Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1473–1476, April 1975

Isolation of a Low-Molecular-Weight Serum Component Antigenically Related to an Amyloid Fibril Protein of Unknown Origin

(systemic amyloidosis/amyloid fibril precursor/isoelectric focusing/ immunoabsorbent chromatography/monospecific amyloid fibril protein antibodies)

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Communicated by Donald S. Fredrickson, January 28, 1975

ABSTRACT **An amyloid fibril protein of unknown origin from a patient with systemic amyloidosis has been purified to homogeneous charge and size by gel filtration and two step isoelectric focusing. From crude antisera to the initial heterogeneous fibril protein, monospecific antibodies have been obtained by immunoabsorption with the immobilized purified amyloid protein. These antibodies have been used to identify an antigenically related serum component in whole sera of patients with and without amyloidosis. Chromatography on Sephadex G-200 in phosphate buffered saline of a patient's whole serum yields a component with an apparent molecular weight of approximately 200,000. Guanidine denaturation of this high-molecular-weight serum component followed by Sephadex G-100 column chromatography in 5 M guanidine affords an antigenically reactive protein with an apparent molecular weight of about 12,500. The antigenic similarity and molecular weight of the latter protein indicates that it could act as the smallest serum precursor of the tissue fibril protein in this group of cases of amyloidosis.**

It has now been clearly established that a large group of cases of human systemic amyloidosis is frequently caused by the deposition in tissues of a "cross-β" pleated sheet fibrillar protein derived solely from a homogeneous light polypeptide chain of an immunoglobulin protein and/or a variable fragment of its NH_2-terminal region as the result of proteolysis or other physicochemical mechanisms (1). In patients with systemic amyloidosis of immunoglobulin origin, sensitive immunochemical techniques have detected a homogeneous immunoglobulin component in the urine or serum that has been shown to have antigenic determinants in common with the patient's amyloid fibril protein (1). A second type of systemic amyloidosis occurs in which the component of the β-pleated sheet protein fibrils found in tissues has no apparent relationship to any known immunoglobulin (2–4). This protein (AA) has been shown to have a homologous amino acid sequence in different patients, but to vary in molecular weight (3, 4). The fibrils composed of this protein have been designated amyloid fibrils of unknown origin, AUO. In those fibril preparations thus far examined, the AA protein has been found to be associated with a lesser proportion of protein of light polypeptide chain origin (5). Recent immunochemical studies have also detected in the serum of this group of patients, as well as of many other patients with and without amyloidosis, the presence of a protein (SAA) that has antigenic determinants in common with the fibrillar tissue AUO protein (6). The SAA component has been described as having a molecular weight less than that of IgG (approximately 100,000) and an α_1–α_2 electrophoretic mobility, but further characterization of this putative serum precursor of the AA protein has not been performed. It is the purpose of this paper to describe the isolation of the antigenically related serum protein and to present some of its properties.

Abbreviations: AUO, whole amyloid fibrils composed primarily of protein AA; AA, Sephadex G-100 purified major amyloid fibril protein; AAE, isoelectric focused purified major amyloid fibril protein; NHS, normal human serum; PS, patient's serum; SAA, serum related AA component; SAAL, low molecular weight SAA component; PBS, 0.1 M phosphate buffered pH 7.2 saline.

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MATERIALS AND METHODS

Amyloid fibrils from the spleen of a patient with systemic amyloidosis and rheumatoid arthritis were concentrated, denatured in 6 M guanidine–0.1 M Tris·HCl buffer at pH 8.5 that contained 50 mM dithiothreitol and the major amyloid fibril protein was purified by sequential gel filtration on Sephadex G-100 columns in 5 M guanidine hydrochloride–1 M acetic acid and 0.1 M ammonium bicarbonate (3). Homogeneity of molecular weight of the protein was assessed by sodium dodecyl sulfate-polyacrylamide (10%) disc gel electrophoresis at pH 7.1. The complete amino-acid sequence of this protein has been reported (3). Isoelectric focusing of the protein separated on Sephadex G-100 column chromatography in guanidine was performed in two steps on an LKB-8100-10 (110 ml) column (7) containing a sucrose gradient of 0–50% (w/v) in deionized 4 M urea. The first step utilized 4% carrier ampholytes (10 ml of pH 3.5–5.0 and 2.4 ml of pH 5–8). A final potential of 1000 V was applied for 96 hr at 8° to a solution containing 26 mg of protein. Fractions of 0.9 ml were collected and recorded at 280 nm. The major absorbing fractions from this column were pooled. In the second step, these pooled fractions were applied directly to a similar isoelectric focusing column, containing additional 0.33% carrier ampholytes pH 4–6, and identical electrophoretic conditions were employed to isolate from this second column a major protein, AAE. Charge homogeneity of the protein was evaluated by means of polyacrylamide (7.5%) disc gel electrophoresis in 4 M urea at pH 7.1, and partial sequence analysis was obtained

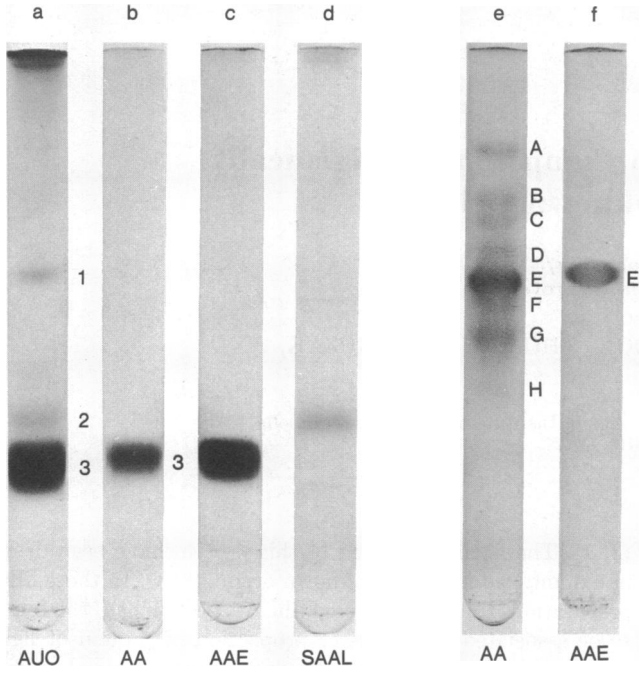

FIG. 1. Polyacrylamide disc gel electrophoresis of AA and related serum proteins at various stages of purification by size and charge. Gels a to d are sodium dodecyl sulfate-polyacrylamide (10%) gels with 10–80 μg of protein applied and e to f are 4 M urea-polyacrylamide (7.5%) gels with 20–25 μg of protein applied. Numbers in gel a indicate (1) immunoglobulin lambda light polypeptide chain, (2) protein of unknown composition, (3) major AA fibril protein. Letters A to H in gel e indicate multiple proteins from which the major protein E was purified by isoelectric focusing, gel f.

in the Beckman model 890 automatic amino-acid Sequencer. Immobilization by coupling of the AAE protein to agarose beads was performed using the cyanogen bromide activation method of Porath as modified by Cuatrecasas (8). A similar technique was employed to couple 200 ml of normal human serum to 200 ml of packed Sepharose 4 B for use as an immunoabsorbent.

Antisera to AUO fibril proteins were obtained by inoculating, in the foot pads and back of New Zealand white male rabbits, 1.5 mg of denatured amyloid fibril concentrate suspended in 1.5 ml of 1 M urea in normal saline and emulsified with an equal volume of complete Freund's adjuvant. Booster injections of 1 mg were administered at three- and six-weeks post-immunization, and at approximately monthly intervals after the second booster dose. Peak antibody production was observed at weeks fifteen and sixteen of the immunization schedule. Both double immunodiffusion and immunoelectrophoresis were performed in barbital buffer, ionic strength 0.03, pH 8.6 in 1.5% agarose on glass slides. An antiserum unreactive to normal human serum (anti-AA) was obtained by passage of crude anti-AUO antiserum through the normal human serum-agarose immunoabsorbent column. Monospecific antibodies to AAE protein (anti-AAE) were obtained by passage of a crude anti-AUO antiserum through an immunoabsorbent column of AAE-agarose, washing with 0.1 M phosphate pH 7.2 buffered saline (PBS) and elution of the specific antibodies with 0.5 M acetic acid. Antisera to heavy and light immunoglobulin chains and to C-reactive protein were obtained from Behring Diagnostics, Woodbury, N.J., and antisera to complement components and the amyloid "P-component" (9) were donated through the courtesy of Dr. H. Müller-Eberhard, Scripps Clinic, La Jolla, Calif., and Dr. J. Sri Ram, National Institutes of Health, Bethesda, Md., respectively.

Sera from 181 patients without amyloidosis were evaluated by immunodiffusion with both anti-AA antiserum and monospecific anti-AAE antibodies to determine the presence of the circulating SAA protein that shares antigenic determinants with the tissue amyloid AA protein. The serum of a patient who had Hodgkin's disease without amyloidosis gave an intense precipitin line of identity with AAE protein and was evaluated further. Two and one half milliliters of this patient's serum, was applied to a Sephadex G-200 column (2.0 × 112 cm) equilibrated with PBS and run at a constant pressure of 15 cm with 2.9 ml fraction volumes collected. All effluent fractions of the column were evaluated for antigenic reactivity by immunodiffusion with anti-AA antisera and anti-AAE antibodies and antisera directed against albumin and the heavy chain determinants of IgM and IgG. This patient's serum was similarly applied to a Sepharose 6 B column (2.0 × 112 cm) equilibrated with PBS using identical chromatography conditions and immunologic evaluation of the fractions obtained. Molecular weight determinations were performed using the method of Andrews (10). An aliquot of this patient's serum was dialyzed against distilled water, lyophilized, redissolved in 5 M guanidine for 24 hr at 27°, and applied to a Sephadex G-100 column (5 × 100 cm) equilibrated with 5 M guanidine and run at a constant pressure of 15 cm, with 10 ml fraction volumes collected. Individual fractions were dialyzed against distilled water, lyophilized, and tested in double immunodiffusion with both anti-AA antisera and anti-AAE antibodies. Molecular weight determination of the isolated serum protein having AAE determinants, SAAL, was performed on sodium dodecyl sulfate-polyacrylamide (15%) disc gel electrophoresis at pH 7.1 by a modification of the method of Weber and Osborn (11).

RESULTS AND DISCUSSION

Two step isoelectric focusing of a homogeneous molecular weight AUO amyloid fibril protein AA, isolated by Sephadex G-100 chromatography, afforded a major, uniformly charged protein, AAE, with a pI of 5.1 (Fig. 1). Automated amino-acid sequence analysis revealed AAE to have the same NH_2-terminal amino acids, i.e., Arg-Ser-Phe-Phe-Ser- as in previously reported AA proteins (1–4). The AAE protein failed to react with antisera to normal human serum, "P-component," heavy or light immunoglobulin polypeptide chains, C-reactive protein, or complement components C_{1q}, C_3, C_4, C_5, C_6, or to antisera to two lambda and two kappa immunoglobulin light chains that had been derived from amyloid fibril proteins as previously reported (12).

In previous studies, antisera to crude denatured AUO fibrils (anti-AUO) were used to detect, in a wide variety of pathologic as well as normal sera, a component having antigenic determinants in common with the tissue AA protein (6, 13). Coupling of the AAE protein to cyanogen bromide-activated agarose provided an immunoaborbent capable of selecting antibodies monospecific for this protein (anti-AAE) from crude antisera to the denatured AUO fibrils. These monospecific antibodies permitted unequivocal identification of an antigenic relationship between AUO amyloid fibrils and

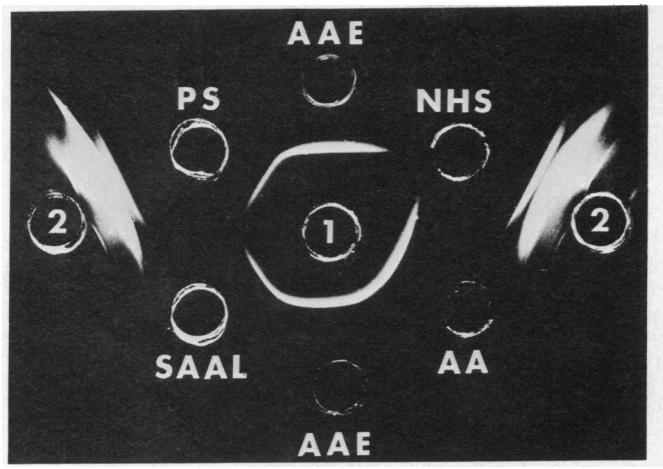

FIG. 2. Double immunodiffusion showing precipitin lines of identity between gel filtration purified (AA) and isoelectric focused purified (AAE) amyloid proteins and the patient's serum (PS) and 12,500-molecular-weight serum component (SAAL) when tested with anti-AA antiserum in well 1. No reaction to normal human serum (NHS) is noted. Anti-NHS antiserum in well 2 reacts only with NHS and patient's serum. Identical results were obtained with anti-AAE antibodies.

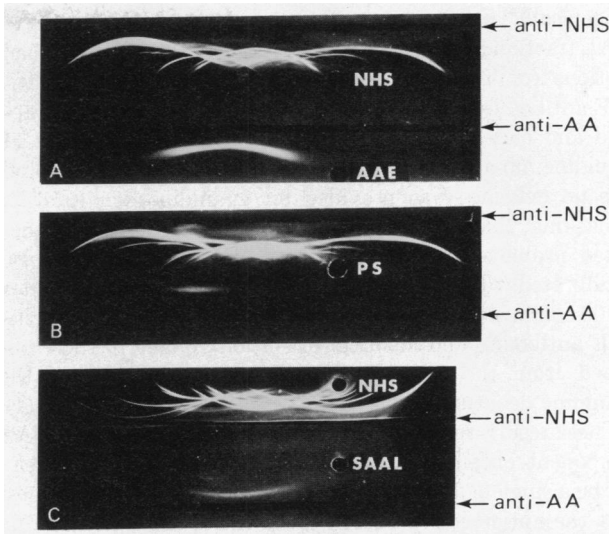

FIG. 3. Immunoelectrophoresis of isoelectric focused purified fibril protein (AAE), patient's serum (PS) and the low-molecular-weight serum fraction (SAAL) tested against anti-AA and anti-NHS antiserum. Note slight tailing of precipitin line in B to β region. Identical results were obtained with monospecific anti-AAE antibodies.

serum components. In addition, when crude anti-AUO antiserum was applied to a normal human serum immunoabsorbent column, antiserum (anti-AA) was obtained which gave a precipitin line on double diffusion with the AA protein that was undiminished in intensity when compared to that of the unabsorbed antiserum; they failed to react with any normal serum component. This result is at variance with the reported loss of antigenic reactivity of a crude anti-AUO antiserum after absorption with an equal volume of normal human serum and the reported identification in all normal sera of an antigenically related AA component demonstrated by radioimmunoassay using crude anti-AUO antiserum absorbed with normal fetal serum (6).

By the use of both anti-AA antiserum and the monospecific anti-AAE antibodies a protein, SAA, was also detected in the serum of a patient with Hodgkin's disease that had antigenic determinants in common with the AAE protein and on electrophoresis an α_1–α_2 mobility (Figs. 2 and 3). This confirms previously reported data (6, 13). Fractionation of this patient's serum on a Sephadex G-200 column revealed SAA to be eluted beginning after the major fractions containing IgM and just prior to the final fractions containing IgG. To insure that the SAA component was not incorporated in the void volume, the serum fractionation was performed on a Sepharose 6 B column in PBS. SAA again was eluted in the same relationship to the appearance of IgM and IgG. The calculation of the apparent molecular weight of SAA gave a value of 200,000 ± 20,000 in contrast to previously reported values of about 100,000 (6, 13). Sephadex G-200 column chromatography of the sera of three other patients, one with amyloidosis associated with rheumatoid arthritis, also gave molecular weights of approximately 200,000. The SAA fractions from G-200 column chromatography were pooled, lyophilized, dissolved in 5 M guanidine, and applied to a Sephadex G-100 column equilibrated in 5 M guanidine. The only fractions which reacted with the anti-AA antiserum and anti-AAE antibodies in double immunodiffusion were eluted in a region corresponding to the fractionation volume of cytochrome *c*. Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis of the antigenically reactive protein, SAAL, revealed a single protein band (Fig. 1), and repeated determinations with calibrated sodium dodecyl sulfate-polyacrylamide (15%) disc gels gave a molecular weight of 12,500 ± 500. Double immunodiffusion revealed that the tissue fibril AAE protein and this low-molecular-weight serum component (SAAL) were

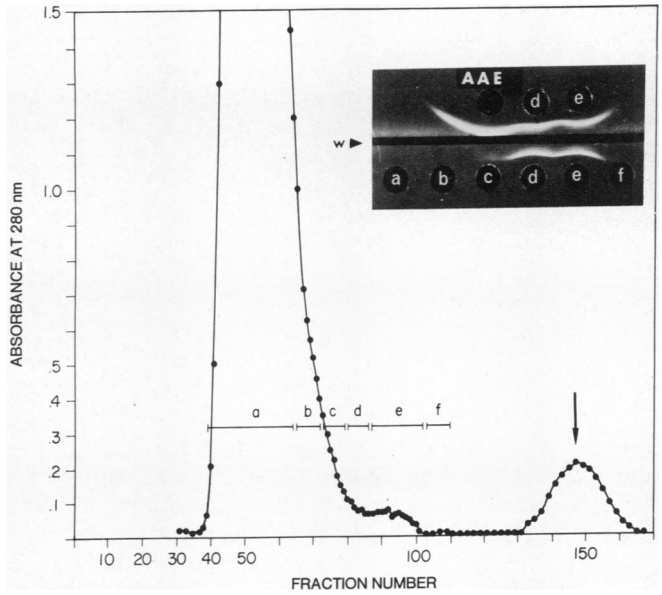

FIG. 4. Fractionation of 840 mg of lyophilized and 5 M guanidine denatured patient's serum applied to a Sephadex G-100 column (5 × 100 cm) in 5 M guanidine·HCl. Arrow indicates N-ϵ-DNP lysine marker. Letters a to f correspond to pooled fractions applied to wells in double diffusion (photograph insert) and tested against anti-AA antiserum in trough (W). The serum protein fractions d and e (SAAL) gave a line of identity with the purified AAE protein obtained by isoelectric focusing. Anti-AAE antibodies gave identical results.

antigenically identical, the immunoelectrophoresis of the SAAL fraction having an α_2–β mobility (Figs. 2 and 3). When fractions from the Sephadex G-200 column unreactive to anti-AAE antibodies, e.g., the albumin reactive fractions, were applied and eluted from the Sephadex G-100 column in 5 M guanidine, no anti-AA or anti-AAE reactivity was noted and thus no evidence was provided for guanidine denaturation unmasking "hidden" AA related determinants in the other eluted proteins. When the patient's whole serum was also equilibrated with 5 M guanidine, and applied to the Sephadex G-100 column, fractions reactive to anti-AA antisera and anti-AAE antibodies and identical in elution volume to those obtained from the Sephadex G-100 chromatography of the guanidine denatured SAA component were observed (Fig. 4).

These results indicate that the high-molecular-weight SAA component obtained by Sephadex G-200 fractionation may not be a single polypeptide chain. They also strongly suggest that the antigenically related lower molecular weight serum protein, SAAL, can act as the smallest precursor of the AUO amyloid fibril protein, AA, since its molecular weight is greater than 9145, which is that of the largest AA protein reported (14). These results do not in themselves permit the conclusion that the antigenically reactive 200,000-molecular-weight SAA component is a polymer. An alternative possibility is that in the patient's whole serum SAAL is complexed to a high-molecular-weight carrier protein to form the SAA component.

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