measured at 280 mμ and 3 ml/tube was collected.

N-terminal residues were identified by the dansyl chloride method (11). Manual sequence determination was performed by the dansyl-Edman procedure (10). Automatic amino acid sequence determination was performed with a Beckman Model 890 sequencer by the method of Edman and Begg (12). Released derivatives were identified by gas chromatography with a Hewlett-Packard gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) and by amino acid analysis of the hydrolyzed derivatives. Peptide maps were done on performic acid-oxidized trypsinized samples (13).

Complete reduction and alkylation of amyloid fibrils. Purified water extracted amyloid fibrils (20 mg/ml) were completely reduced in 5 M guanidine with 0.05 M dithiothreitol at room temperature for 1 hr. The reduction was terminated by the addition of iodoacetic acid in 20% excess (10). After 1 hr the completely reduced and alkylated amyloid fibrils were chromatographed on a Sephadex G-100 column (5 × 200 cm) equilibrated in 5 M guanidine 1 N acetic acid (3).

RESULTS

Fig. 1 lists the complete amino acid sequence of ASF from a patient with FMF. It was obtained as detailed

TABLE I
Tryptic Peptides Isolated from the Carboxy-Terminal CNBr Fragment of ASF

Peptide	Mobility	Residue No.	Sequence
T_1	−1.0	25 + 47	Arg
T_2	0.3	26 – 34	*Glu-Ala-Asn-Tyr-Ile-Gly-Ser-Asp-Lys
T_3	−0.4	35 – 39	Tyr-Phe-His-Ala-Arg
T_4	0.0	40 – 46	Gly-Asn-Tyr-Asp-Ala-Ala-Lys
T_5	0.0	48 – 52	Gly-Pro-Gly-Gly-Ala
T_6	−0.3	48 – 53	Gly-Pro-Gly-Gly-Ala-Arg
T_7	0.0	54 – 62	Ala-Ala-Glu-Val-Ile-Ser-Asn-Ala-Arg
T_8	0.4	54 – 60	Ala-Ala-Glu-Val-Ile-Ser-Asn
T_9	0.0‡	63 – 67	Glu-Asn-Ile-Gln-Arg
T_{10}	−0.4	68 – 71	Leu-Thr-Gly-Arg
T_{11}	0.8	72 – 76	Gly-Ala-Glu-Asp-Ser

* The arrows indicate the residues identified by manual sequence determination.

‡ The mobility of peptide T_9 was also measured after the first step of the Edman degradation. It was −0.4 which indicated that the first residue was an acid and the second and fourth residues were amides.

below. The presence of methionine at residues 17 and 24 permitted the use of CNBr to cleave the chain at these sites. The precipitate obtained after CNBr cleavage was shown to correspond to the amino terminal fragment of the protein from residues 1–17 by amino acid analysis. The elution pattern of the supernatant on Sephadex-G-25 is shown in Fig. 2. The smaller peak which eluted late was identified as the peptide from residue 18–24 by amino acid analysis. The first peak corresponded to the carboxy-terminal fragment from residue 25–76. It was subjected to automatic sequence analysis which yielded unambiguous results up to residue 55 and identified several additional high yield residues up to resdue 68. Recovery of the first steps obtained in good yield was about 35%. The complete amino acid sequence of this fragment was obtained by manual sequence analysis of the 11 tryptic peptides which were ordered on the basis of the results obtained with the sequencer (Table I). The last tryptic peptide (residue 72–76) was placed at the carboxy terminus since it could not be positioned elsewhere in the sequence.

Serine was detected as the N terminal of the whole molecule although a small amount of arginine was also present in position 1 (Fig. 1). This type of heterogeneity may be the result of proteolytic digestion of a larger precursor molecule. Recovery of the first phenylalanine (step 3) ranged from 35 to 60% in different experiments. Residue 53 remains uncertain since the sequencer yielded tryptophan while the corresponding tryptic peptide ended with arginine.

The partial amino acid sequences of the three other ASF proteins has been determined with the sequencer and one of these has been reported previously (5). The number of residues sequenced is shown in Table II. Also included is the partial amino acid sequence of two amyloid proteins sequenced by Benditt, Eriksen, Hermodson, and Ericsson (7), and Ein, Kimura, and Glenner (8). All six proteins had identical amino acid sequences in the regions studied with the exception of the protein from a patient with tuberculosis which was sequenced furthest and which had a valine-alanine substitution at residue 52 just before the termination of the sequence analysis.

TABLE II

Amino Acid Sequences of ASF's Prepared from Amyloid Fibrils from Six Patients

Amyloid preparation	Reference	Number of residues sequenced	Substitution and position
FMF	This paper	76	Prototype
Tuberculosis	This paper	54	Val/Ala-52
Bronchiectasis	This paper	11	None
Hodgkin's disease	(5)	33	None
Tuberculosis	(7)	24	None
Rheumatoid arthritis	(8)	15	None

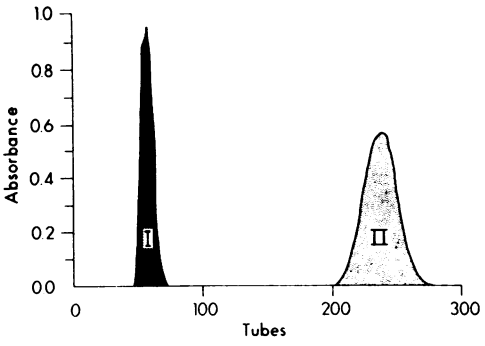

FIGURE 3 Gel filtration of the reduced and alkylated FMF amyloid fibrils on Sephadex G-100 in 5 M guanidine 1 N acetic acid. Peak I consists of light chain-related fragments and peak II is the ASF protein. Absorbance was measured at 280 mμ and 4.5 ml per tube was collected.

In an effort to characterize the remaining protein of the amyloid fibril two approaches were employed. Firstly, two preparations of amyloid fibrils were completely reduced, alkylated, and subjected to filtration on Sephadex G-100. The elution pattern of the completely reduced and alkylated native amyloid fibrils is shown in Fig. 3. 100% of the fibrils was recovered in the two peaks as determined by dry weight. Peptide map analyses of the first peak resembled a map of lambda and some kappa light chain peptides as prepared in our laboratory (Fig. 4). This probably represents polymerized light chains. In contrast, peptide map analysis of peak II was identical to a map of ASF. When maps of peak I and peak II were superimposed they yielded a map that was identical to the map of the native amyloid (Fig. 4). Secondly, the residue after extraction of the ASF was subjected to peptide map analysis. The maps were similar to peak I and also resembled those of light chains.

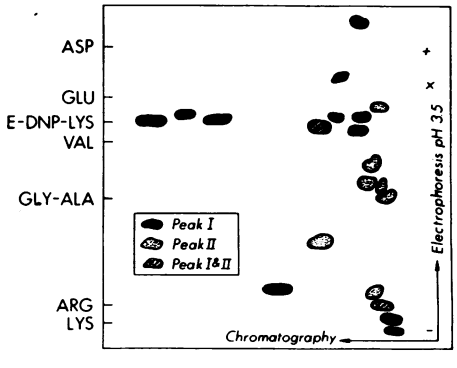

FIGURE 4 Composite diagram of peptide maps prepared from peak I (light chain-related proteins) and peak II (ASF) prepared as in Fig. 3.

DISCUSSION

The results of the present study demonstrate that a protein having no homology with any known immunoglobulin is a major component of certain types of amyloid fibrils.[2] This newly described protein has been found as the major component in amyloid fibrils from patients with the secondary and FMF-associated amyloidoses, and appears to be virtually identical in all of them. The significance of the observed interchange in one protein is difficult to evaluate since it occurred just before the termination of the sequence analysis but it raises the possibility that there may be slight differences among different proteins.

The discovery of this protein suggests that there may be at least two chemically distinct types of amyloid fibrils (4–8). On one hand, preparations from patients with the secondary and FMF-associated amyloidosis consist primarily of the newly described ASF but contain in addition, variable quantities of proteins related to immunoglobulins. Preliminary studies of several peptic-tryptic cysteic acid-containing peptides from this component suggest that it is chemically heterogeneous and does not represent a monoclonal type of immunoglobulin chain. In contrast, as demonstrated by Glenner, Terry, Harada, Isersky, and Page (3), preparations obtained from patients with myeloma-associated and primary amyloidosis consist mainly of fragments containing the variable region of monoclonal light chains. We do not as yet have enough information to be certain of the presence or absence of the newly described protein in this type of amyloid although we have not been able to isolate it from amyloid fibrils from three patients with myeloma or macroglobulinemia.

The heterogeneity of the amino terminus with the presence of arginine or serine as the first residue, raises the possibility that ASF is a product of proteolytic digestion of a larger molecule. This is particularly intriguing in light of the fact that amyloid-like fibrils can be formed in vitro by digestion of certain Bence Jones proteins with pepsin (14). The exact nature of ASF and its origin remain to be determined.

Addendum. After this paper was accepted for publication, Hermodson et al. (15) reported the amino acid sequence of a protein purified from monkey amyloid fibrils. The sequence of the monkey protein is similar to that of the human ASF and differs in only 12 amino acid residues.

[2] No significant homology was found with any known protein when a computer search of amino acid sequences was performed by Dr. M. Dayhoff.

ACKNOWLEDGMENTS

We would like to thank Mrs. Vesna Ivanovic for her valuable technical assistance.

This work was supported by U. S. Public Health Service Grants AM 01431, AM 02594, AM 05064, NIH Training Grant 5 TO 5GMO 1668 from the National Institute of General Medical Sciences and the Michael and Helen Schaffer Fund.

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