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Isolation and characterization of the amyloid-related apoprotein (SAA) from human high density lipoprotein

(amino acid composition/NH_2-terminal amino acid sequence/isoelectric point/molecular weight/protein AA)

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ABSTRACT Two apoproteins immunologically related to the 9000-dalton abnormal tissue constituent known as amyloid protein AA were isolated from the lipoprotein density interval 1.125–1.21 g/cm^3 (HDL_3) of a pool of human serums by delipidation, gel filtration, and ion-exchange chromatography. Lesser amounts of the same apoproteins were isolated from the density interval 1.063–1.125 g/cm^3 (HDL_2). These apoproteins, designated $apoSAA_1$ and $apoSAA_2$, have molecular weights near 11,500, almost identical amino acid compositions, and slightly different isoelectric points. Their amino acid sequences are identical as far as determined (30 residues), except that $apoSAA_2$ lacks the NH_2-terminal arginine found in $apoSAA_1$. The sequence is homologous with that of amyloid protein AA, which thus has residing in the plasma high density lipoproteins a potential precursor whose biological significance and function remain to be determined.

Certain amyloid-containing tissues, in particular those associated with inflammation, yield upon extraction a substance that includes as a major constituent a unique polypeptide, originally called amyloid protein A (1) and now termed amyloid protein AA (2). It has been found that antiserum prepared against protein AA reacts with a 100 to 200-kilodalton (kDal) α-globulin present in normal as well as pathological human serums (3, 4). Although different methods of assay have led to different estimates of the normal serum concentration of the AA-related antigenic material (SAA), there is general agreement that increased levels are found in association with a variety of pathological conditions (5–9).

Treatment of native serum with formic or stronger acid converts the bulk of the AA-related antigenic material into a low molecular weight (10,000–15,000) protein, sometimes called SAAL, the putative precursor of the still smaller tissue-derived protein AA (10–12). In a study of human serum in which an increased level of SAA had been induced by typhoid vaccination of the donor, we showed that the high molecular weight species was a part of the high density lipoproteins, mainly HDL_3, and that acid treatment of the HDL_3 released a significant portion of the SAA as a 10- to 15-kDal species (13). We found in mouse plasma a similar association of SAA with HDL, further characterized the protein, and proposed the name apoSAA for the low molecular weight species (14). Another group of investigators has recently described an association between SAA and HDL in rabbit serum (15).

We report here on the isolation of apoSAA from the HDL of a pool of human serums, and on the characterization of the purified material, including NH_2-terminal amino acid sequences.

MATERIALS AND METHODS

Isolation and Delipidation of HDL. A pool of patient serums with an elevated level of AA-immunoreactivity (stored at −18°C until used) was the starting material for this investigation. For the removal of the low density lipoproteins, a batch of pooled serum adjusted to solvent density 1.063 g/cm^3 by the addition of solid KBr was centrifuged for 20 hr at 50,000 rpm and 10°C in a Beckman 60 Ti rotor; the top quarter (8.5–9.0 ml) was aspirated from each of the eight tubes. The pooled infranates, adjusted to solvent density 1.125 g/cm^3 by the addition of KBr, were centrifuged for 21 hr at 55,000 rpm and 10°C in the 60 Ti rotor; the top quarter layers were aspirated and pooled (HDL_2 fraction). The pooled infranates at solvent density 1.125 g/cm^3 were adjusted to solvent density 1.21 g/cm^3 by the addition of KBr and centrifuged for 22 hr under the conditions just described; the top quarter layers were aspirated and pooled (HDL_3 fraction). The HDL_2 and HDL_3 fractions were dialyzed exhaustively in Spectrapor no. 1 tubing (6- to 8-kDal cutoff; Spectrum Medical Industries, Los Angeles) against distilled water, and lyophilized. The lyophilized products were delipidated at 0°C with 3:2 (vol/vol) ethanol/ether by extraction/centrifugation repeated three times over a period of 2 days, washed twice by centrifugation with anhydrous ether, and dried in a stream of N_2 (HDL_2 and HDL_3 apoproteins).

Gel Chromatography of HDL_2 and HDL_3 Apoproteins. These preparations, dissolved in 6 M urea/0.01 M HCOONa/HCOOH, pH 3.0, were fractionated on a 5.1 × 91 cm Sephadex G-100 column by upward flow of the formate/urea buffer at approximately 22°C; the column was calibrated by using α-chymotrypsinogen A, ribonuclease A, and glucagon (Sigma) as molecular size markers. Effluent fractions containing the principal peak of AA-immunoreactivity (10–15 kDal) were pooled, dialyzed exhaustively in Spectrapor no. 3 tubing (3.5-kDal cutoff) against 0.5% acetic acid, and lyophilized.

Anion-Exchange Chromatography of Low Molecular Weight HDL Apoproteins. The 10- to 15-kDal AA-immunoreactive components of the HDL_2 and HDL_3 apoproteins, dissolved in 8 M urea/0.015 M Tris/HCl, pH 8.2, were fractionated on a 1.6 × 64 cm column of DEAE-cellulose (Whatman DE-52, prewashed with 0.1 M HCl and 0.1 M NaOH and finally equilibrated against 8 M urea/0.015 M Tris/HCl, pH 8.2). The chromatograms were developed with a linear gradient

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Abbreviations: AA or protein AA, tissue-derived amyloid protein A; SAA, serum component related to AA; SAAL, low molecular weight protein derived from SAA by acid treatment of whole serum; HDL_2 and HDL_3, high density plasma lipoproteins isolated at solvent densities 1.063–1.125 and 1.125–1.21 g/cm^3, respectively; apoSAA, protein component of SAA extracted from HDL apoproteins; kDal, kilodalton; pI, isoelectric point.

(total volume, 580 ml) between 0.015 M and 0.1 M Tris, in 8 M urea, pH 8.2, at 21°C ± 0.5°C. Pooled effluent fractions of interest were dialyzed and lyophilized as described in the immediately preceding section.

Analytical Procedures. ApoSAA was estimated by radioimmunoassay as described (13), using human protein AA as standard and rabbit antiserum thereto as antibody. Before assay, chromatographic effluent samples in 6 M urea at pH 3 were neutralized and diluted with 3 vol of 0.25 M Na_2HPO_4, and those in 8 M urea at pH 8.2 were diluted with 4 vol of phosphate-buffered saline. ApoSAA levels were computed from the linear portion of a standard curve obtained by a log/logit transformation of the data (16). Results are expressed as AA-equivalent immunoreactivities in mass units of protein AA.

Double immunodiffusion was carried out in Hyland Immuno-Plates (Travenol Laboratories, Costa Mesa, CA), using antiserum concentrated 3-fold by vacuum dialysis against phosphate-buffered saline; antigens were dissolved by heating for 2 min at 100°C in 0.2% $NaDodSO_4$/0.01 M sodium phosphate, pH 7.4, then diluted with 3 vol of 0.28% polyoxyethylene sorbitan monolaurate (Tween 20)/0.01 M sodium phosphate, pH 7.4, to a final antigen concentration of approximately 0.25 mg of lyophilized preparation per ml.

Polyacrylamide gel electrophoresis, in acid/urea and $NaDodSO_4$/urea systems, was done essentially as described (17). Isoelectric focusing was done by the method of Gidez *et al.* (18); the gels were stained according to Vesterberg and Hansén (19), except that the concentration of Coomassie blue G-250 was increased 2.5-fold and the staining was done at 60°C for 1 hr. The pH gradient in the gels was determined by measuring the pH of water extracts of serial 3-mm slices of gel after focusing.

Amino acid compositions were determined as described (1). Samples (50–200 μg) were hydrolyzed in constant-boiling HCl at 108–110°C for 24 hr; no corrections were applied for hydrolytic losses of serine and threonine. Tryptophan was estimated from tyrosine/tryptophan ratios ascertained from ultraviolet absorption spectra of samples in 0.2 M NaOH by comparison with similar spectra of known-concentration mixtures of *N*-acetyltyrosine ethyl ester and *N*-acetyltryptophanamide (20, 21).

NH_2-terminal amino acid sequences were determined in the laboratory of K. A. Walsh and L. H. Ericsson (Department of Biochemistry, University of Washington) by automated Edman degradation in a Beckman 890 C sequencer (Beckman program 122974), using a 0.25 M Quadrol buffer system (22). Phenylthiohydantoin derivatives were identified by two high-pressure liquid chromatographic methods (23, 24).

EXPERIMENTAL OBSERVATIONS

From an initial volume of 260 ml, the pooled serum yielded 320 mg of HDL_3 apoproteins with an immunoreactivity equivalent to 570 μg of AA, and 125 mg of HDL_2 apoproteins with an immunoreactivity equivalent to 180 μg of AA. Although the quantitation of apoSAA is subject to considerable uncertainty (25), these figures give an idea of the magnitudes involved in these experiments.

In gel filtration of the HDL_3 apoproteins in 6 M urea at pH 3 (Fig. 1), the major part of the AA-immunoreactivity appeared in the 10- to 15-kDal range and a minor amount appeared in a lower molecular weight range. Repeated experiments revealed not more than trace amounts of activity associated with the major chromatographic peaks, representing the principal HDL_3 apoproteins in what are probably monomeric and polymeric states; aggregation of apolipoprotein A-I, and presumably of other apolipoproteins, appears to be promoted by lyophilization (26, 27).

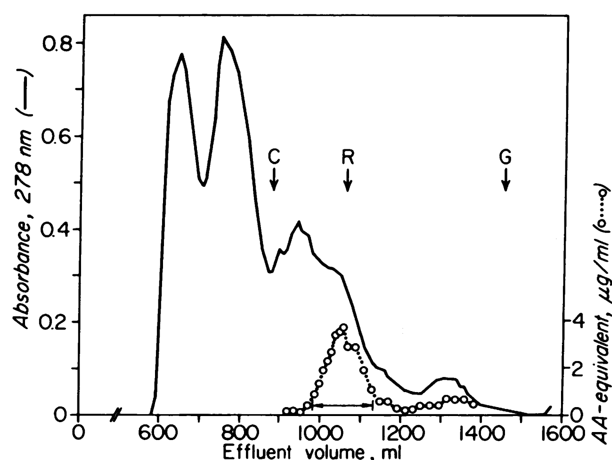

FIG. 1. Sephadex G-100 chromatography (in 6 M urea/0.01 M HCOONa/HCOOH, pH 3) of HDL_3 apoproteins (320 mg) from pooled patient serum. Flow rate, 44 ml/hr; effluent fraction volume, 10 ml; vertical arrows indicate elution positions of molecular weight standards (C, α-chymotrypsinogen; R, ribonuclease; G, glucagon); double-ended arrow indicates pooled effluent fractions.

In the corresponding profile of the HDL_2 apoproteins the principal AA-immunoreactivity appeared at the same location in which it was observed in the HDL_3 apoproteins; the peak was smaller and was superimposed on a somewhat different pattern of protein components.

Ion-exchange chromatography of the apoSAA-rich component of the HDL_3 apoproteins in a Tris concentration gradient (DEAE-cellulose, 8 M urea, pH 8.2) revealed the bulk of the AA-immunoreactivity in association with two major protein peaks (named $apoSAA_1$ and $apoSAA_2$, in the order of their elution) in the latter half of the chromatogram; lesser amounts of reactivity were detected elsewhere in the chromatogram (Fig. 2). The serrate appearance of the AA-immunoreactivity profile in the $apoSAA_2$ region does not necessarily indicate discrete peaks of activity, inasmuch as the data represent single samples assayed in a system perturbed by the presence of urea at 1 M concentration. The prime purpose of the assay was location of major peaks of activity.

In $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3A)

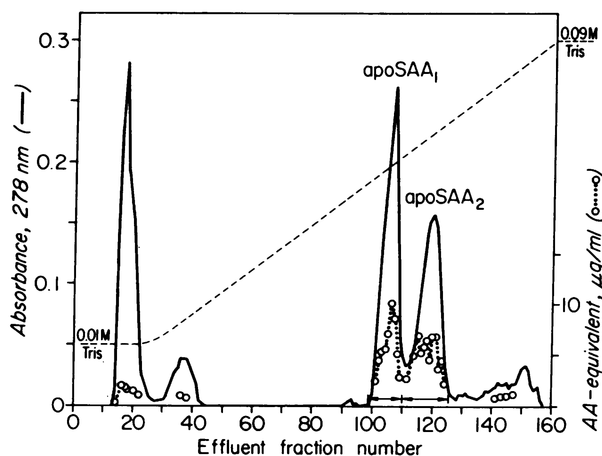

FIG. 2. DEAE-cellulose chromatography of apoSAA-rich fraction (25 mg) of HDL_3 apoproteins of pooled patient serum. Linear Tris chloride gradient in 8 M urea, pH 8.2; flow rate, 8 ml/hr; effluent fraction volume, 3.7 ml; double-ended arrows indicate pooled effluent fractions.

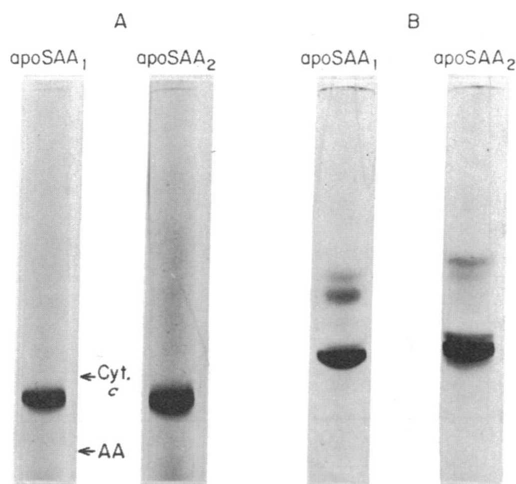

FIG. 3. Electrophoretic analysis of apoSAA purified by DEAE-cellulose chromatography; downward migration. (A) Samples (13 μg), in 8 M urea/2% $NaDodSO_4$/1% dithiothreitol/0.01 M (Tris) phosphate, pH 6.8, heated 2 min at 100°C and applied to 7.5% acrylamide gels in 8 M urea/0.1% $NaDodSO_4$/0.1 M (Tris) phosphate, pH 6.8; anode at bottom; arrows indicate migratory positions of horse heart cytochrome *c*, 12.4 kDal (Sigma), and monkey AA, 8.6 kDal (28); stain, Coomassie blue R-250 according to Swank and Munkres (29). (B) Samples (15 μg), in 8 M urea/35% (vol/vol) acetic acid, applied to 7.5% acrylamide gels in 5 M urea/10% (vol/vol) acetic acid; cathode at bottom; stain, Coomassie blue G-250 according to Vesterberg and Hansén (19).

$apoSAA_1$ and $apoSAA_2$ migrated essentially as a single band corresponding to a molecular weight estimated to be 11,300 by comparison with molecular weight standards. In acid/urea/polyacrylamide gel electrophoresis (Fig. 3*B*), $apoSAA_1$ and $apoSAA_2$ each exhibited one principal band and several much fainter bands; the principal bands had very nearly equal mobilities. By isoelectric focusing (Fig. 4), the isoelectric point (pI) of the principal component of $apoSAA_1$ was estimated to be 6.1–6.2, and that of the principal component of $apoSAA_2$, 5.6–5.7. Each preparation exhibited minor or faint bands of lower pI than that of the principal component.

Ion-exchange chromatography of the apoSAA-rich component of the HDL_2 apoproteins in the described Tris concentration gradient yielded AA-immunoreactive peaks corresponding to the $apoSAA_1$ and $apoSAA_2$ peaks derived from the HDL_3 apoproteins; the similarity was substantiated by isoelectric focusing. Because the yields were lower, the apoSAA preparations derived from the HDL_2 apoproteins were not further characterized.

In double immunodiffusion against rabbit antiserum (13) to amyloid protein AA derived from human tissue (17), $apoSAA_1$ and $apoSAA_2$ were indistinguishable; the two preparations formed a precipitin line of identity that also included tissue-derived protein AA (Fig. 5).

The amino acid compositions of $apoSAA_1$, $apoSAA_2$, SAAL (obtained from acid-treated whole serum), and tissue-derived protein AA are compared in Table 1. The compositions of the two apoSAA proteins are seen to be very nearly the same, and in only two instances, arginine and glutamic acid, do the residue percentages for individual amino acids fall by more than a fraction of a residue outside the ranges of values for individual amino acids compiled from five published amino acid compositions of human SAAL. The tyrosine to tryptophan ratio in each apoSAA protein was determined spectrophotometrically to be greater than 1:1 and less than 3:1. It seems certain that each of the proteins contains five tyrosine residues (nearest integer for a protein of molecular weight 11,300 and mean residue weight 110) and therefore should contain at least two but not more than four tryptophan residues, three being a likely value. Uncertainty regardithe background absorption in the ultraviolet spectra precluded a closer estimate of the tyrosine to tryptophan ratios. Judged on the basis of the isoelectric focusing and acid/urea electrophoretic patterns, $apoSAA_1$ is apparently the purer of the two preparations. Its molecular weight calculated from the nearest-integer amino acid composition is 11,468, which, although a provisional figure, agrees well with the value 11,300 obtained by $NaDodSO_4$ gel electrophoresis. There is a sufficient number of each kind of amino acid residue in $apoSAA_1$ to formulate a complete 83-residue (largest known) protein AA molecule, with the exception of one arginine and one isoleucine residue. These deficits may be real, the consequence of genetic polymorphism, or merely apparent, the result of experimental error of one kind or another. Of the approximately 20 amino acid residues that account for the molecular weight preponderance of $apoSAA_1$ over AA, the major portion consists of glutamic acid, leucine, lysine, and proline, whereas 8 of the common amino acids appear not to be represented.

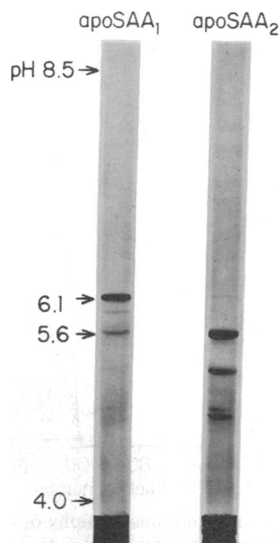

FIG. 4. Isoelectric focusing of apoSAA between pH 4 and 8.5. Samples (25 μg), in 8 M urea/0.2 M Tris/HCl, pH 8, applied to 7.5% acrylamide gels in 6.8 M urea.

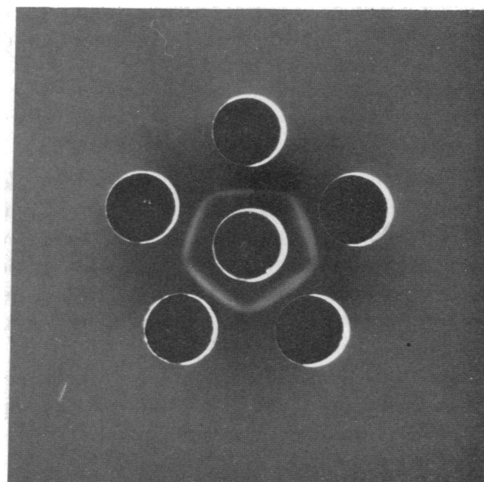

FIG. 5. Double immunodiffusion of apoSAA and amyloid protein AA versus rabbit antiserum to AA. Antiserum in center well, AA in top well, $apoSAA_1$ in right and lower right wells, $apoSAA_2$ in left and lower left wells.

Table 1. Amino acid compositions

Amino acid	Residues per 100 residues found			Integral values, residues per molecule		
	apoSAA₁*	apoSAA₂*	SAAL†	apoSAA₁	AA‡	apoSAA₁ − AA
Lys	5.8	6.4	4.3–7.2	6	3	3
His	2.3	2.3	2.6–4.0	2	2	0
Arg	7.2	6.3	8.2–9.0	7	8	−1
Asp	11.4	10.7	11.2–12.9	11	10	1
Thr	2.1	2.7	1.5–3.7	2	1	1
Ser	7.9	7.6	6.2–7.8	8	7	1
Glu	12.2	13.3	9.4–10.6	12	7	5
Pro	4.3	3.5	3.5–4.3	4	1	3
Gly	10.8	10.3	8.5–11.5	11	9	2
Ala	13.3	12.6	13.2–15.7	13	13	0
Cys	Trace	Trace	Trace–1.8	0	0	0
Val	2.2	3.2	1.8–3.4	2	2	0
Met	1.8	1.7	1.5–1.8	2	2	0
Ile	2.4	2.5	1.6–2.7	2	3	−1
Leu	4.9	5.3	3.9–6.6	5	2	3
Tyr	4.7	5.0	2.7–5.1	5	4	1
Phe	6.8	6.7	5.6–6.6	7	7	0
	100.1	100.1				
Trp	≈3§	≈3§	Not done	3	2	1
				102	83	19
			Calculated molecular weight	11,468	9303	

* Means of three independent analyses (Trp excluded).
† Range of published values (11, 12, 30).
‡ From sequence analysis (31).
§ Spectrophotometric estimate (see text).

The NH_2-terminal amino acid sequences of apoSAA₁ and apoSAA₂ (Fig. 6) are identical except that apoSAA₂ lacks the NH_2-terminal arginine of apoSAA₁, a difference compatible with the amino acid compositions. The unidentified residues in positions 19 and 22 of apoSAA₁ will undoubtedly prove to be the same as the arginine and serine residues found in the corresponding positions of apoSAA₂. The apoSAA₁ sequence, as far as it has been determined, is identical with that of human amyloid tissue protein AA reported by various groups (31–34) and also, for at least the first 20 residues, with that of SAAL (30). It has been stated that the amino acid sequences of SAAL and AA are identical through the first 63 residues (35).

DISCUSSION

The molecular size and chemical structure of the apoSAA that we have isolated from the HDL_3 of human serum indicate that apoSAA is the same as the low molecular weight form of SAA (SAAL) isolated by others from acid-treated whole serum (10–12). The NH_2-terminal amino acid sequence homology between tissue amyloid protein AA and apoSAA, and their identical antigenic behavior in double immunodiffusion *versus* antiserum to protein AA, indicate a close relationship between the abnormal tissue constituent and the HDL apoprotein characterized here.

Human HDL apoproteins with a low content of threonine, as well as other features of amino acid composition characteristic of amyloid proteins AA and apoSAA, have recently attracted the notice of two groups of workers not engaged in the study of amyloidosis. First, two threonine-poor proteins, having different molecular weights (10,000–12,000 and 22,000–25,000) but amino acid compositions similar to those of protein AA and apoSAA, were discovered in the HDL_3 of plasma obtained from individuals treated with amphotericin B for coccidiomycosis infection (36). The smaller of these proteins, whose sequence has not yet been determined but whose pI is approximately 6.0, could be the same as our apoSAA₁. The larger protein, with a pI of approximately 6.5, was obtained by mercaptoethanol reduction of a 40-kDal species of undetermined structure; it does not obviously correspond to any of the AA-immunoreactive constituents in our HDL preparations and its nature and relationships remain somewhat of an enigma. More recently, several threonine-poor proteins were detected in the plasma HDL of four patients receiving parenteral fluids for a variety of life-threatening disorders (37); these proteins have molecular weights from 8000 to 11,000, pI values from 5.0 to 8.0, and amino acid compositions very similar to the composition of human apoSAA. NH_2-terminal amino acid sequences, reported for three of the proteins to a maximum of 16 residues (38), are completely homologous with the sequence of apoSAA. The protein of pI 6.1, designated SV-D4, and the protein of pI 5.7, lacking the NH_2-terminal arginine and designated SV-D5, appear to be the same as our apoSAA₁ and apoSAA₂, respectively. A second sequence, lacking the NH_2-terminal arginine-serine peptide, was also detected in SV-D5. The protein designated SV-D2 (9.9 kDal, pI 6.85) lacked the NH_2-terminal arginine. NH_2-terminal shortenings like these were observed some time ago in the NH_2-terminal sequences of AA proteins (32) and were also observed in the sequence of the 83-residue AA protein cited in Table 1.

Threonine-poor proteins have been found also in the plasma HDL of the African green monkey (vervet): a protein of molecular weight 13,900 and pI 6.94, and another of molecular

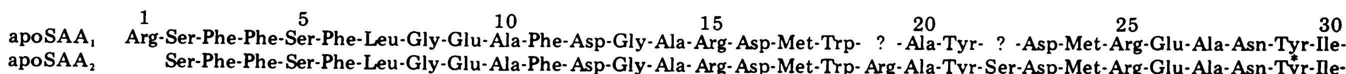
1 5 10 15 20 25 30
apoSAA₁ Arg-Ser-Phe-Phe-Ser-Phe-Leu-Gly-Glu-Ala-Phe-Asp-Gly-Ala-Arg-Asp-Met-Trp- ? -Ala-Tyr- ? -Asp-Met-Arg-Glu-Ala-Asn-Tyr-Ile-
apoSAA₂ Ser-Phe-Phe-Ser-Phe-Leu-Gly-Glu-Ala-Phe-Asp-Gly-Ala-Arg-Asp-Met-Trp-Arg-Ala-Tyr-Ser-Asp-Met-Arg-Glu-Ala-Asn-Tyr*-Ile-

FIG. 6. NH_2-terminal amino acid sequence of apoSAA. Unidentified residues indicated by question mark. *, Tentative identification.

weight 11,500 and pI 6.44 (39). Wide variations were observed in amounts of these proteins among groups of animals that were maintained on diets that differed with respect to type of fat and level of cholesterol, but no consistent relationship was observed between dietary regimens and plasma concentrations of the threonine-poor proteins. Curiously, no tryptophan was found in either of these proteins, and methionine (a single residue) was found in only one of them; otherwise their amino acid compositions resemble the composition of human apoSAA. Amyloid tissue protein AA isolated by us from the liver of a diseased monkey (macaque) was found to contain two methionine and three tryptophan residues in a total of 76 amino acid residues (28).

The available evidence for the origin of material in plasma antigenically related to protein AA is somewhat conflicting and suggests that several tissue or cell types may be responsible. The strongest evidence indicates that hepatocytes are a significant source of apoSAA (40, 41). Other studies suggest splenic (42) and placental (43) tissue, cultured embryonal fibroblasts (44), plasmacytoid cells of reactive lymphoid tissues (45), and polymorphonuclear leukocytes (46) as sources of such material. The question of origin(s) still remains to be answered.

On the basis of recent studies in our laboratory showing a rapid rate of disapearance of apoSAA from the plasma of mice injected with apoSAA-rich HDL (47), we suggest the possibility that SAA is involved in the removal of certain noxious agents or cell debris from the plasma or tissues and that its low level in normal plasma is a reflection of rapid turnover, a specific role in HDL function being thus indicated for the amyloid-related apoprotein.

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