Isoproteins of human apolipoprotein A-II: isolation and characterization

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Abstract  In human serum, polymorphism of apoA-II predominantly in HDL could be demonstrated. HDL apoA-II was composed of four isoproteins, each with a molecular weight of 8600 (reduced form) and identical immunological properties. The isoproteins are designated apoA-II-1 (pl 5.16), apoA-II-2 (pl 4.89) corresponding to the already known apoA-I monomer band, apoA-II-3 (pl 4.58), and apoA-II-4 (pl 4.31). The amino acid compositions of the A-II isoproteins were virtually identical with the published data for apoA-I. Treatment with acid phosphatase, alkaline phosphatase, or neuraminidase before electrophoresis did not alter the apoA-I pattern. The apoA-I isoprotein pattern was studied in ten male and ten female normolipidemic volunteers, in two patients with Tangier disease, and in three patients with abetalipoproteinemia. The isoelectric focusing patterns of apoA-I appeared virtually identical in all subjects. However, in Tangier disease, due to the low apoA-I concentration, only apoA-I-1 and apoA-I-2 were detectable, and in abetalipoproteinemia a different relative distribution pattern of the individual isoforms was found as compared to normal HDL. Our studies indicate that apoA-I, similar to apoA-I, exists in several isoforms. The relationship of these isoforms to each other is at present unclear. They may originate from relatively basic isoforms that are modified in charge by post-translational processes such as proteolytic cleavage, sequential deamidation, or other mechanisms. — G. Schmitz, K. Ilsemann, B. Melnik, and G. Assmann. Isoproteins of human apolipoprotein A-II: isolation and characterization. J. Lipid Res. 1983. 24: 1021–1029.

Supplementary key words  HDL, • isoelectric focusing • crossed immunoelectrophoresis • gel blotting • abetalipoproteinemia

Apolipoprotein A-II (apoA-II) is one of the major protein components of human serum high density lipoproteins (HDL) (1, 2). It consists of two identical polypeptide chains, each containing 77 amino acid residues of known sequence (3–5). The two polypeptide chains are crosslinked by a single disulfide bond at cysteine-6 (3). The minimum molecular weight of the dimer is 17,380. The amino-terminal residue of each chain is pyrrolidine carboxylic acid, and the carboxy-terminal residue is glutamine. The protein contains no carbohydrates and has been shown to lack histidine, arginine, and tryptophan (3, 6, 7). ApoA-II readily recombines with phospholipids (phosphatidylcholine, sphingomyelin) to form protein-phospholipid complexes (8–12), which leads to an increase of a-helicity from 25 to 48%. In addition, apoA-II associates with other apoproteins (e.g., apoA-I) by protein–protein interaction (2, 11) or forms apoprotein complexes (e.g., with apoE or apoA-I Milano) as a mixed disulfide (13, 14). An interrelationship between apoA-I and apoA-II in the interaction with HDL (15, 16) or phospholipid vesicles (17) has been reported and it could be demonstrated that 2 mol of apoA-II can displace 1 mol of apoA-I from the HDL surface.

It has been recently demonstrated that apoA-I, the major apoprotein of HDL, is composed of at least six isoforms that differ in charge but not in molecular weight (18, 19). It appears from those studies that the basic isoforms of apoA-I are synthesized and modified to more acidic forms that are the major apoA-I components in plasma. These findings suggest a complicated pattern of processing, involving proteolytic cleavage and charge modification, before the major isoprotein form of apoA-I present in plasma is attained.

However, similar isoforms of apoprotein A-II have not yet been described. In our recent studies on the HDL1/HDL2 interconversion and the formation of HDL1 (20, 21), some unknown apoprotein bands were detected in isoelectric focusing gels of HDL subfractions that might be related to apoprotein A-II isoforms. We have therefore investigated whether apoprotein A-II isoforms exist in human lipoprotein fractions.

MATERIALS AND METHODS

Patients

Blood was drawn from ten male and ten female normolipidemic volunteers and three patients with abetalipoproteinemia.

Abbreviations: apoA-II, apolipoprotein A-II; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; IEF, isoelectric focusing; pl, isoelectric point; SDS, sodium dodecyl sulfate.
lipoproteinemia (patient I, 20 years old, male, total cholesterol: 0.70 mmol/l, triglycerides: 0.05 mmol/l; patient II, 29 years old, male, total cholesterol: 0.70 mmol/l, triglycerides: 0.08 mmol/l; patient III, 24 years old, female, total cholesterol: 0.54 mmol/l, triglycerides: 0.01 mmol/l) after a 14-hr fast. Blood of two patients with the homozygous form of Tangier disease was obtained from patient IV, 49 years old, male, total cholesterol: 1.29 mmol/l, triglycerides: 3.95 mmol/l; patient V, 52 years old, female, total cholesterol: 2.46 mmol/l, triglycerides: 2.71 mmol/l. More detailed clinical data of these patients have been described previously (22, 23). In all studies, serum was obtained by blood clotting and immediately used for further analyses.

Preparation of apoHDL3

Na3 was added at a concentration of 0.01 M after serum separation as an antibacterial agent. HDL3 was prepared from serum by sequential preparative ultracentrifugation in a Beckman L8-70 ultracentrifuge at 4°C using a 60 Ti rotor (24). Density adjustments were made with solid KBr. VLDL, LDL, and HDL2 were separated at d 1.125-1.21 g/ml at 250,000 g for an additional 48 hr. The HDL3 fractions, d 1.25-1.21 g/ml, were dialyzed exhaustively against 0.05 M Tris, 0.15 M NaCl, pH 7.4, for 48 hr; HDL3 was prepared by density adjustment to 1.21 g/ml and spinning at 250,000 g for an additional 48 hr. The HDL3 fractions, d 1.25-1.21 g/ml, were dialyzed exhaustively against 0.05 M Tris, 0.15 M NaCl, 0.01 M NaN3, 0.05 M EDTA, pH 7.4, at 4°C. The HDL3 fractions were delipidated with ethanol-diethylether 3:1 (v/v) at -20°C as described elsewhere (25).

Enzymatic treatments of HDL3 (18)

Five-hundred-microliter aliquots of HDL3 (1 mg of protein) in 0.1 M sodium acetate buffer pH 5.5, were treated either with 0.02 units of neuraminidase from C. perfringens (Sigma) at 37°C for 1 hr or with 300 μg of acid phosphatase at 25°C for 1 hr. In addition, 500 μl of HDL3 in 0.1 M Tris-HCl buffer, pH 8.0, was incubated with 2 units of alkaline phosphatase at 25°C for 1 hr.

Isoelectric focusing

Analytical isoelectric focusing of apoHDL3. Analytical isoelectric focusing was performed on 7.5% polyacrylamide gels according to the method of Pagnan et al. (26) with 2% ampholytes pH 4–6 (Ampholine, LKB) in a Bio-Rad Model 220 flat-bed electrophoresis cell. The delipidated HDL3 fractions were dissolved in 1% dodecylsulfate (Eastman-Kodak), 2% ampholytes pH 4–6 and 0.01 M Tris-HCl, pH 8.2. For reduction of disulfide bonds, β-mercaptoethanol (Serva) was added to the sample buffer at a concentration of 1%. The mixture was incubated 15 min at room temperature. The focusing was carried out on 1.5-mm slab gels running overnight for 15 hr at 200 V starting with power limited to 2.25 W per plate. For the last 1.5 hr of the run, voltage was increased to 600 V. Apoproteins were visualized by staining with Coomassie Brilliant Blue G-250 (Bio-Rad) (27). A considerable increase in detection sensitivity was achieved by applying the photochemically derived silver stain according to Merril (28).

Analytical isoelectric focusing of whole serum. Analytical isoelectric focusing of whole serum was performed according to the method of Menzel, Kladetzky, and Assmann (29). Six microliters of serum was incubated with 50 μl of 0.01 M Tris-HCl, pH 8.2, 1% dodecylsulfate (Kodak), 2% ampholytes (pH range 4–6, LKB) and 5 μl β-mercaptoethanol (Serva) for 1 hr at room temperature. After addition of 10 μl of 80% sucrose, the sample was applied on the gel. The electofocusing was carried out under the same conditions as described above.

Preparative isoelectric focusing. Apoprotein A-II isoforms were obtained quantitatively by preparative flat-bed electrophoresis in granulated gels with the LKB 2117 Multiphor system using a pH gradient from 4–6 (30). The gel slurry from which the gel bed was prepared contained 2.5 g Ultradex (LKB), 3 ml of ampholytes pH 4–6 (Ampholine, LKB) and 45 ml of 8 M urea. Electrophoresis was carried out overnight for 15 hr at 2000 V and a constant starting power of 8 W. As electrode solution, 0.4 M ethylenediamine was used at the cathodic side and 0.2 M sulfuric acid at the anodic side. Separated apoprotein zones were collected with a spatula, using as a template a stained filter paper print made from the gel surface. The apoproteins were eluted from the gel medium with 1% dodecylsulfate, 0.01 M Tris-HCl, pH 8.2; centrifugation at 3000 rpm and collection of the supernatant protein solution were repeated three times. The apoproteins in the supernatant were precipitated over a period of 1 hr with 10% trichloroacetic acid (TCA), washed three times with cold 10% TCA, and finally twice with acetone to obtain a TCA-free protein precipitate.

Two-dimensional polyacrylamide gel electrophoresis

For the two-dimensional electrophoresis, a discontinuous SDS system with 20% polyacrylamide separating gels similar to that of Neville was used (31). After cutting individual polyacrylamide gel strips from the analytical focusing gels, these strips were immersed in 0.002 M ethylmorpholine-HCl, pH 8.5, 0.2% SDS, 0.1% β-mercaptoethanol, 0.001% bromphenol blue, and 4% sucrose. The gel strips were incubated for 15 min at room temperature and applied closely onto the stacking gels of the SDS system for running in the second dimension. The electrophoresis was carried out using the Bio-Rad electrophoresis cell Model 220 with initially 15
mA per plate and voltage limited to 200 V. After the front marker had reached the separating gel, the current was increased to 30 mA per plate. The following molecular weight markers were applied: trypsin inhibitor (lung) (Serva) 6500; α-lactalbumin 14,000; soybean trypsin inhibitor 20,000; carbonic anhydrase 30,000; ovalbumin 43,000; bovine serum albumin 67,000; phosphorylase b 94,000 (Pharmacia). Gels were stained with ovalbumin 43,000; bovine serum albumin 67,000; phosphorus was increased to 30 mA per plate. The following conditions and molecular weight standards were the same as mentioned above.

Preparation of apolipoprotein antisera

Antisera against apoA-II, C-II, and C-III were prepared in our laboratory as described elsewhere (32). Rabbits were immunized at multiple intradermal sites with 1 mg of apoprotein mixed with complete Freund’s adjuvant. The animals were boostered at intervals of 3 weeks with half the dose of antigen in incomplete Freund’s adjuvant. Bleedings were taken after 12 weeks. The antisera were monospecific as tested by crossed immunoelectrophoresis using commercially available antibodies were used from Behring-Marburg (anti apoA-II, batch No. S 539) and Immuno-Diagnostics (anti apoA-II, batch No.: 641/06). Antibodies made against apoA-II did not cross-react with apoA-I, apoC-II, or apoC-III antibodies in these studies.

Crossed immunoelectrophoresis

After isoelectric focusing, the apoprotein A-II bands were identified by crossed immunoelectrophoresis using the moulding-in technique (33). Polyacrylamide gel strips of 1 mm were cut and immersed in barbital buffer, pH 8.6, ionic strength μ = 0.02 for 10 min and then placed near the smaller edge of 84 × 94 mm gel Bond films (FMC Corporation Marine Colloids Division). Around the polyacrylamide strips, 1% melted agarose (Bio-Rad) in the same barbital buffer was poured. After gelling, an agarose gel segment of 6.5 cm length was cut out parallel to the polyacrylamide gel strip. This area was filled with monospecific apoprotein A-II antiserum in melted agarose at 52°C. Crossed immunoelectrophoresis was performed in a Bio-Rad electrophoresis Cell Model 1415 at 2.5 V per cm for ca. 15 hr with the same barbital buffer. After washing and drying, the plates were stained with 0.2% Coomassie R-250 (Bio-Rad) in 50% methanol–12% acetic acid and destained in 20% ethanol.

Polyacrylamide gel blotting

Electrophoretic transfer of apoproteins from isoelectric focusing and SDS polyacrylamide gels to unmodified nitrocellulose, subsequent reaction with monospecific antibodies against apoprotein A-II as well as C-II and C-III, and visualization with fluorescence-labeled protein A was performed according to the method of Burnette (34).

Cyanogen bromide cleavage of apoA-II isoforms

CNBr-cleavage was done by solubilizing the apolipoproteins in 70% formic acid and incubating them with a 400-fold molar excess of CNBr (Sigma) for 6 hr at 30°C. The resulting fragments were lyophilized, dissolved in a small volume of IEF-sample buffer, and focused using a pH gradient from 3–7.

Tryptic cleavage of apoA-II isoforms

(TPCK)-Trypsin (Worthington Cat. No.: 3742 TRTPCK 31 C 796) was added to apoA-II dissolved in 0.1 M ammonium bicarbonate buffer, pH 7.7, containing 0.02% Na2S3. The enzyme–protein ratio was 1:100 (w/w). The digestion was done in a waterbath at 37°C for 16 hr. The tryptic peptides were separated on high-performance cellulose thin-layer plates (Merck, West Germany). The chromatography solvent was butanol–acetic acid–water 4:1:1. The plates were dried with warm air, sprayed with the electrophoresis buffer water–pyridine–acetic acid 897:100:3, and run in an LKB 2117 multiphor system (4°C) in the second dimension at 500 V with methyl green as a marker. After drying, the plates were sprayed with ninhydrin reagent consisting of 1 ml acetic acid, 1 ml pyridine, and 98 ml of a 0.3% solution of ninhydrin in acetone or with fluorescamine spray reagent (Hoffmann-la Roche). The color of the ninhydrin-positive spots developed in 2–5 min at 80°C in an oven.

Amino acid analysis

Amino acid analysis was performed on an LKB 4150 automatic amino acid analyzer according to the procedure of Brewer et al. (3). Amino acid hydrolysis of the apoA-II isoforms was carried out at 110°C for 15 hr in constant-boiling HCl after addition of 0.05 mg/ml phenol. A column packed with Ultrapac 11 (Li+-form) working with a five-buffer system (pH range 2.80–3.55 and Li+-concentration range 0.2–1.65 m) was used.

RESULTS

The apoprotein patterns of the HDL subfractions HDL2 and HDL3, both from normolipidemic individ-
uals and patients affected with dyslipoproteinemia, were systematically analyzed by two-dimensional gel electrophoresis in order to investigate whether the unknown apoprotein bands demonstrated before (20, 21) were related to apoprotein A-II isoproteins.

As shown in Fig. 1, we could demonstrate that apoHDL₃ from normal individuals contains four apoA-II-monomer isoproteins: apoA-II-1, apoA-II-2, apoA-II-3, and apoA-II-4 (Fig. 1A) with identical molecular weight (Fig. 1B) and immunological properties (Fig. 1C) of apoA-II. In fluorescence blots from the isoelectric focusing gels, the four bands were only visualized when a monospecific antibody against apoA-II was used. These results demonstrate that besides the major apoA-II band normally detectable in reduced isoelectric focusing gels of apoHDL₃ and which is here called apoA-II-2, three minor A-II bands exist, a more basic isoform A-II-1 and two more acidic isoforms, A-II-3 and A-II-4.

In order to exclude a pseudopolymorphism of apoA-II due to proteolytic digestion or carbamylation during the preparation of apoHDL₃, we analyzed the apoA-II isoproteins in isoelectric focusing gels (pH 4–6) after direct application of 6 μl of whole fresh serum onto the gels (reduced with β-mercaptoethanol). As shown in Fig. 2, in crossed immunoelectrophoresis (Fig. 2A), in anti-A-II fluorescence blots (Fig. 2B), and in silver-stained two-dimensional gels (Fig. 2C), the isoforms of apoA-II...
Fig. 2. Analysis of the apoprotein A-II isoforms from fresh whole normal serum after a 14-hr fast. The figure is a representative analysis of 20 normolipidemic volunteers. Panel A demonstrates the crossed immunoelectrophoresis after isoelectric focusing of 6 μl of reduced fresh normal serum against apoprotein A-II antibody. Four peaks 1–4 with a line of identity are visible. Panel B shows the corresponding anti-A-II fluorescence blot of a gel strip obtained from isoelectric focusing of whole serum. The four bands are clearly distinguishable. Panel C demonstrates the two-dimensional SDS electrophoresis on 20% polyacrylamide gel of a gel strip obtained from isoelectric focusing of whole serum. The section shows the apoprotein A-II area after sensitive silver staining. The numbers 1–8 indicate the following apoprotein spots: 1, A-II-1; 2, A-II-2; 3, A-II-3; 4, A-II-4; 5, C-II-1-0; 6, C-II; 7, C-II-1-; and 8, C-II-2.

could be detected in whole fresh serum. Due to the small volume of applied material, apoA-II-4 was only present in a very low amount.

The HDL₃ subfractions from three patients with abetalipoproteinemia, as demonstrated in Fig. 3 (A, B, and C), contained relatively higher amounts of the apoA-II isoforms A-II-1, A-II-3, and A-II-4 as compared to HDL₃ from normal individuals. It is obvious (Fig. 3D) that these patients have a low concentration of apoC-III-1 and apoC-III-2 and that apoA-II-3 and apoA-II-4 almost comigrate with these two C-apoproteins in the isoelectric focusing gels. Silver-stained sections of two-dimensional gels from the abetalipoproteinemia HDL₃ fractions with and without treatment with neuraminidase are demonstrated in Fig. 3D and E. As can be seen from panel D left and right (without neuraminidase) and panel E left and right (treated with neuraminidase) in Fig. 3, there is no difference in the pattern of apoA-II isoforms (No. 1, 2, 3, 4) after neuraminidase treatment. However, there are profound

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Fig. 3. Distribution of the apoprotein A-II isoforms in apoHDL₃ of a patient with abetalipoproteinemia (1, 6). The analysis of the two other homozygotes for abetalipoproteinemia (II, 6; III, 9) showed exactly the same characteristics. Panel A shows the crossed immunoelectrophoresis after isoelectric focusing of apoHDL₃ against anti-apoA-II. Four peaks with a line of identity are visible. These peaks correspond to the four bands on the isoelectric focusing gel pH 4-6 (panel B) and to its one-dimensional anti-A-II fluorescence blot (panel C). Panel D demonstrates a silver-stained section of the second dimension SDS gel from the above isoelectric focusing strip. The solid spots on the right side of panel D, identified by blotting with monospecific antibodies against apoA-II, apoC-II, and apoC-III, represent the four isoforms of apoA-II. The numbers indicate: 1, C-II-1; 2, C-II-2; 3, C-III-0 and corresponding spots that have been previously reported (35); 4, C-III-1 and corresponding spots; 5, C-III-2 and corresponding spots; and 6, C-III-3. The remaining spots are unidentified proteins. Panel E represents the same sample as demonstrated in panel D after neuraminidase treatment. The numbers correspond to panel D.

changes in the pattern of the C-apoproteins (for detail see legend to Fig. 3). When Tangier sera were applied on the gels (not shown here), only the A-II-1 and A-II-2 isoforms could be detected in anti-A-II fluorescence blots.

Treatment of HDL₃ with acid phosphatase and alkaline phosphatase before electrophoresis (not shown) did not alter the apoA-II pattern, indicating that protein phosphorylation is not the basis for the apoA-II isoproteins.

In order to test whether individual isoforms of apoA-II can be separated from a mixture of the A-II monomers, the apoA-II monomer band was cut from an ordinary SDS gel of normal apoHDL₃ (Fig. 4A) and subsequently separated on an isoelectric focusing gel pH 4-6 (Fig. 4B). As can be seen, three of the four A-II isoforms can be identified on the isoelectric focusing gel. ApoA-II-4 was not identified here due to the limited sensitivity of the Coomassie staining. Furthermore, the focusing pattern was not changed by repeated focusing, as demonstrated in Fig. 4C and D.

The apparent isoelectric points of each of the A-II isoproteins are given in Table 1. As demonstrated before for the isoforms of apoprotein A-I (18), isoproteins A-II-1 through A-II-4 are separated from each other by one charge unit.

The apoA-II isoforms were isolated by preparative isoelectric focusing from apoHDL₃ fractions and their
peptide maps after CNBr- and tryptic cleavage were determined. This analysis is shown in Fig. 5 and indicates that the apoA-II isoforms have similar one-dimensional CNBr- (Fig. 5 left) and tryptic peptide maps (Fig. 5 right) as the major A-II isoform A-II-2.

The amino acid compositions of the main isoforms A-II-1, A-II-2, and A-II-3 were virtually identical (not shown). All three fractions closely resemble the published data of the apoA-II amino acid composition (3). However, it was very difficult to get acceptable masses of the apoA-II-1, A-II-3, and A-II-4 isoproteins without contaminating C-II and C-III isoproteins.

DISCUSSION

Our studies demonstrate for the first time distinct apoA-II isoprotein components. Four apoA-II isoproteins exist, designated here apoA-II-1, apoA-II-2, apoA-II-3, and apoA-II-4. These isoproteins were identified by two-dimensional electrophoresis, immunological techniques (crossed immunoelectrophoresis, fluorescence blotting), CNBr- and tryptic peptide maps, and amino acid analyses. These isoforms have been demonstrated to be commonly present in human serum, predominantly in the HDL₃ fraction.

The isoproteins have the same molecular weight on SDS gels, immunological properties identical to the major isoform of apoA-II (apoA-II-2), and the same one-dimensional peptide maps upon isoelectric focusing (pH 3–7) after CNBr-cleavage. The tryptic peptide maps and the amino acid composition of the four isoproteins were virtually identical and agree with the published

### Table 1. Apparent isoelectric points of apoHDL₃ apoproteins

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<th>Apoprotein</th>
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<td>A-I-1</td>
<td>5.60</td>
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<td>A-I-2</td>
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<tr>
<td>A-I-3</td>
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<tr>
<td>A-II-1</td>
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<td>A-II-4</td>
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data of apolipoprotein A-II (3). Treatment with acid phosphatase, alkaline phosphatase, or neuraminidase before electrophoresis did not alter the apoA-II pattern, indicating that neither protein phosphorylation nor covalently bound sialic acid is the basis for the apoA-II isoproteins. ApoA-II polymorphism is not related to artifactual conditions generated by carbamylation or deamidation (7), since all isoforms could be demonstrated by direct focusing of native serum (29).

The different distribution pattern of the apoA-II isoforms in abetalipoproteinemia is at present unexplained but may be due to alterations and/or abnormalities in the subfraction distribution pattern of HDL in these patients (35). The observation of the anomalous distribution of the apoA-II isoforms in abetalipoproteinemia serum, however, underlines our conclusion of the in vivo origin of the polymorphs of this apoprotein.

In a recent paper by Zannis et al. (36) concerning the characterization of the major apolipoproteins secreted by hepatoma cells, in addition to the major isoform A-II-2 a more basic unknown apoprotein was identified in the culture medium, with a molecular weight similar to that of A-II. This was named band X and discussed as a polymorphic form of apoA-II or apoC-III. This indicates that apoA-II similar to apoA-I (18) may originate from relatively basic isoproteins that are modified by post-translational processes. The site of the charge modification may be in the lymph or plasma rather than within the cells, because the more acidic forms of apoA-II were not secreted by the hepatoma cells (37). The charge modification of apoproteins may be important in the regulation of apolipoprotein and lipoprotein metabolism.

Further studies should be carried out to clarify whether the nature of charge modifications is due to proteolytic processes, sequential deamidations, or other mechanisms.

REFERENCES


