Identification of disulfide-linked apolipoprotein species in human lipoproteins

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Abstract We wished to determine whether apolipoprotein C-II$_{Toronto}$, a mutant form of apolipoprotein C-II that contains a C-terminal cysteine residue, exists as a monomeric species or as multiple disulfide-linked species in plasma lipoproteins. The plasma lipoproteins from a heterozygous carrier and two homozygous carriers of apoC-II$_{Toronto}$ were investigated. The mutant apolipoprotein was found in homodimeric form and as heterodimers with apolipoprotein A-II, apolipoprotein B-100, and apolipoprotein E. Of particular interest was the demonstration of the existence of the disulfide-linked species apolipoprotein B-100:A-II and B-100:C-II$_{Toronto}$ in the very low density and low density lipoproteins in subjects who were carriers of apoC-II$_{Toronto}$. We also observed that apoE3:C-II$_{Toronto}$ and apoE3:A-II dimers were present in the chylomicrons and very low density lipoproteins of these subjects. The observation of the existence of apolipoprotein B-100:A-II was extended to other hypercholesterolemic and hypertriglyceridemic subjects. The highest proportion of apolipoprotein B-100:A-II was observed in the very low density lipoproteins of hypertriglyceridemic subjects. The concentration of this species was significantly higher in hyperlipidemic subjects than in normolipidemic controls. These results demonstrate that the molecular species of cysteine-containing apolipoproteins are complex and should be considered in studies of human lipoprotein composition and function.—**Connelly, P. W., G. F. Maguire, C. Vezina, R. A. Hegele, and J. A. Little.** Identification of disulfide-linked apolipoprotein species in human lipoproteins. *J. Lipid Res.* 1993. **34:** 1717–1727.

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Among the naturally occurring apolipoproteins (apo) in human plasma, apoA-II, apoE2, apoE3 (1), apoB-100 (2, 3), apo[a] (4), and apoD (5) are known to contain cysteine residues. Rare mutants, such as apoA-I$_{Milano}$, the first mutant apolipoprotein to be described, have also been reported to contain cysteine residues (6). ApoA-II is typically present in high density lipoproteins (HDL) as a homodimer (1), while apoE3 (with a single cysteine, Cys112) and apoE2 (with Cys112 and Cys158) exist as monomeric and dimeric species (7). Heterodimers of apoA-II and apoE were previously reported (8–10). These heterodimers have been isolated from very low density lipoproteins (VLDL) and HDL. The apoE was found to have a significantly reduced ability to bind to receptors when present as the apoE:A-II complex and the binding ability was normalized by the reduction of this complex (8, 9). ApoE is an important ligand in the removal of chylomicrons from the circulation under normal conditions (1). ApoB is known to contain 25 cysteine residues (2, 3). Studies of proteolytic fragments of apoB have identified 16 of these as half cystine (11). It is not known whether the specificity of the disulfide species is invariant. Thus, there are potentially nine cysteine residues per mole of apoB-100 that could form disulfide linkages with other cysteine containing apolipoproteins. Of these nine, two are thought to be exposed on the surface of normal low density lipoproteins (LDL) (12). Using fluorescence labeling, Sommer et al. (13) have reported evidence for one cysteine residue at the surface of LDL and two cysteine residues in a more hydrophobic environment.

We have reported that in the first family to be described with apoC-II deficiency, the homozygotes and heterozygotes are characterized by the presence of a mutant nonfunctional apoC-II, apoC-II$_{Toronto}$ (apoC-II-T) (14, 15). Homozygotes present with chylomicronemia due to the lack of apoC-II, the apolipoprotein activator of lipoprotein lipase (16). It is known that the apoC-II-T homozygotes are also homozygous for apoE3 (15–17). The question of the characterization of the heterodimeric species of apolipoproteins is directly important in the sub-

Abbreviations: apo, apolipoprotein; C-II-T, C-II$_{Toronto}$; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; EDTA, ethylenediamine tetraacetate; SDS, sodium dodecyl sulfate; PBS, 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl; HC, hypercholesterolemia; HTG, hypertriglyceridemia.

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jects that we have identified with apoC-II-T, as this apolipoprotein contains a single carboxyterminal cysteine residue. Particularly important to these investigations was the use of a protocol whereby apolipoprotein cysteine residues could be derivatized in intact lipoproteins to prevent artefactual disulfide formation. The apolipoprotein species were characterized by isoelectric focusing, sodium dodecyl sulfate polyacrylamide electrophoresis, Western blot, and immunoassay. Studies were also carried out on hyperlipidemic subjects and normolipidemic controls.

EXPERIMENTAL PROCEDURES

Subjects

Blood was obtained from two apoC-II-T homozygotes (one male, one female) and one apoC-II-T heterozygote (female). The clinical description of these patients has been previously reported (14–17). Blood was also obtained from a patient with Type III hyperlipoproteinemia who was homozygous for apoE2 as judged by isoelectric focusing of apoVLDL. Blood samples were taken from 13 hyperlipidemic subjects attending the St. Michael's Hospital Lipid Clinic and from 9 normolipidemic subjects drawn from Hospital staff volunteers. The protocols were approved by the Institutional Review Board.

Treatment of plasma

Blood was collected from subjects after an approximately 12-h fast. The blood was drawn into tubes containing Na_2-EDTA and plasma was prepared by centrifugation at 15,000 rpm for 15 min at 5°C. An aliquot of the plasma was immediately pyridylethylated by addition of 9 μl 4-vinyl pyridine/ml plasma to give an 80 mM solution (18). Blood was collected from hyperlipidemic subjects without anticoagulant and the serum was isolated for subsequent analyses.

Plasma lipoproteins were isolated from both 4-vinyl pyridine-treated and nontreated plasma by sequential ultracentrifugation using a Beckman 80Ti rotor in an L8-80 centrifuge (Beckman Instruments, Fullerton, CA). Chylomicrons were isolated by centrifugation at 19,000 rpm for 30 min at 22°C. The chylomicrons were centrifuged a second time at 19,000 rpm for 60 min at 22°C. VLDL, intermediate density lipoproteins (IDL) (1.006–1.019 g/ml), and LDL (1.019–1.063 g/ml) were isolated by centrifugation at the stated densities at 45,000 rpm for 16.5 h at 10°C. VLDL was suspended in 1.006 g/ml NaCl, 0.01% Na_2-EDTA, pH 7.5, and reisolated under the same conditions. HDL (1.063–1.21 g/ml) was isolated by centrifugation at 45,000 rpm for 48 h at 10°C.

As a control for effectiveness of pyridylethylation, isolated lipoproteins were reduced with 60 mM β-mercaptoethanol for 1 h at 37°C, before addition of 4-vinyl pyridine to 80 mM final concentration.

All samples were dialyzed against 0.01% Na_2-EDTA, pH 7.5, prior to electrophoresis.

Sodium dodecyl sulfate electrophoresis of apolipoproteins

Aliquots of chylomicrons were lyophilized, and extracted using ethanol–diethyl ether 3:1 (19). When chylomicrons were absent, whole plasma and other lipoprotein fractions did not require extraction before electrophoresis. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed in 3.5% acrylamide: 18% glycerol gels as previously described (20). After electrophoresis, proteins were either stained or transferred to nitrocellulose membranes and immunoblotted as described (20, 21).

To investigate the presence of apoB-100:C-II-T and apoB-100:A-II, LDL was isolated from plasma treated with 4-vinyl pyridine and electrophoresed on an SDS slab gel under nonreducing conditions. That portion of the gel containing apoB-100 was excised and divided into two halves, both of which were soaked for 30 min in 0.1 M sodium phosphate buffer, pH 7.0, 2% SDS. One-half of the excised gel was reduced by addition of 60 mM β-mercaptoethanol to the buffer. The gel slices were then placed side by side on another SDS slab gel and once again electrophoresed. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Millipore) at 100 mA for 16 h and immunoblotted with rabbit anti-human antibodies. Goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories, Richmond, CA) was used as the second antibody.

Isoelectric focusing of apolipoproteins

Aliquots of VLDL were separated by isoelectric focusing as previously described (14, 15). After electrophoresis, proteins were either stained with Coomassie brilliant blue G250 or transferred onto a polyvinylidene difluoride membrane. Prior to transfer, the gel was soaked for 30 min in 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. Proteins were transferred in 25 mM Tris, 192 mM glycine, pH 8.3, for 3 h at 500 mA. Proteins were detected on the membrane as described (14, 15) using goat anti-rabbit IgG alkaline phosphatase conjugate as the second antibody.

Immunoaffinity chromatography

Polyclonal antibodies to apoB and apoA-II were raised in rabbits by injection of LDL (1.03–1.05 g/ml) or apoA-II purified by ion exchange chromatography after isoelectric focusing of HDL. Antibodies, specific for apoB and apoA-II, were immunoaffinity-purified by adsorption of the respective antiserum with LDL or HDL linked to Affigel-15, according to the manufacturer's instructions (Bio-Rad Laboratories). The specificity of the antibodies was verified by immunoblotting techniques (21).

Affinity-purified antibody to apoA-II was coupled to Affigel-10 (Bio-Rad) at a ratio of 8 mg of protein per ml of gel. To estimate the concentration of apoB complexed to apoA-II, LDL (d 1.006 to d < 1.063 g/ml) from a hypercholesterolemic subject (850 μg of protein) was applied to the anti-apoA-II immunoadsorber at 5 ml/h in 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA. After collecting the unbound fraction, the column was washed with the starting buffer until the absorbance at 280 nm was less than 0.02. The bound fraction was eluted at 20 ml/h with 3 M NaSCN and desalted immediately on a desalting column (Econo-Pac 10DG, Bio-Rad). After concentration in dialysis bags, using polyvinyl-pyrrolidone (BDH, Toronto, ON) outside the bag, the bound fraction was dialyzed in 10 mM phosphate, 0.15 M NaCl, pH 7.4.

Immunoassay of apolipoproteins

Microtiter plates (NUNC Maxisorp, GIBCO BRL, Burlington, Ontario, Canada) were coated with capture antibody, either anti-apoB or anti-apoA-II, by incubating the wells with 100 μl of affinity-purified antibody (5 μg/ml) in 0.05 M sodium bicarbonate containing 0.02% sodium azide, for 2 h at room temperature. After washing with 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl (PBS), any remaining binding sites were blocked by incubation with 300 μl of 0.5% bovine serum albumin (RIA grade, Sigma, St. Louis, MO) in PBS containing 0.05% Tween-20 (Bio-Rad Laboratories) for 30 min. Standard curves of LDL or apoA-II and of a calibrated serum consisted of six serial doubling dilutions in 0.1% bovine serum albumin in PBS containing 0.05% Tween-20 (assay buffer), each in duplicate. Plasma or lipoprotein samples were analyzed as three serial doubling dilutions in assay buffer. Aliquots (100 μl) of the dilutions were transferred to the coated plates and the plates were incubated for 1 h at room temperature. The wells were washed with PBS–0.05% Tween-20 and then incubated for 1 h with affinity-purified anti-apoB or anti-apoA-II, conjugated to alkaline phosphatase (Boehringer Mannheim, GmbH, Germany) (22). After washing the wells with PBS–0.05% Tween-20, the substrate, *p*-nitrophenyl phosphate (Bio-Rad Laboratories) was added and incubated for 30 min. The reaction was stopped with 0.4 M NaOH, and the color development was read at 405 nm. The data were analyzed with the Titersoft program (Flow ICN, Costa Mesa, CA), which allowed various curve-fitting options and calculated concentrations of the unknown samples from the standard curve.

To assay apoB-100:A-II, wells were coated with anti-apoA-II and the bound apoB was detected with anti-apoB–alkaline phosphatase conjugate. An LDL preparation, in which the apoB complexed to apoA-II had been estimated by immunoaffinity separation on anti-apoA-II Affigel, was used as a standard. Total apoB was assayed in different wells on the same plate with anti-apoB-coated wells.

RESULTS

A general property of cysteine residues is that they can form disulfides under oxidizing conditions. When blood is drawn, it is possible for cysteine residues to artefactually crosslink within and between proteins. The study of apolipoprotein cysteines presents the additional challenge that cysteines can theoretically be oriented towards the aqueous environment or buried within the hydrophobic lipid environment. Consequently, some cysteines may be inaccessible to sulfhydryl reagents (23). Thus, to assess the status of cysteine residues, we wished to have a reagent that would derivatize all free cysteines and for which the reaction could be qualitatively assessed. An example of the suitability of 4-vinyl pyridine for derivatization of apoE2 is shown in **Fig. 1.** The IDL of a patient with Type III hyperlipoproteinemia was isolated and treated with β-mercaptoethanol (+BME), untreated (−BME), or isolated from plasma treated with 4-vinyl pyridine (+4VP) and subjected to SDS-glycerol gel electrophoresis. Fig. 1 shows the result of immunoblot with anti-apoE. Monomeric apoE and a low molecular weight band that may be a proteolytic peptide of apoE were seen in the presence of reducing agent (+BME). A series of oligomers with molecular weights consistent with apoE2:A-II (40 kDa),

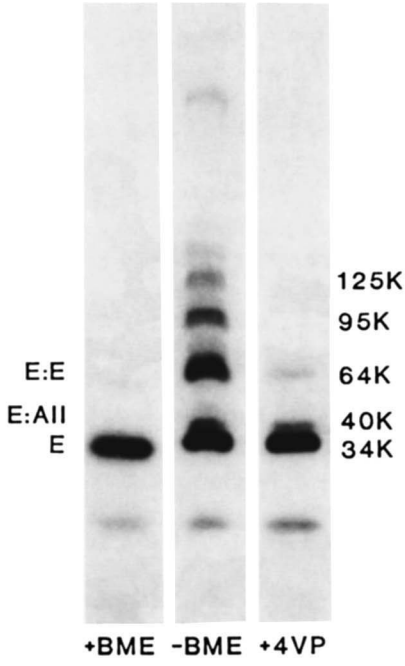

Fig. 1. Western blot with anti-apoE of the apolipoproteins of IDL after separation by sodium dodecyl sulfate-glycerol polyacrylamide electrophoresis. The IDL was isolated from a patient with Type III hyperlipoproteinemia and an apoE2/2 phenotype. IDL isolated from untreated plasma was electrophoresed after reduction with β-mercaptoethanol (+BME) or without reduction (−BME). IDL isolated from plasma treated with 80 mM 4-vinyl pyridine (+4VP) was electrophoresed without reduction. Abbreviations: E:E, apoE2:E2 dimer; E:AII, apoE2:A-II dimer; E, apoE2 monomer; K, kDa.

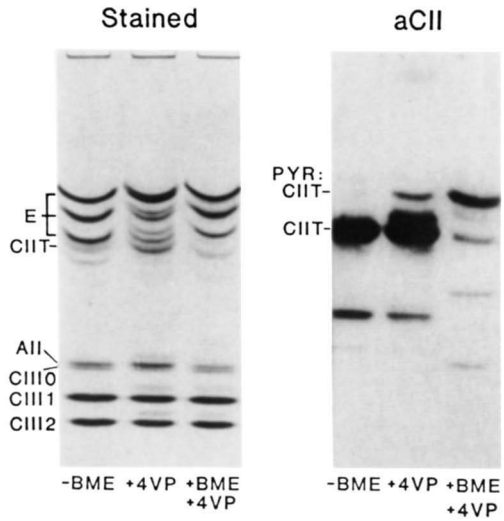

Fig. 2. Isoelectric focusing of homozygous C-II-deficient VLDL apolipoproteins. VLDL isolated from untreated plasma was either reduced prior to electrophoresis (−BME) or reduced and derivatized with 4-vinyl pyridine prior to electrophoresis (+BME, +4VP). VLDL isolated from plasma treated with 80 mM 4-vinyl pyridine was dialyzed to remove excess 4-vinyl pyridine and reduced with β-mercaptoethanol prior to electrophoresis (+4VP). The panel labeled "Stained" shows the apolipoproteins detected with Coomassie Blue stain. The panel labelled "aCII" shows the apolipoproteins detected by Western blot with anti-apoC-II. Abbreviations: E, apoE; CIIT, apoC-II-T; AII, apoA-II; CIII0, apoC-III-0; CIII1, apoC-III-1; CIII2, apoC-III-2; PYR:CIIT, pyridylethyl-C-II-T.

apoE2:E2 (64 kDa), apoE2:E2:E2 (95 kDa), and apoE2:E2:E2:E2 (125 kDa) were detected under non-reducing conditions (−BME). Pretreatment with 4-vinyl pyridine (+4VP) blocked the formation of the majority of these oligomers and showed most of the apoE2 to be present as a monomer, with apoE2:A-II and apoE2:E2 dimers present at detectable levels. This showed the importance of a positive control for the suitability of the derivatizing agent and also showed that 4-vinyl pyridine is effective at derivatizing free sulfhydryls of apoE in intact lipoproteins.

When cysteine is derivatized with 4-vinyl pyridine, a positive charge is introduced into the protein. This allowed validation of the reactivity of apolipoprotein cysteines by isoelectric focusing. The results for a VLDL isolated from a patient homozygous for apoC-II-T and apoE3 are shown in **Fig. 2.** All samples were reduced prior to electrophoresis. Each lane is labeled to indicate the treatment of the isolated VLDL prior to delipidation and electrophoresis. The lanes labeled "Stained" show the apolipoproteins as detected using Coomassie Blue staining of the gel, and the lanes labeled "aCII" show Western blot with anti-apoC-II antibody. About one-half of the apoE appeared to be derivatized when the intact VLDL was treated with 4-vinyl pyridine in the absence of β-mercaptoethanol (+4VP). Each of the apoE isoforms was essentially completely charge-shifted when the intact VLDL was treated with β-mercaptoethanol and 4-vinyl pyridine as seen by protein stain (+BME, +4VP).

Immunoblots showed two isoforms of apoC-II-T (−BME) as previously reported (14). Treatment of the intact VLDL with 4-vinyl pyridine (+4VP) showed two major and two minor bands, consistent with only a fraction of the apoC-II-T having a free cysteine in the intact VLDL. Treatment of the intact VLDL with β-

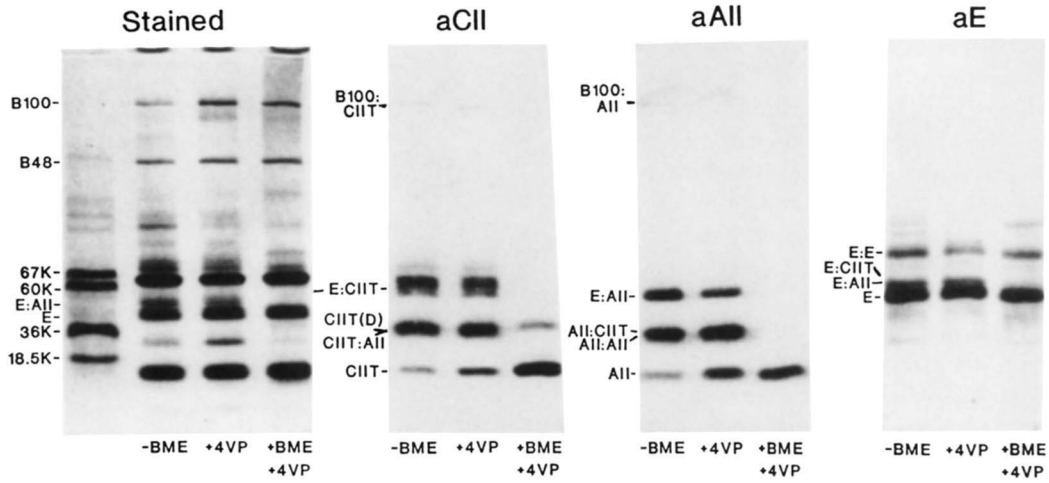

Fig. 3. Sodium dodecyl sulfate-glycerol polyacrylamide gel electrophoresis of homozygous C-II-deficient chylomicron apolipoproteins. Chylomicrons isolated from untreated plasma were either reduced with 60 mM β-mercaptoethanol and derivatized with 80 mM 4-vinyl pyridine (+BME, +4VP) or electrophoresed without reduction (−BME). Chylomicrons isolated from plasma treated with 80 mM 4-vinyl pyridine were electrophoresed without reduction (+4VP). The panel labeled "Stained" shows the apolipoproteins detected with Coomassie Blue stain. The first lane contains molecular weight markers. The panel labeled "aCII" shows the apolipoproteins detected by Western blot with anti-apoC-II antibody. The panel labeled "aAII" shows the apolipoproteins detected by Western blot with anti-apoA-II antibody. The panel labeled "aE" shows the apolipoproteins detected by Western blot with anti-apoE antibody. Abbreviations: B100, apoB-100; B48, apoB-48; CIIT(D), apoC-II-T:C-II-T dimer. Other abbreviations as in Figs. 1 and 2.

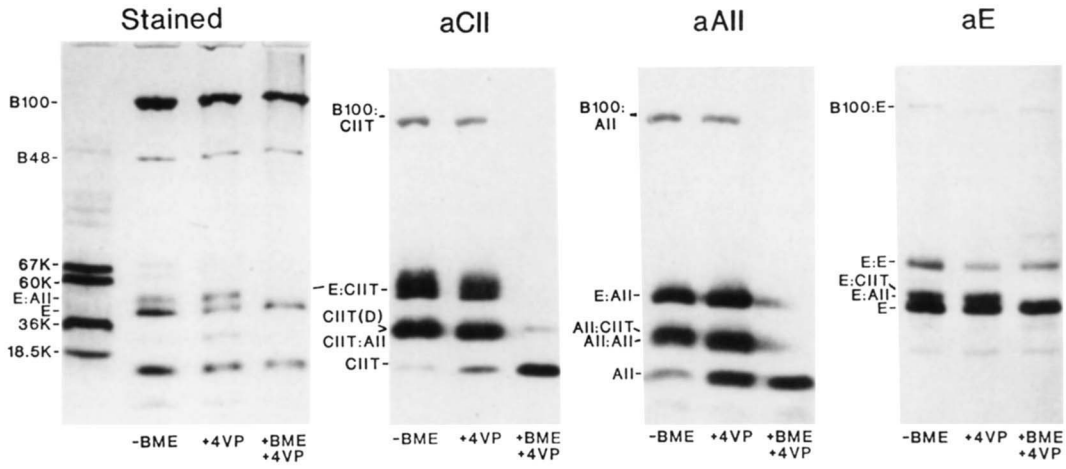

Fig. 4. Sodium dodecyl sulfate-glycerol polyacrylamide gel electrophoresis of homozygous C-II-deficient VLDL apolipoproteins. VLDL isolated from untreated plasma were either reduced with 60 mM β-mercaptoethanol and derivatized with 80 mM 4-vinyl pyridine (+BME, +4VP) or electrophoresed without reduction (−BME). VLDL isolated from plasma treated with 80 mM 4-vinyl pyridine were electrophoresed without reduction (+4VP). The panel labeled "Stained" shows the apolipoproteins detected with Coomassie Blue stain. The first lane contains molecular weight markers. The panel labeled "aCII" shows the apolipoproteins detected by Western blot with anti-apoC-II antibody. The panel labeled "aAII" shows the apolipoproteins detected by Western blot with anti-apoA-II antibody. The panel labeled "aE" shows the apolipoproteins detected by Western blot with anti-apoE antibody. Abbreviations as in Figs. 1 and 2.

mercaptoethanol prior to derivatization with 4-vinyl pyridine (+BME, +4VP) showed essentially complete charge-shift of apoC-II-T, consistent with all apoC-II-T cysteine residues being accessible and reactive with 4-vinyl pyridine under these conditions. Similar results were obtained for chylomicrons and LDL. Thus, under the conditions of pyridylethylation, free sulfhydryls of apoE, apoC-II-T, and apoA-II (not shown) were accessible and reactive in the intact lipoprotein.

SDS-glycerol polyacrylamide gel electrophoresis was carried out with samples run in parallel under three conditions. Treatment "−BME" indicates isolation of lipoproteins under ordinary conditions in the absence of any sulfhydryl reducing agents and includes disulfide-linked apolipoproteins present in the native lipoprotein, plus those formed artefactually during manipulation. Treatment "+4VP" indicates addition of 4-vinyl pyridine to plasma and isolation of lipoproteins in the presence of 4-vinyl pyridine. This condition shows the disulfide-linked apolipoproteins present in the native lipoprotein. Treatment "+BME, +4VP" indicates reduction of the isolated lipoprotein with β-mercaptoethanol with subsequent addition of 4-vinyl pyridine. This condition serves as a positive control showing that apolipoprotein cysteine residues were reactive with 4-vinyl pyridine in the intact lipoprotein.

Apolipoprotein species of chylomicrons

Protein stain of the chylomicron apolipoproteins separated by SDS electrophoresis showed that apoB-100 and apoB-48 were present as well as albumin, apoE, apoC-II-T, apoC-III and apoA-II (**Fig. 3,** panel "Stained").

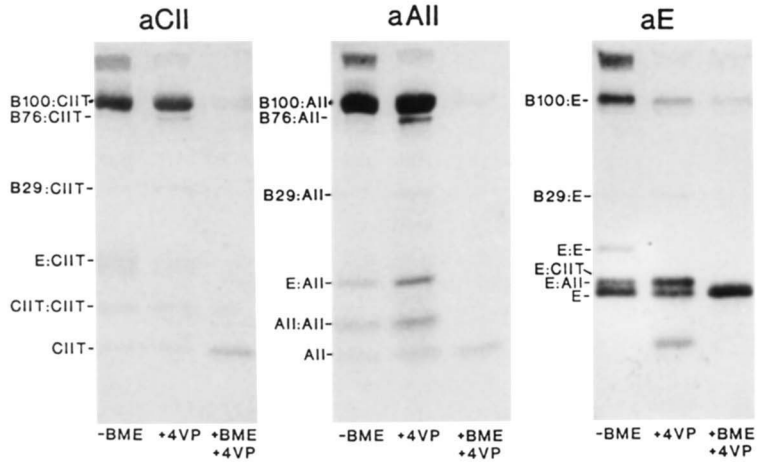

Fig. 5. Sodium dodecyl sulfate-glycerol polyacrylamide gel electrophoresis of homozygous C-II-deficient LDL apolipoproteins. LDL isolated from untreated plasma was either reduced with 60 mM β-mercaptoethanol and derivatized with 80 mM 4-vinyl pyridine (+BME, +4VP) or electrophoresed without reduction (−BME). LDL isolated from plasma treated with 80 mM 4-vinyl pyridine was electrophoresed without reduction (+4VP). The panel labeled "aCII" shows the apolipoproteins detected by Western blot with anti-apoC-II antibody. The panel labeled "aAII" shows the apolipoproteins detected by Western blot with anti-apoA-II antibody. The panel labeled "aE" shows the apolipoproteins detected by Western blot with anti-apoE antibody. Abbreviations: B76, apoB-76; B29, apoB-29. Other abbreviations as in Figs. 1 and 2.

Immunoblot with anti-apoC-II of intact chylomicrons treated with 4-vinyl pyridine (+4VP), (Fig. 3, panel aCII) detected small amounts of apoC-II-T with a monomeric molecular weight and a band with a molecular weight consistent with apoC-II-T:C-II-T (CIIT(D)) dimers or apoC-II-T:A-II dimers. ApoC-II-T:E3 was detected and had an apparently diffuse molecular weight. The reason for this is unknown. A faint band was detected with a molecular weight consistent with apoB-100:C-II-T. Immunoblot with anti-apoA-II (Fig. 3, panel aAII) showed that a small amount of apoA-II was in the monomeric state in the native chylomicrons. The majority of the apoA-II existed as either apoA-II:A-II dimers or apoC-II-T:A-II dimers. A faint band was detected with a molecular weight consistent with apoB-100:A-II. Immunoblot with anti-apoE (Fig. 3, panel aE) showed apoE3 heterodimers with molecular weights consistent with apoE3:A-II and apoE3:C-II-T. There was also reactivity of the anti-apoE antisera with a band in the molecular weight range of an apoE3:E3 dimer. However, the intensity of this band was unchanged after reduction and pyridylethylation.

Apolipoprotein species of VLDL

Protein stain of the VLDL apolipoproteins separated by SDS electrophoresis showed that the major form of apoB was apoB-100 (**Fig. 4,** panel "Stained"). Minor amounts of apoB-48 were also seen. Bands of about 43 kDa (consistent with apoE3:A-II or apoE3:C-II-T), 37 kDa (apoE3), 20 kDa (consistent with apoA-II:A-II and apoC-II-T:C-II-T), and 8 kDa (apoCs and apoA-II) were visible.

Immunoblot with anti-apoC-II revealed a pattern consistent with the majority of apoC-II-T existing in VLDL as dimeric species with only small amounts of monomeric apoC-II-T (Fig. 4, panel aCII). The bands of about 20 kDa that were detected were consistent with apoC-II-T:C-II-T (CIIT(D)) dimers or apoC-II-T:A-II dimers. The broad band of about 43 kDa was consistent with apoC-II-T:E3 dimers. As was seen for chylomicrons, the anti-apoC-II revealed bands in the 43-kDa region that appeared to be more heterogeneous than the bands revealed with the anti-apoE or the anti-apoA-II antiserum. It was not possible to satisfactorily identify the source of this apparent molecular weight heterogeneity. In addition to these bands, there was clearly a significant immunostaining of anti-apoC-II antiserum coincident with apoB-100. This was present in VLDL treated with 4-vinyl pyridine as well as untreated VLDL and the reaction was eliminated upon reduction and pyridylethylation of isolated VLDL. This is consistent with apoC-II-T existing as a disulfide-linked dimer with apoB-100. Immunoblot with anti-apoA-II revealed roughly equal distribution of apoA-II among monomeric apoA-II, apoA-II:A-II or apoA-II:C-II-T dimers and apoA-II:E dimers (Fig. 4, panel aAII, +4VP). Untreated VLDL (−BME) had significantly less monomeric apoA-II, indicating that some dimer formation could occur during isolation or delipidation of VLDL. In addition to these bands, there was clearly a significant immunostaining of anti-apoA-II antiserum coincident with apoB-100. This band was present in 4-vinyl pyridine-treated (Fig. 4, panel aAII, lane +4VP) and untreated VLDL (lane −BME). It was eliminated upon reduction and pyridylethylation of VLDL (Fig. 4, panel aAII, lane +BME, +4VP). This is consistent with apoA-II being disulfide-linked to apoB-100. Immunoblot with anti-apoE (Fig. 4, panel aE, lane +4VP) showed three bands, of 70 kDa, 43 kDa, and 37 kDa. The 43-kDa band was completely absent after reduction and pyridylethylation (lane +BME, +4VP), consistent with this being apoE3:A-II and apoE3:C-II-T dimers. The 70-kDa band was significantly diminished in intensity after reduction and pyridylethylation of the VLDL, consistent with the presence of apoE3:E3 homodimer. Comparison of the amount of the 70-kDa band in lanes −BME and +4VP suggests that apoE3:E3 dimers can form artefactually during the isolation and/or delipidation of VLDL. There also appeared to be traces of anti-apoE immunostaining coincident with apoB-100. However, this reaction was unchanged after reduction and pyridylethylation.

Apolipoprotein species of LDL

Protein stain of the LDL apolipoproteins separated by SDS electrophoresis showed a band for apoB-100 and a diffuse band at the top of the gel. Reduction and pyridylethylation before electrophoresis, showed only the apoB-100 band, suggesting that the diffuse band in the unreduced sample was disulfide-linked apoB-100 (not shown). LDL isolated from 4-vinyl pyridine-treated plasma revealed apoB-100 and three bands with smaller molecular weights equivalent to apoB-76, apoB-29, and apoB-12 (not shown). These bands are similar to fragments observed after kallikrein treatment (24).

Immunoblot with anti-apoC-II gave a very strong reaction to apoC-II coincident with the apoB-100 band (**Fig. 5,** panel aCII, lanes −BME and +4VP). A faint reaction was obtained to apoB-76 and apoB-29 (lane +4VP). ApoC-II-T monomer, apoC-II-T:C-II-T and apoC-II-T:E dimers were also detected. There was a significant decrease in the reaction of anti-apoC-II with the apoB species when the LDL was reduced and pyridylethylated prior to electrophoresis and an increase in the reaction to monomeric apoC-II (lane +BME, +4VP). Immunoblot with anti-apoA-II gave a very strong reaction to apoA-II coincident with the apoB-100. (Fig. 5, panel aAII, lanes −BME and +4VP). There was a reaction to apoB-76 and a light reaction to apoB-29 in lane +4VP. There was also a reaction with apoA-II:E3, apoA-II:A-II, and monomeric apoA-II. In this gel, it was possible to see that the apoA-II:A-II dimer had a different apparent molecular

weight than that of the apoC-II-T:C-II-T dimer. Reduction and pyridylethylation of the LDL resulted in the almost complete loss of apoA-II in the apoB bands and appearance of only monomeric apoA-II (lane +BME, +4VP). Immunoblot with anti-apoE showed a strong reaction with apoB-100 and apoB-100 aggregates (panel aE, lane −BME) that was significantly reduced by treatment with 4-vinyl pyridine (lane +4VP). This is consistent with apoE3 forming artefactual dimers with apoB-100 under the ordinary conditions of LDL isolation. ApoE3:A-II and monomeric apoE3 were the predominant apoE species (lane +4VP). A band of about 8 kDa was detected with anti-apoE when LDL was isolated from plasma treated with 4-vinyl pyridine (+4VP) but not when isolated LDL was treated with β-mercaptoethanol and 4-vinyl pyridine (+BME, +4VP). This suggests that treatment of plasma with 4-vinyl pyridine may have activated a proteolytic cleavage of apoE.

In order to confirm that the reaction to anti-apoC-II and anti-apoA-II was due to the presence of apoB-100:C-II-T and apoB-100:A-II, the apoB-100 band from LDL, isolated from 4-vinyl pyridine-treated plasma, was separated by nonreducing SDS electrophoresis and the apoB-100 band was excised. Portions of the gel containing apoB-100 were treated with β-mercaptoethanol and then layered onto a second SDS gel and subjected to SDS electrophoresis. The proteins were transferred to nitrocellulose membranes and blotted with anti-apoB, anti-apoC-II, and anti-apoA-II. Immunoblot with anti-apoB (not shown) detected a single species of apoB-100, indicating that this band was successfully excised and that the apoB remained intact during the subsequent manipulations. The anti-apoC-II detected two bands, one coincident with apoB-100 and the other with monomeric apoC-II-T (**Fig. 6,** lane aCII, +BME). The anti-apoA-II detected two bands, one coincident with apoB-100 and the other with monomeric apoA-II (Fig. 6, lane aAII +BME).

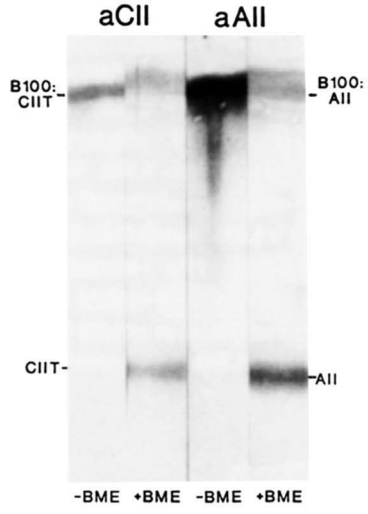

Fig. 6. Sodium dodecyl sulfate-glycerol polyacrylamide gel electrophoresis of heterozygous C-II-deficient low density apolipoproteins. LDL isolated from plasma treated with 80 mM 4-vinyl pyridine was electrophoresed without reduction. Gel containing apoB was excised. One-half of the gel was untreated (−BME), and one-half was soaked in buffer containing 60 mM β-mercaptoethanol (+BME). These gel slices were then placed on a second sodium dodecyl sulfate-glycerol polyacrylamide gel, electrophoresed, and analyzed by Western blot with anti-apoC-II antibody (panel aCII), and anti-apoA-II antibody (panel aAII). Abbreviations as in Fig 5.

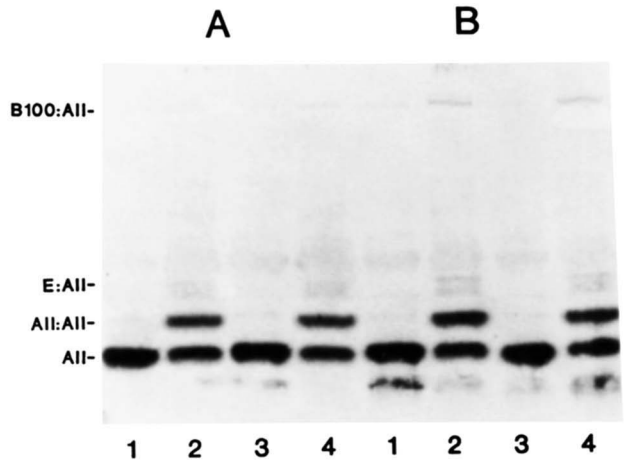

Fig. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of serum, showing apolipoproteins detected by Western blot with anti-apoA-II antibody; (A) from a subject with Type IIB hyperlipoproteinemia; and (B) from a subject with Type IV hyperlipoproteinemia. Lanes 1 and 2 show untreated serum. Lanes 3 and 4 show serum treated with 80 mM 4-vinyl pyridine. Lanes 1 and 3 show samples reduced with 40 mM dithiothreitol prior to electrophoresis, while lanes 2 and 4 show unreduced samples.

Analysis of plasma from normolipidemic and hyperlipidemic subjects for B-100:A-II disulfide-linked species

Our studies were extended to normolipidemic and hyperlipidemic subjects. **Fig. 7** shows the apolipoproteins detected after separation of serum by SDS-glycerol gel electrophoresis and immunoblot with anti-apoA-II. For illustration, the results for one hypercholesterolemic and one hypertriglyceridemic subject are shown. The authentic species that were identified in the presence of 4VP were monomeric apoA-II, apoA-II homodimer, apoE:A-II, and apoB-100:A-II, (lanes A4 and B4).

The VLDL from these subjects is shown in **Fig. 8.** The apoB-100:A-II and apoE:A-II species are clearly present in both subjects. The hypertriglyceridemic subject was an apoE3/E2 heterozygote and an additional species with a molecular weight consistent with apoE2:A-II:A-II was observed (lane B4, Fig. 8).

Immunoassay of lipoprotein fractions

Enzyme-linked immunosorbent assays with a "sandwich" design were developed to quantify disulfide-linked species. Of particular interest was whether apoB-100:C-II-

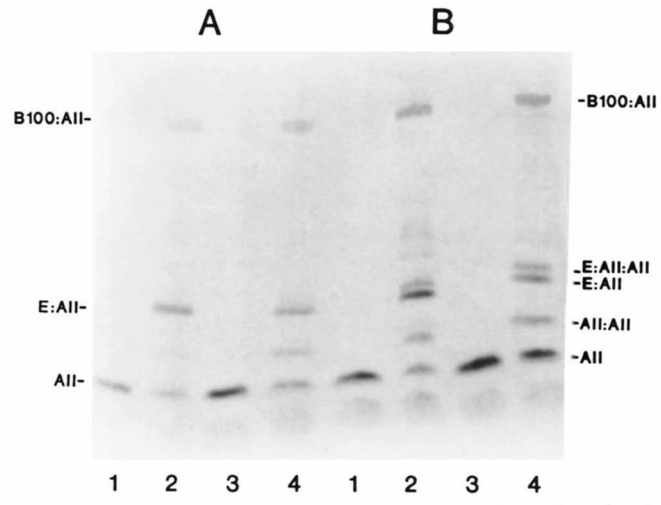

Fig. 8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of VLDL apolipoproteins, with subsequent detection by Western blot with anti-apoA-II antibody: (A) from a subject with Type IIB hyperlipoproteinemia; and (B) from a subject with Type IV hyperlipoproteinemia. Lanes 1 and 2 show apolipoproteins from VLDL isolated from untreated serum. Lanes 3 and 4 show apolipoproteins from VLDL isolated from serum treated with 80 mM 4-vinyl pyridine. Lanes 1 and 3 show samples reduced with 40 mM dithiothreitol prior to electrophoresis, while lanes 2 and 4 show unreduced samples.

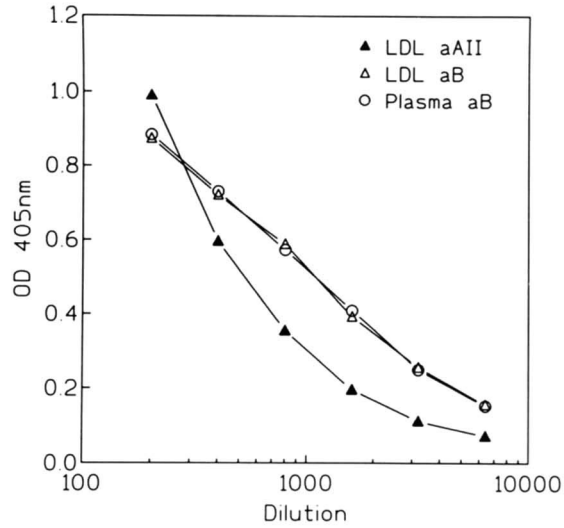

Fig. 9. Comparison of titration curves of apoB bound to anti-apoA-II or anti-apoB. Dilutions of LDL were incubated with microtiter wells coated with anti-apoA-II (filled triangles) or anti-apoB (open triangles). Dilutions of plasma were incubated with wells coated with anti-apoB (open circles). Bound apoB was detected with anti-apoB linked to alkaline phosphatase as described in the Methods section.

T and apoB-100:A-II could be detected by immunoassay. We were able to detect apoB-100:A-II in VLDL and LDL when microtiter plates were coated with anti-apoA-II and the bound apolipoprotein complexes were detected with anti-apoB. As the buffers used in this assay contained Tween-20, lipid–apolipoprotein complexes were disrupted and only apolipoproteins that were covalently linked remained associated. The titration curve of apoB-100 in the apoB-100:A-II complex was not parallel to that of total apoB-100 (**Fig. 9**). Ten percent of the total apoB-100 of this LDL bound to an anti-apoA-II affinity column. This LDL was used as a standard for the assay results presented here.

The characteristics of the hyperlipidemic subjects are shown in **Table 1.** These patients were identified as hypercholesterolemic when the LDL cholesterol was greater than the 95th percentile compared to the Lipid Research Clinics Prevalence tables (25). Hypertriglyceridemic subjects were defined as those with triglyceride greater than the 95th percentile and LDL cholesterol less than the 95th percentile. By definition, the hypercholesterolemic subjects had significantly higher LDL cholesterol than either normal or hypertriglyceridemic (HTG) subjects and the hypertriglyceridemic subjects had higher VLDL than either normal or hypercholesterolemic (HC) subjects.

The mean concentration of apoB-100:A-II in the LDL from the control subjects was 40 ± 11 μg/ml. This was significantly lower than the concentration of apoB-100:A-II in the hyperlipidemic subjects (**Table 2**). The concentration of LDL apoB-100:A-II was not significantly different between the HTG and HC subjects. As a result, the ratio of LDL apoB-100:A-II to LDL B was significantly higher in the HTG subjects compared with the HC subjects (Table 2). The VLDL apoB-100:A-II was not measured in control subjects. The concentration of VLDL apoB-100:A-II was significantly higher in the HTG subjects than in the HC subjects as would be expected given the large difference in VLDL concentrations. In addition,

TABLE 1. Characteristics of subjects analyzed for apoB-100:A-II

Subject[a]	Age	Chol	TG	VLDL-C	VLDL-TG	LDL-C	HDL-C
	yr	*mmol/l*					
Normal	38.1 ± 8	4.78 ± 1.0	1.53 ± 0.9	0.33 ± 0.21	1.08 ± 0.83	2.88 ± 0.8	1.56 ± 0.4
HTG	42.4 ± 7	6.99 ± 2.4	9.43 ± 4.7	4.12 ± 2.2	8.46 ± 4.0	2.2 ± 0.7	0.68 ± 0.1
HC	40.2 ± 14	7.5 ± 0.7	2.04 ± 0.6	0.58 ± 0.3	1.38 ± 0.6	5.58 ± 0.6	1.35 ± 0.3

[a] Normal subjects were six women and three men; HTG subjects were two women and three men; HC subjects were six women and two men.

TABLE 2. Concentration of apoB-100:A-II in VLDL and LDL

Subject	VLDL B-100:A-II	VLDL B	VLDL Ratio	LDL B-100:A-II	LDL B	LDL Ratio
	μg/ml	*μg/ml*		*μg/ml*	*μg/ml*	
Normal	NM	48.3 ± 22.6	NM	40 ± 11	722 ± 210	0.06 ± 0.01
HTG	138 ± 84	327 ± 170	0.40 ± 0.1	169 ± 100	502 ± 184	0.33 ± 0.1
HC	18 ± 8	89 ± 38	0.22 ± 0.09	135 ± 58	1132 ± 132	0.12 ± 0.04

Abbreviations: VLDL ratio, VLDL B-100:A-II/VLDL B; LDL ratio, LDL B-100:A-II/LDL B; NM, not measured. Significant difference for fractions containing apoB-100:A-II:VLDL B-100:A-II, HTG vs. HC $P = 0.003$; LDL B-100:A-II, Normal vs. HC $P = 0.003$; Normal vs. HTG $P = 0.0009$; VLDL ratio, HTG vs. HC $P = 0.013$; LDL ratio, Normal vs. HTG $P = 0.0001$; HTG vs. HC $P = 0.003$. All P values derived from nonparametric tests of significance.

the ratio of VLDL apoB-100:A-II to VLDL apoB was significantly higher in the HTG subjects compared with the HC subjects (Table 2).

We did not succeed in detecting apoB-100:A-II in whole plasma or when LDL was assayed in the presence of HDL (results not shown). Thus the apoA-II of HDL competes with apoB-100:A-II. This immunochemical behavior was further illuminated by a comparison of the immunoassay values for apoA-I and apoA-II in HDL before and after reduction and pyridylethylation. The reduction and pyridylethylation had no effect on the amount of apoA-I detected. However, there appeared to be one-half as much apoA-II as in untreated HDL (not shown). This indicates that monomeric apoA-II gives a significantly lower immunochemical response in this sandwich immunoassay than apoA-II:A-II dimer and may be an additional reason for the interference by apoA-II of HDL.

DISCUSSION

We have demonstrated that the apolipoprotein species present in hyperchylomicronemia associated with apoC-II deficiency due to the presence of apoC-II-T are multiple, complex, and dependent upon the lipoprotein class. The initial objective was to test whether the mutant apoC-II, apoC-II-T, which contains a cysteine residue, exists in lipoproteins in a monomeric or dimeric state. In the process of investigating this question we made observations about the state of apoE3, apoA-II, and apoB-100 that are likely to be significant in a broader context. Using electrophoresis in a single dimension and Western blot, the identification of each species was dependent upon the limits of resolution of dimeric species by molecular weight in our system. For example, apoC-II-T homodimers were only resolved from apoA-II homodimers in LDL (Fig. 5). This molecular weight region for the apolipoproteins of chylomicrons and VLDL was positive by Western blot for apoC-II-T and apoA-II. We have, for the purposes of this study, presumed that all possible combinations of homodimeric and heterodimeric species were present in these bands (Figs. 3 and 4). The identification of apoC-II-T:E heterodimers is based upon the observation of apolipoproteins with molecular weight of about 43 kDa were positive by Western blot for apoC-II. The reaction by Western blot for apoC-II in this region was diffuse. It is not apparent why the bands in this region lacked sharpness, but it suggests that there may be an additional basis for heterogeneity that we have not yet identified. Thus we provided evidence that apoC-II-T exists primarily in dimeric form and that in chylomicrons and VLDL it forms disulfides with other apolipoproteins known to contain sulfhydryl groups, i.e., apoA-II, apoE3, and apoB-100. The current tests clearly establish the qualitative nature of these species. It is difficult to make rigorous comparisons of the relative amounts of each of these disulfide-linked species. The relative intensity of the bands detected by Western blot reflects the immunogenicity and efficiency of transfer of protein from the polyacrylamide gel, the efficiency of binding of protein to the nitrocellulose membrane, and the actual mass of protein. It is probably only appropriate to consider distinctions between species present in trace quantities versus readily detectable quantities. Of particular interest was the observation that apoC-II-T formed complexes with apoB-100. These complexes were present in VLDL and LDL. The existence of apoB-100:C-II-T raises the possibility that these species are metabolized differently than either apoC-II-T homodimers or apoB-100 without apoC-II-T.

Consistent with the original report of apoE:A-II dimers, we found these dimers in VLDL. In addition, we observed these dimers in the d 1.006–1.063 g/ml LDL. The existence of these dimers in VLDL as well as HDL (8–10) has importance in the conceptualization of the metabolic significance of apoE:A-II dimers. Thus the apoA-II that can be present in chylomicrons and VLDL may be more likely to form these heterodimers which could impair the function of chylomicron and VLDL-associated apoE. This may also indirectly lead to a decrease in HDL apoA-II, if the apoA-II of chylomicrons and VLDL is a source of HDL apoA-II.

Of broader interest was the detection of apoB-100:A-II in hyperlipidemia, particularly hypertriglyceridemia.

Thus we hypothesize that apoB-100:A-II is common to hypertriglyceridemic states. It is known that the LDL of hypertriglyceridemic subjects has altered binding characteristics in studies using human fibroblasts (26). We speculate that apoB-100:A-II would have a decreased binding affinity for the LDL receptor. This may increase catabolism of this LDL by alternative receptor-dependent pathways. Clearly this could be of potential significance in the development of atherosclerosis.

Disulfide-linked species of apoB-100 and mutant apolipoproteins or apoA-II have not been previously reported. A subfraction of apoB lipoproteins containing apoA-II has been isolated by anti-apoA-II affinity chromatography of VLDL from subjects with Tangier disease or Type V hyperlipoproteinemia (27). This fraction of lipoprotein would contain by definition any apoB-100:A-II if it existed in the subjects studied. The apoA-II-containing VLDL was found to have a pseudo first-order rate constant for triglyceride hydrolysis that was one-half of that of the apoA-II-free VLDL from these subjects (27). This suggests that apoA-II serves as a marker of a metabolically distinct VLDL.

The existence of disulfide-linked species of apolipoproteins in lipoproteins may have a number of significant consequences. First, it is clear that the apoE of apoE:A-II does not function as an effective ligand for the apoB,E receptor. Second, apoB-100:A-II may also lack function if the apoA-II is sufficiently close to the receptor binding domain of apoB-100. ApoB-100:[a] has been reported to bind to LDL receptors with a reduced affinity (28, 29) that can be attributed to the presence of apo[a]. Recent studies of the kinetics of the binding of apoB-100 to LDL receptors suggest that this is a complex process (30). The observations reported here may provide a basis for testing the importance of the cysteine residues of apoB-100 in the binding to the LDL receptor. Third, the time course of formation of these disulfide-linked species and their site of formation is unknown. It may be that they are formed during lipoprotein assembly within hepatocytes. If this is the case, they may reflect abnormal assembly of lipoproteins. Alternatively, the disulfide-linked species may form after the lipoproteins are secreted. This latter possibility has been shown for recombinant apo[a] expressed in transgenic mice that were then injected with human LDL (31). However, it appears that the conditions for isolation of the LDL did not include reagents that would derivatize free sulfhydryls. Thus some of the formation of apoB-100:[a] may have occurred during manipulation of the plasma in vitro.

The presence of the apoB-100:A-II may reflect a slow process and thus may be an indication of the residence time of the lipoproteins. If the formation of apoB-100:A-II is a rapid process, then it would reflect the relative molar ratios of the reactants. Development of more sophisticated assays will allow us to determine the total amount of apoA-II, apoE, and apoB-100 present as heterodimers. With these assays we will be able to test other hyperlipidemic populations for the concentration of these species and address such questions as the rate of change in their concentration after different dietary or drug treatments.

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