Characterization of disulfide-linked heterodimers containing apolipoprotein D in human plasma lipoproteins

Francisco Blanco-Vaca,[1] David P. Via, Chao-yuh Yang, John B. Massey, and Henry J. Pownall

Baylor College of Medicine and The Methodist Hospital, 6565 Fannin Street, M.S.A601, Houston, TX 77030

Abstract Human plasma apolipoprotein (apo) D is a glycoprotein with an apparent molecular weight of 29,000 M_r. It is present, mainly, in high density lipoproteins (HDL) and very high density lipoproteins (VHDL). Western blot analysis of HDL and VHDL using rabbit antibodies to human apoD revealed major immunoreactive bands at 29,000 and 38,000 M_r, with minor bands ranging from 50,000 to and 80,000 M_r. Only the 29,000 M_r band corresponding to apoD remained when the electrophoresis was conducted under reducing conditions, demonstrating that apoD is cross-linked to other proteins via disulfide bonds. The broad pattern of immunoreactivity was also observed under nonreducing conditions when the blood was collected into a solution of sulfhydryl-trapping reagents, or when these reagents were added to the isolated lipoproteins. These results indicated that the disulfide bonds were not the result of disulfide exchange during the experimental procedures. On the basis of amino acid sequencing and reactions to antibodies, the 38,000 M_r band was identified as an apoD-apoA-II heterodimer. The apoD-apoA-II was also demonstrated in plasma. In both HDL and plasma, the apoD-apoA-II heterodimer constituted the major form of apoD. Disulfide-linked heterodimers of apoD and apoB-100 were also found in low and very low density lipoproteins, and in whole plasma. ■■ It is concluded that a fraction of human apoD, like other cysteine-containing apolipoproteins, exists as a disulfide-linked heterodimer with other apolipoproteins in all major human lipoprotein fractions.—Blanco-Vaca, F., D. P. Via, C-y. Yang, J. B. Massey, and H. J. Pownall. Characterization of disulfide-linked heterodimers containing apolipoprotein D in human plasma lipoproteins. *J. Lipid Res.* 1992. **33:** 1785–1796.

Supplementary key words HDL • VHDL • apolipoprotein A-II • apolipoprotein B-100 • α_2-microglobulin superfamily

Apolipoprotein (apo) D is a glycoprotein with an apparent molecular weight between 26,000 and 32,000 M_r (1, 2). Plasma apoD concentrations vary from 6 to 12 mg/dl (1, 2). Most, if not all of it, is associated with lipoproteins, especially high and very high density lipoproteins (HDL and VHDL) (2–4). ApoD does not contain the amphiphilic α-helical regions that characterize other soluble apolipoproteins (5, 6). In contrast, it shares homology with human plasma retinol-binding protein and a growing number of members of the α_2-microglobulin protein superfamily, all of which transport hydrophobic lipid ligands (5–9).

The physiological role of apoD is not known. ApoD, together with apoA-I, lecithin:cholesterol acyltransferase (LCAT), and the cholesteryl ester transfer protein (CETP) form a pre-β-HDL subclass which is thought to be involved in the early steps of the cholesterol transport from peripheral tissue to the liver (10–12). The presence of apoD mRNA in interstitial and connective tissue fibroblasts of all organs (13, 14), and the accumulation of apoD in the regenerating peripheral nerve suggested that apoD also carries cholesterol or its derivatives within extravascular compartments (15–17). However, the identification of GCDPF-24, a progesterone-binding protein present in high concentrations in the cyst fluid of women with breast gross cystic disease, as apoD, has strongly suggested a new ligand for this protein (18). Furthermore, GCDPF-24 does not bind cholesterol and there is no correlation between GCDPF-24 and cholesterol concentrations in cystic fluid (19, 20). These findings bring into question a role for apoD in reverse cholesterol transport. On the basis of the homology between apoD, insecticyanin, and bilin binding protein, it has also been proposed

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Abbreviations: apo, apolipoprotein; BME, β-mercaptoethanol; CETP, cholesteryl ester transfer protein; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoproteins; HPLC, high performance liquid chromatography; M_r, electrophoretic mobility relative to the standards; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; VHDL, very high density lipoproteins; VLDL, very low density lipoproteins.
[1]To whom correspondence should be addressed.

that apoD is a carrier of heme-related compounds (9). It remains to be shown whether apoD, like other members of the α_2- microglobulin superfamily, carries more than one ligand (21-23).

Plasma apoD concentrations correlate positively with those of apoA-I (24, 25). The cross-reactivity of monoclonal antibodies to apoD with other proteins in HDL, VHDL, and low density lipoproteins (LDL) has raised doubts about the significance of these measurements (26, 27). A disulfide link between apoD and apolipoprotein B-100 has been observed in LDL, but not in HDL and VHDL, where most of the cross-reactivity appears (27). Considering that apoD contains at least one free sulfhydryl group and the absence of data indicating that it is synthesized as a polyprotein precursor (5, 6), we searched for disulfide links between apoD and other apolipoproteins within all major human plasma lipoprotein classes.

MATERIALS AND METHODS

Materials

Hydroxylapatite, β-mercapthoethanol (BME), acrylamide, N,N'-methylene-bis-acrylamide, glycine, sodium dodecyl sulfate (SDS), Tris, and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories (Richmond, CA). Pre-stained protein molecular weight standards were from Bethesda Research Laboratories (Gaithersburg, MD). Protein A-Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). The 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt, iodoacetamide, 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), hydrogen peroxide, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). Goat polyclonal antibodies to apoA-II were purchased from Calbiochem (La Jolla, CA). The peroxidase-labeled anti-IgG to rabbit, mouse, and goat IgG were from Organon Teknika-Cappel West Chester, PA). Carrier-free Na[^{125}I] was from Amersham (Arlington Heights, IL). L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington Biochemicals (Malvern, PA).

Lipoprotein isolation

Plasma or blood was obtained from healthy fasted donors at The Methodist Hospital Blood Donor Center (Houston, TX). EDTA, sodium azide, and apoprotinin were added to the plasma (28). To inhibit disulfide bond formation during handling, some blood was collected directly into DTNB (100 mg/ml DTNB, 0.1 M phosphate buffer, pH 7.0); alternatively, the plasma was treated with iodoacetamide (0.36 g/ml, 1 M phosphate, pH 8.0) (29).

Lipoproteins were isolated from human plasma by sequential flotation (30). Very low density lipoproteins (VLDL), intermediate plus low density lipoproteins

(IDL + LDL), and HDL, respectively, were isolated at densities of < 1.006 g/ml, 1.006–1.063 g/ml, and > 1.063 < 1.21 g/ml). VHDL was isolated as the clear zone between HDL and the lipoprotein-free plasma fraction. Lipoproteins were also isolated by ultracentrifugation on a potassium bromide density gradient where the densities were determined by refractometry (31). Isolated lipoproteins were dialyzed against 0.15 M NaCl, 0.05 M Tris, 0.002 M EDTA, pH 7.4, at 4°C. ApoHDL was prepared by delipidation of HDL with diethyl ether–ethanol 3:1 (v/v) (32). Protein concentrations were measured by the method of Bradford, using bovine serum albumin as the standard (33).

Isolation of apolipoproteins

Protein homogeneity, defined as the presence of a single band in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), was determined after staining with Coomassie Brilliant Blue R-250 or/and with silver. ApoD was isolated by a previously published procedure consisting of chromatography of apoHDL on a hydroxylapatite column in 2 M urea, 1 mM K_2HPO_4, pH 8.0 (34). ApoD eluted in the unretained fraction. Partially purified apoD–apoA-II heterodimer was obtained by overloading apoHDL on a hydroxylapatite column, followed by repeating hydroxylapatite chromatography or by Mono Q chromatography using a linear gradient (0 to 1 M NaCl in 10 mM Tris, 4 M urea, pH 7.4). ApoA-I, apoA-II, and apoE were isolated as previously described (35, 36).

Trypsinolysis, peptide isolation and amino acid sequence analysis

Tryptic cleavage of partially purified apoD–apoA-II (1 mg/ml in 0.1 M ammonium bicarbonate, pH 8.0) was performed with TPCK-treated trypsin at enzyme–substrate ratio 1:50. The mixture was left at room temperature for 5 h. The tryptic hydrolysate was then subjected to high performance liquid chromatography (HPLC) separation on a Beckman system (Beckman Inc., Fullerton, CA) that included a 165 Variable Wavelength Detector, a 421 Controller, and two 114 M Solvent Delivery Modules (28). A Vydac C_{18} reversed-phase column (250 × 4.6 mm), heated to 50°C, was used to isolate the peptides at a flow rate of 1.5 ml/min with a trifluoroacetic acid (TFA) system (buffer A: 0.1% TFA in water, v/v; buffer B: 0.08% TFA in 95% acetonitrile and 5% water, v/v/v). Sequence analysis of HPLC-purified peptides was determined with a gas-phase sequencer from Applied Biosystems (Applied Biosystem Inc., Foster City, CA) (28).

### Immunochemical and electrophoretic procedures

ApoD antiserum was produced by subcutaneous immunization of a rabbit with purified human apoD. The antiserum reacted with apoD and HDL in a noncompeti-

tive enzyme-linked immunosorbent assay (ELISA) and on Western blots (37). It did not cross-react with bovine serum albumin, human apoA-I, or human apoA-II, in dot and Western blot analysis. The rabbit IgG fraction containing antibodies to apoD was isolated by Protein A Sepharose CL-4B chromatography (38) and was homogeneous on SDS-PAGE. The anti-apoD IgG recognized its antigen in VLDL, LDL, HDL, and VHDL, giving essentially parallel curves in a competitive ELISA (37). Based upon a titration, 40 μg/ml of anti-apoD IgG was used in all Western blots. The concentrations of apolipoproteins and anti-apoD IgG were determined spectrophotometrically. The characteristics of the monoclonal antibodies to human apoA-II (M-26 and M-32), and polyclonal antibodies to human apoE and to human LDL have been described (39–41). Affinity-purified polyclonal antibodies to electrophoretically separated apoD and apoA-II were obtained by releasing antibody from nitrocellulose transfers. After dialysis in PBS, pH 7.4, the antibodies were used for Western blots (17).

The SDS-PAGE were conducted according to Laemmli (42). Usually 12% or 15% acrylamide was used to separate the apolipoproteins of HDL or VHDL. A linear 3.5% to 15% gradient of acrylamide was used to analyze the apolipoprotein compositions of VLDL and IDL + LDL and all the lipoprotein fractions isolated by density gradient ultracentrifugation. When needed, proteins were reduced before SDS-PAGE with 5% BME. Free sulfhydryl groups were blocked with iodoacetamide and/or DTNB (29). One or both were added to the sample and incubated for 90 min in darkness at room temperature prior to SDS-PAGE. DTNB and iodoacetamide were sometimes added to blood or plasma before isolation of the lipoproteins (29). The apparent molecular weights of the proteins were assigned by comparison with pre-stained standards. The gels were stained with Coomassie Brilliant Blue R-250 and/or with silver, or electroblotted onto nitrocellulose sheets (43). These were blocked and incubated with the appropriate antibodies to apoD, apoA-II, LDL, or apoE. According to the animal species in which the first antibody was raised, the antigen was detected using horseradish peroxidase-labeled anti-rabbit, -mouse, or -goat IgG. To measure the percentage distribution of apoD in HDL and in plasma, anti-apoD IgG was labeled with ^{125}I by the chloramine T procedure (44), and the counts corresponding to the apoD monomer and the apoD–apoA-II heterodimer were determined using a Betascope 603 Blot Analyzer (Betagen Corporation, Waltham, MA).

RESULTS

Fig. 1 shows the electrophoretic migration of a preparation of human apoD obtained as the non-retained frac-
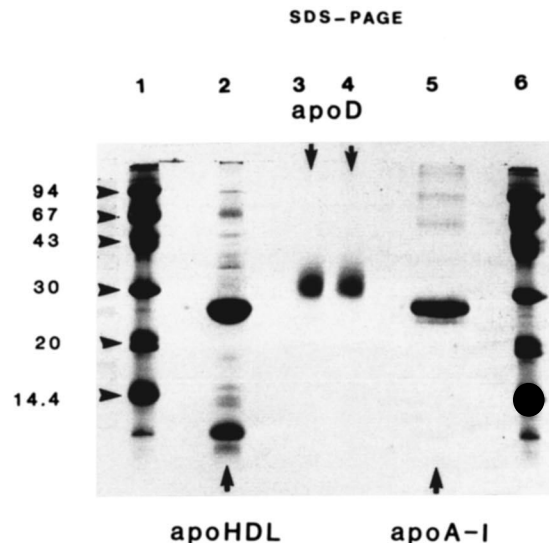
Fig. 1. SDS-PAGE of purified apoD stained with Coomassie Brilliant Blue R-250. Lanes 1 and 6, migration of standard molecular weight proteins. Lane 2, apoHDL. Lanes 3 and 4, apoD obtained as the nonretained fraction obtained after apoHDL hydroxylapatite chromatography. Lane 5, apoA-I. All the samples (30 μg protein each lane) were reduced with 5% BME.

tion after hydroxylapatite chromatography. This material was used to raise antibodies in rabbits. The electrophoretic behavior of apoD in lipoproteins, analyzed by Western blot analysis using antibodies to apoD, was different under reducing and nonreducing conditions. Under nonreducing conditions, immunoreactive bands of 29,000 M_r, 38,000 M_r, 50,000 M_r and 60,000 M_r were observed in the HDL fractions (**Fig. 2, A**). Also, immunoreactive bands at the stacking gel, together with a 65,000 M_r and 29,000 M_r bands, were noted in the LDL fractions (Fig. 2, A). Essentially only the 29,000 M_r band corresponding to apoD remained when the lipoproteins were tested under reducing conditions (Fig. 2, B). Only lane 1 corresponding to the most dense HDL fraction (d 1.21 g/ml) contaminated with plasma proteins showed an additional 65,000 M_r immunoreactive band. This was shown to be due to the cross-reaction of the peroxidase goat IgG anti rabbit IgG with human plasma IgG (Fig. 2, B, lane 1), explaining also the origin of the 150,000 M_r band seen in nonreduced conditions (Fig. 2, A, lane 1). For that reason, the rest of the experiments were performed using affinity-purified peroxidase antibodies adsorbed with human IgG. Collective consideration of these results supports the existence of disulfide bonds between apoD and other proteins in HDL and LDL.

Disulfide-linked dimers containing apoD in HDL and VHDL

Western blot analysis of HDL and VHDL revealed major immunoreactive bands of 38,000 M_r and 29,000

WB anti apoD

A   NR LIPOPROTEINS

B   R LIPOPROTEINS

C
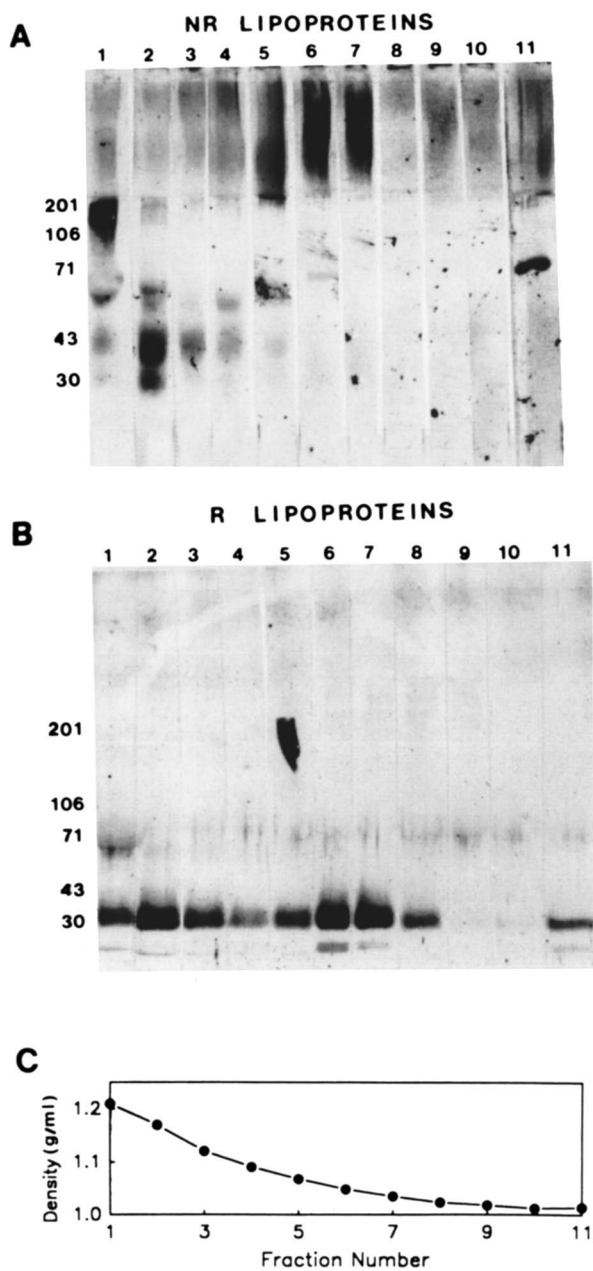
Fig. 2. Western blots (WB) of lipoprotein fractions isolated by density gradient ultracentrifugation using antibodies to apoD. A: Nonreduced (NR) lipoproteins. All lanes were loaded with 20 μg of protein except lanes 8 (15 μg), 9 (3 μg), and 10 (5 μg). B: Reduced (R) (5% BME) lipoproteins. In all lanes, the mass of protein used was twice that used in A. In both A and B, the migration of standard molecular weight proteins is shown at the left. C: Densities (g/ml) of the isolated lipoprotein fractions.

M_r. Addition of 22 mM iodoacetamide (final concentration) to the HDL samples 90 min before SDS-PAGE did not alter the immunoreactivity of nonreduced HDL. The pattern typical of nonreduced HDL remained after the

samples were boiled with the SDS-PAGE buffer without BME for 5 min and incubated in 22 mM iodoacetamide under a nitrogen stream. No differences were observed in weekly Western blot analyses of HDL that were stored at 4°C for 4 weeks (data not shown). This shows that the disulfide links formed by apoD cannot be prevented by sulfhydryl reagents, and that once they are formed they are stable over a time frame that is much longer than that of the isolation procedure.

The reduction-dependent pattern of immunoreactivity was not observed when similar experiments were performed using a mixture of purified human apoA-I, monomeric apoA-II, apoE, and apoD in concentrations that are representative of those found in plasma. In this case, only the 29,000 M_r band corresponding to apoD appeared (**Fig. 3**). These results established that disulfide bond formation between apoD and other apolipoproteins is not easily reproduced in the absence of a lipid or lipoprotein surface.

To rule out disulfide exchange between apolipoproteins during isolation as a source of heterodimer formation, freshly collected blood was divided into two parts. One
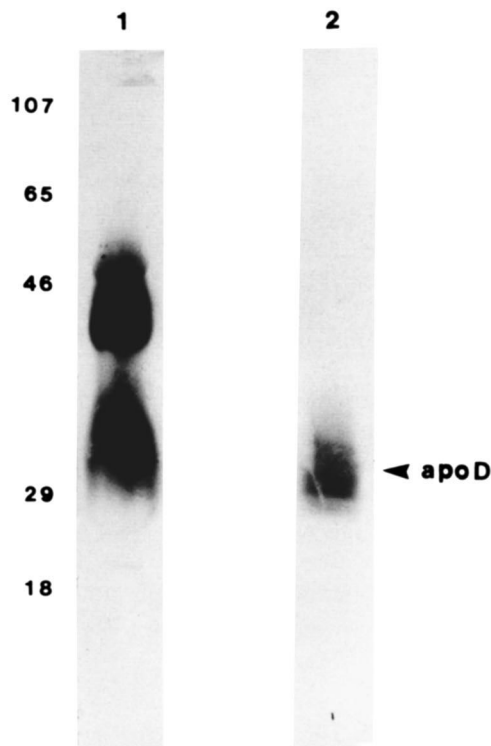
Fig. 3. Western blot (WB) using antibodies to apoD. Lane 1, 40 μg of nonreduced HDL. Lane 2, 10 μg of apoA-I, 4 μg of monomeric apoA-II, 1 μg of apoE, and 1 μg of apoD, incubated 3 h at 37°C prior to the addition of nonreducing SDS-PAGE sample buffer. The migration of standard molecular weight proteins is shown at the left.

part was treated during the first minute after collection with DTNB or iodoacetamide (29), and the other was not. In each case the same pattern of the nonreduced HDL and VHDL was observed (**Fig. 4, A**). The most prominent bands corresponded to the 29,000 M_r and 38,000 M_r, with traces of reactivity appearing between 50,000 M_r and 80,000 M_r (Fig. 4, A).

To test the hypothesis that the 38,000 M_r band was a disulfide complex of apoD and apoA-II, a series of Western blots were conducted using affinity-purified polyclonal antibodies (Fig. 4, B) and monoclonal antibodies (M-26 and M-32) to human apoA-II (Fig. 4, C and D). The pattern of immunoreactivity obtained was independent of the antibody used, and of the presence or absence of DTNB or iodoacetamide. In all cases immunoreactive bands of 9,000 M_r, 18,000 M_r, 38,000 M_r, 46,000 M_r, 60,000 M_r, and 70,000 M_r were observed. Most of the bands of molecular weight higher than 9,000 M_r disappeared with the addition of 5% BME, indicating the cleavage of disulfide bonds between apoA-II and other HDL-apolipoproteins. However, some reactivity corresponding to the 18,000 M_r and 38,000 M_r bands was observed after reduction regardless of which antibody was used.

The minor immunoreactive bands in HDL and VHDL to apoD antibodies ranged from 50,000 to 80,000 M_r.

These showed marked interindividual differences in their immunoreactivity, although they were consistently more evident in HDL isolated by density gradient ultracentrifugation than by sequential flotation. In the latter case, these were usually seen in VHDL rather than in HDL. The composition of these was not determined. No apoD–apoE heterodimer was found in HDL fractions isolated by gradient density ultracentrifugation and analyzed by Western blot. Under nonreducing conditions, HDL exhibited immunoreactive bands of 37,000 M_r, 46,000 M_r, and 72,000 M_r. Only the 37,000 M_r band remained after the addition of BME (data not shown). This pattern of immunoreactivity is consistent with the reported existence of apoE–apoA-II heterodimer and apoE homodimer (29, 45).

Amino acid sequence analysis of the apoD–apoA-II heterodimer

ApoD–apoA-II heterodimers were isolated in the non-retained fraction after overloading apoHDL on to a hydroxylapatite column. Typically, part of the 29,000 M_r apoD monomer eluted first (fractions 13–17, **Fig. 5**) and was succeeded by the rest of the 29,000 M_r apoD monomer and the 38,000 M_r apoD–apoA-II heterodimer (fractions 19–29). However, these preparations also contained low molecular weight proteins including apoA-II
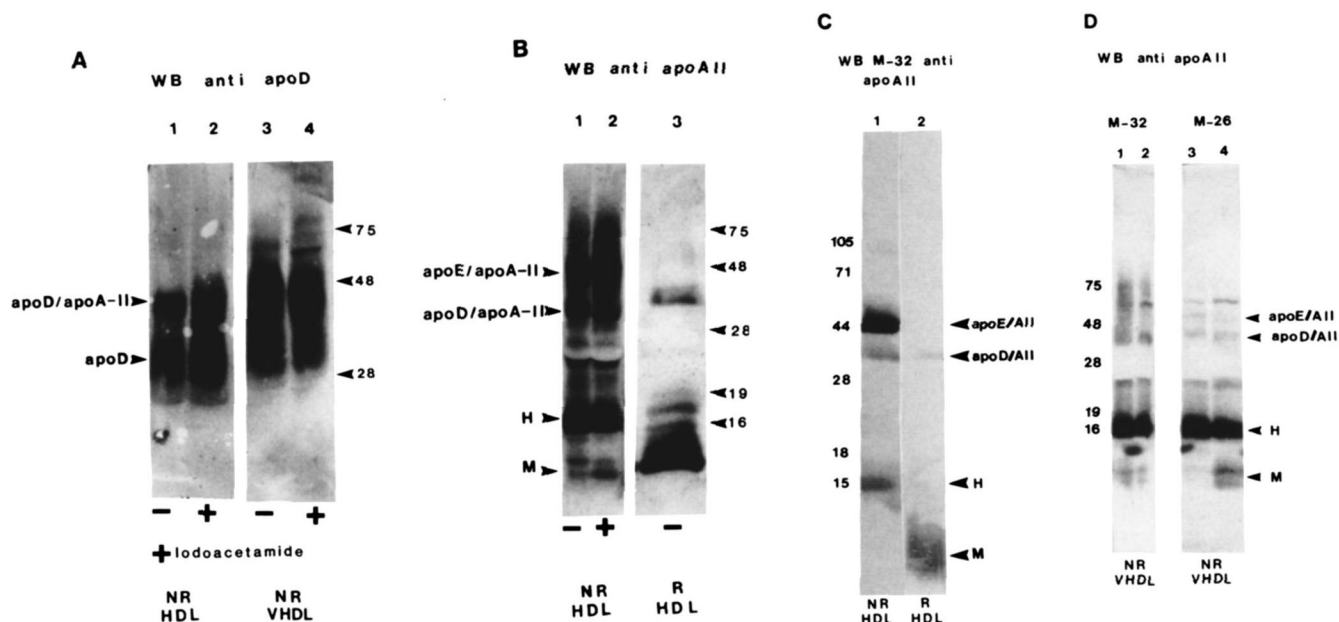
Fig. 4. A: Western blots (WB) using antibodies to apoD of nonreduced (NR) HDL (lanes 1 and 2) and VHDL (lanes 3 and 4) isolated from blood collected in a final concentration of 97 mM iodoacetamide (+) or with no additions (−). The positions of apoD and apoD–apoA-II heterodimer are shown by arrows. Minor immunoreactive bands of higher molecular weight of unknown identity (see text for discussion) can be seen, especially in VHDL. B: Nonreduced HDL (lanes 1 and 2) and reduced (R) HDL (lane 3) tested with affinity-purified polyclonal antibodies to apoA-II. The positions of the apoA-II monomer (M), apoA-II homodimer (H), apoD–apoA-II and apoE–apoA-II heterodimers are shown by arrows. In both A and B, the migration of standard molecular weight proteins is shown at the right. C: Nonreduced HDL (lane 1) and reduced HDL (lane 2) tested with M-32 monoclonal antibodies to apoA-II. D: Nonreduced VHDL tested with M-32 and M-26 monoclonal antibodies to apoA-II. The positions of the apoA-II monomer (M), apoA-II homodimer (H), apoD–apoA-II, and apoE–apoA-II heterodimers is shown. In both C and D, the migration of standard molecular weight proteins is shown at the left. In A, 30 μg of protein was applied to each lane. In B, C, and D, 15 μg of protein was applied.
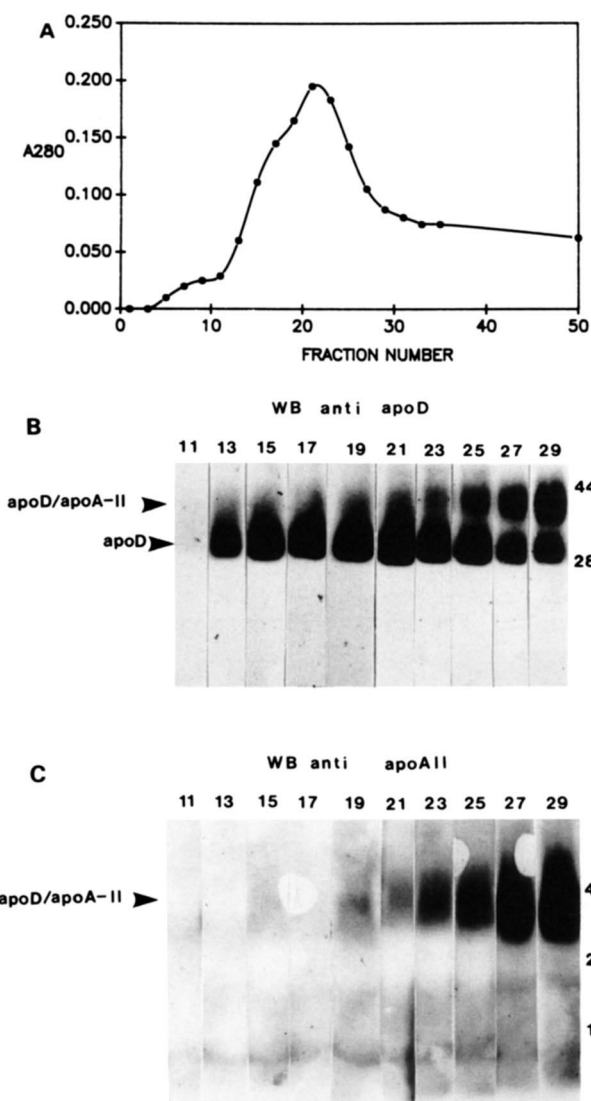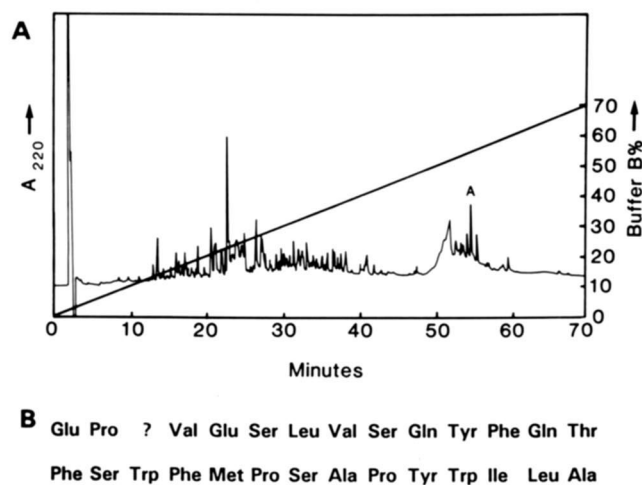
Fig. 5. A: Elution profile of a nonretained fraction after hydroxylapatite chromatography that yielded a partially purified preparation of apoD–apoA-II heterodimer. B and C: Western blots (WB) of representative fractions obtained by hydroxylapatite chromatography, in nonreducing conditions, tested with anti-apoD and polyclonal antibodies to apoA-II. The positions of apoD monomer and apoD–apoA-II heterodimer are shown by arrows. Protein (1.5 μg) was loaded in each lane. The migration of standard molecular weight proteins is shown at the right.

Glu Pro ? Val Glu Ser Leu Val Ser Gln Tyr Phe Gln Thr

Phe Ser Trp Phe Met Pro Ser Ala Pro Tyr Trp Ile Leu Ala

Fig. 6. A: Chromatogram of the tryptic peptides of partially purified apoD–apoA-II heterodimer obtained by high performance liquid chromatography as described under Materials and Methods. B: Fourteen cycles of amino acid sequence analysis of the peak A shown in the panel A. Two amino acid residues were obtained in each cycle.

(Fig. 6, A, peak A). Two amino acid residues were obtained in each cycle, except in cycle 3, in which only one was demonstrated (Fig. 6, B). The sequences obtained match that of residues 4–17 of apoA-II and 89–102 of apoD.

Quantification of apoD monomer and apoD–apoA-II heterodimer in HDL

The distribution of apoD monomer and apoD–apoA-II heterodimer was studied in HDL from five normolipidemic individuals; on the average, 45% occurred as the apoD monomer whereas 54% was found in an apoD–apoA-II heterodimer (**Table 1**). The distribution covered a large range and did not correlate in any obvious way with plasma triglyceride or cholesterol levels in our small population. There was no apparent difference between the distribution of apoD monomer and heterodimer in the HDL of males and females. Moreover, the

dimers, and traces of apoA-I. Rechromatography on hydroxylapatite or Mono Q chromatography separated apoD–apoA-II heterodimer from all other proteins, except from the apoD monomer.

To confirm that the 38,000 M_r band corresponded to the apoD–apoA-II heterodimer, 1 mg of partially purified apoD–apoA-II heterodimer was digested with trypsin, the peptides generated were purified by HLPC, and the amino-terminal sequence of each peak was determined. As shown in **Fig. 6,** fourteen cycles of sequence analysis were performed for the peak eluted at 54% of buffer B

TABLE 1. Percentage distribution of apoD monomer and apoD–apoA-II heterodimer in HDL

Subject	Sex	TC[a]	T[b]	ApoD	ApoD–apoA-II
		mg/dl		%	%
1	M	150	57	42	58
2	M	200	164	54	45
3	F	155	49	38	62
4	M	210	81	45	55
5	F	160	115	48	52
				45 ± 6[c]	54 ± 6[c]

[a]TC, total plasma cholesterol expressed in mg/dl.
[b]TC, plasma triglyceride expressed in mg/dl.
[c]Mean ± standard deviation.

distributions of monomer and heterodimer within HDL_2 and HDL_3 were similar (data not shown).

Disulfide-linked dimers of apoD in VLDL and LDL

VLDL and LDL were isolated from blood or plasma treated with DTNB or iodoacetamide. When these VLDL and LDL were analyzed under nonreducing conditions, bands reactive to anti-apoD appeared at the stacking gel and at 29,000 M_r (**Fig. 7, A**). The same high molecular weight band recognized by anti-apoD also reacted with antibodies to human LDL at a migration distance characteristic of apoB-100 (Fig. 7, B). Minor immunoreactive bands between 50,000 and 200,000 M_r, together with a 37,000 M_r band that was particularly visible in VLDL, were also detected by antibodies to LDL. While the former were probably due to limited proteolysis of apoB-100, the 37,000 M_r band likely corresponded to apoE. When the VLDL and LDL were reduced and visualized with anti-apoD, the 29,000 M_r band corresponding to apoD was the major immunoreactive band
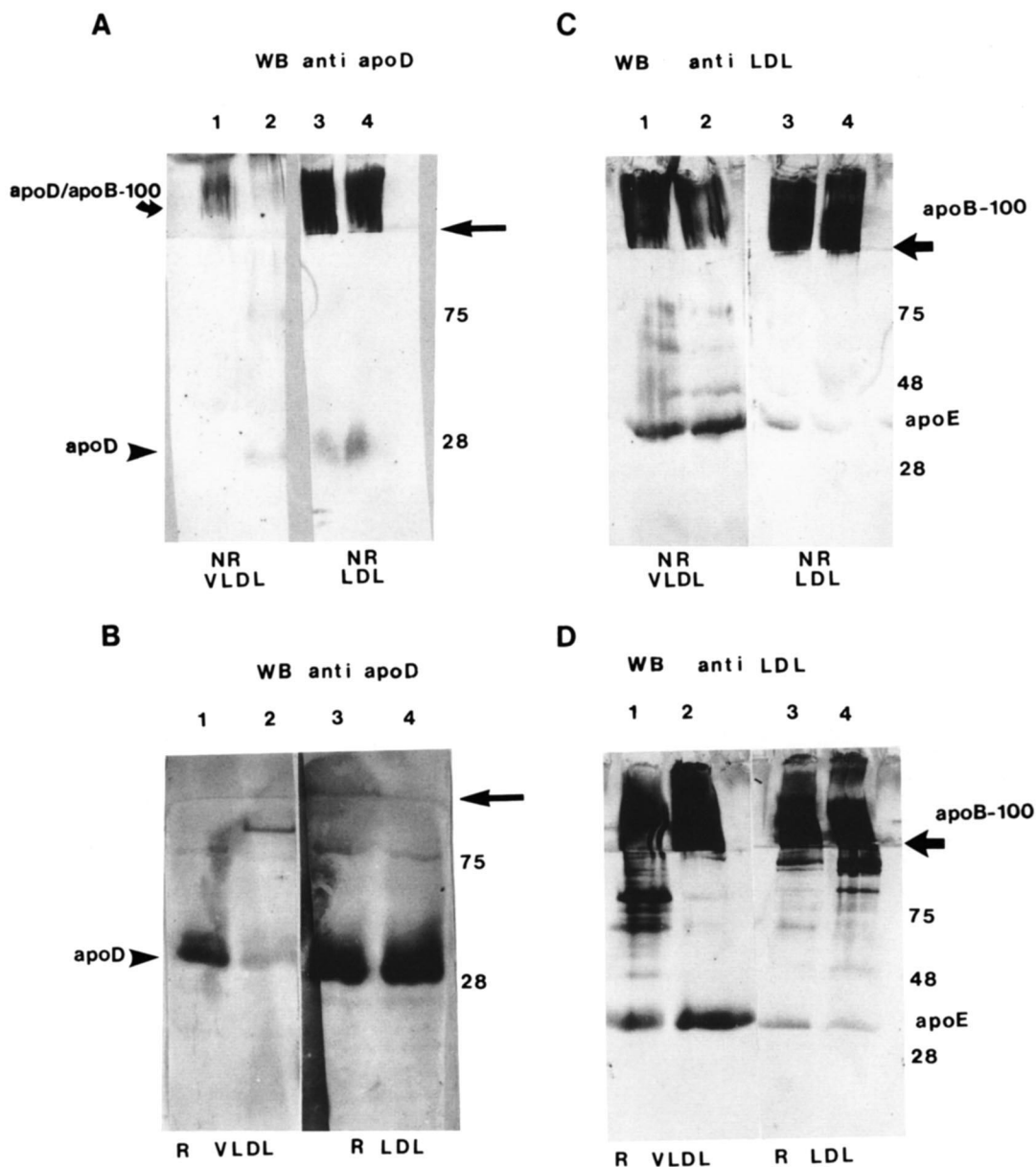
Fig. 7. Western blots (WB) using antibodies to apoD (A and B) or antibodies to LDL (C and D) of VLDL and LDL isolated from blood or plasma treated with 28 mM DTNB (lanes 1 and 3) and 97 mM iodoacetamide (lanes 2 and 4). A: Nonreduced (NR) VLDL (lanes 1–2) and LDL (lanes 3–4) (20 μg/lane). The migration of apoD–B-100 heterodimer and apoD is shown. B: Reduced (R) VLDL (lanes 1–2) and LDL (lanes 3–4). C and D: same as A and B, respectively, except that 20 μg of protein was applied in each lane. In A, B, C, and D, the arrows signal the border between the stacking and separating gels. The migration of standard molecular weight proteins is shown at the right.

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(Fig. 7, C). No major changes were found when reduced VLDL and LDL were tested with antibodies to LDL, although there was an increase in the immunoreactive bands due to partial proteolysis of apoB-100 (Fig. 7, D).

Disulfide-linked dimers of apoD in plasma

Studies on nonreduced plasma confirmed the existence of the apoD–apoA-II and apoD–apoB-100 heterodimers, but did not reveal other homo and/or heterodimers (**Fig. 8**). Other bands between 43,000 M_r and 500,000 M_r appeared in the Western blots of plasma using rabbit IgG anti-human apoD, but they were proven to be nonspecific because incubation with nonimmune rabbit IgG caused the same pattern of reactivity. This nonspecific reactivity was found only in plasma samples, and appeared even after using affinity-purified antibodies and up to 15% Tween-20 or 15% Triton X-100 in the antibody and wash solutions.

The distribution of apoD monomer and apoD–apoA-II heterodimer was studied in the plasma of two male individuals (numbers 1 and 2 in Table 1) by counting the radioactivity associated with each band, as previously done with HDL. One plasma aliquot from one individual was treated with iodoacetamide. The treatment did not alter the distribution percentage of apoD monomer and apoD–apoA-II heterodimer in plasma; 33 ± 1% of apoD was found in the monomer form, while 67 ± 1% was in the apoD–apoA-II heterodimer form. No apoD–apoB-100 was detected using this technique.

WB
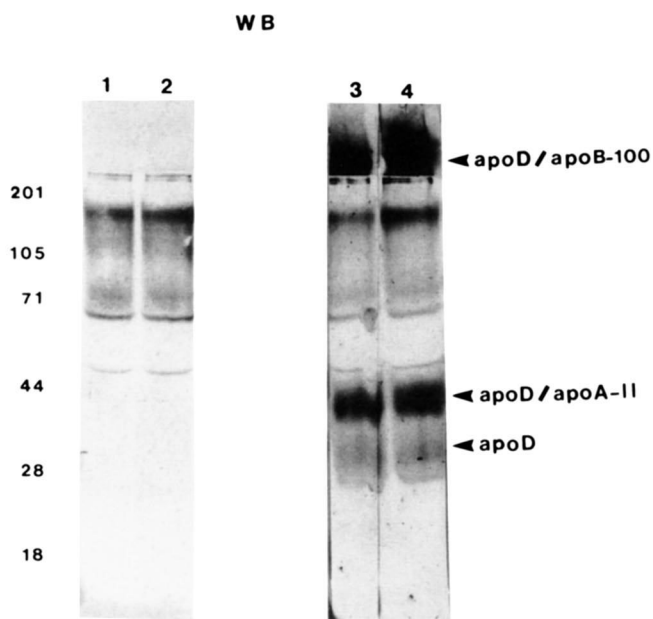
Fig. 8. Western blot (WB) using nonimmune rabbit IgG (lanes 1 and 2) and rabbit IgG anti-human apoD (lanes 3 and 4) of 3 μl of non-reduced plasma. The migration of standard molecular weight proteins is shown at the left.

DISCUSSION

ApoD dimer formation in vivo

Human apoD contains five cysteines (6), so there is one obligatory free sulfhydryl group in the monomeric species. The various kinds of complexes formed by apoD were studied by Western blot analysis using antibodies to apoD and to several other cysteine-containing apolipoproteins. The polyclonal antibodies to apoD were monospecific; Western blot analysis of all lipoprotein classes under reducing conditions revealed only the 29,000 M_r apoD band (Fig. 2). HDL and VHDL fractions under nonreducing conditions showed immunoreactive bands of 29,000 M_r, 38,000 M_r, 50,000 M_r, and 60,000 M_r (Fig. 2, A). Nonreduced LDL fractions showed bands of > 200,000 and 29,000 M_r (Fig. 2, A). These results indicated that apoD occurs as a monomer and in disulfide-linked complexes with other proteins.

Nonreduced HDL, isolated by sequential ultracentrifugation, exhibited major immunoreactive bands of 29,000 M_r and 38,000 M_r. The same was found in VHDL with other minor bands between 50,000 M_r and 80,000 M_r. All tests indicated that the disulfide bridges were not formed during storage at 4°C or during SDS-PAGE. No differences were found in HDL or VHDL isolated from blood taken in presence or absence of DTNB or iodoacetamide (Fig. 4, A). The pattern of immunoreactivity described was found in the HDL of all twelve individuals studied, even when blood was collected into DTNB or iodoacetamide (29). On the basis of these experiments, it is likely that the disulfide linked species identified by Western analysis exist in HDL and in VHDL in vivo.

Identification and quantification of the apoD–apoA-II heterodimer from HDL and VHDL

Given its molecular weight (38,000 M_r) and its reactivity against antibodies to both apoD and apoA-II, the major disulfide complex was identified as an apoD–apoA-II heterodimer (Fig. 4, B, C, and D). The 38,000 M_r band, like the 18,000 M_r band corresponding to the apoA-II homodimer, decreased when the samples were reduced but was still visible when tested with affinity-purified polyclonal antibodies (Fig. 3, B), or with M-32 (Fig. 4, C). Thus, the remaining reactivity seen under reducing conditions was likely to be due to an incomplete reduction or, alternatively, a partial reoxidation during the SDS-PAGE. The fact that some of the 38,000 M_r band could be detected using reducing conditions with antibodies to apoA-II and not with antibodies to apoD is due to the higher sensitivity obtained with the former under our experimental conditions (Fig. 5, B and C). Other apoA-II disulfide-mediated complexes were also found (Fig. 4, B, C and D). These had 46,000 M_r, 60,000 M_r, and 70,000 M_r bands and probably correspond to heterodimers of apoA-II with apoE (29) and with lipoprotein-

associated coagulation inhibitor factor (46).

Amino acid sequencing confirmed the existence of the apoD–apoA-II heterodimer linked by a disulfide bond because *a)* the sequences obtained were identical to those reported for residues 89–102 of apoD and residues 4–17 of apoA-II; *b)* two amino acid residues were obtained in each cycle, at 1:1 molar ratio; and *c)* residue number 6 of apoA-II that could not be identified would correspond to the cysteine that is involved in the disulfide bond with apoD (Fig. 6). Further evidence that the peptides were not generated from apoA-II dimers is that all of these eluted between 0 and 40% buffer B, whereas the peak A originated from the apoD–apoA-II heterodimer eluted later.

Various methods, including immunoaffinity and ultracentrifugal flotation, have been used to show that apolipoproteins occur on the same particle. However, these techniques cannot be interpreted unambiguously because the proteins are exchangeable and can appear in the same density or size range without being on the same particle. Quantification of the distribution of apoD in HDL showed that most of it (54 ± 6%) is in the apoD–apoA-II heterodimer form. This is a key finding that supports previous reports that most of apoD is in HDL particles that contain both apoA-I and apoA-II (10, 47).

Minor disulfide dimers in HDL and VHDL

The identities of the minor apoD heterodimers between 50,000 M_r and 80,000 M_r observed in HDL and VHDL could not be determined (Fig. 4, A). Although there was interindividual variation, the minor species were always more apparent in HDL isolated by density gradient ultracentrifugation than in those isolated by sequential ultracentrifugation. This could have been due to preferential losses of these proteins during the ultracentrifugation. Recently, Weisgraber and Shinto (45) identified the apoE3 homodimer in plasma; previously it was not detected because of selective loss during ultracentrifugation. However, we did not detect these complexes in studies on plasma where the apoD–apoB-100 was demonstrated (Fig. 8). Considering that no more than 10% of apoD is in VLDL and LDL (2, 4), the minor disulfide dimers are likely to account for less than 10% of plasma apoD.

To determine whether apoD homodimers are components of these 50,000–80,000 M_r bands, the isolation and amino acid sequence analysis will be needed. Other than homodimers of apoE and its heterodimer with apoA-II, no other dimeric species were found in HDL and VHDL using antibodies to apoE. However, given the genotypic differences of human apoE and their effects on apoE concentration and apoE-lipoprotein binding (48), a more comprehensive study would be necessary before one can rule out the existence of an apoD–apoE heterodimer. Heterodimers of apoD–LCAT and apoD–

CETP are also possible but were not studied in detail (47, 49, 50).

Identification of apoD heterodimers in VLDL and LDL

ApoD–apoB-100 heterodimers were also found in LDL and VLDL. These were first described by Camato et al. in LDL (27). We have thus extended the original observation by providing evidence that the heterodimers are not products of disulfide exchange and that they also exist in VLDL.

Identification of apoD heterodimers in plasma

The studies on plasma using the peroxidase detection confirmed the existence of the apoD–apoA-II and apoD–apoB-100 heterodimers (Fig. 8). No other apoD-containing homo or heterodimers were found. Quantification of the apoD monomer and apoD–apoA-II heterodimer in plasma of two individuals using ^{125}I-labeled IgG anti human apoD confirmed that this heterodimer is the major form of apoD in plasma (67 ± 1%), while the balance (33 ± 1%) was monomeric. In whole plasma, the apoD–apoB-100 heterodimer could not be detected, probably due to the lower sensitivity of the one antibody-isotopic method compared with the two antibody–peroxidase method.

The differences between the distribution of the apoD monomer and apoD–apoA-II heterodimer in HDL and plasma could be due to selective loss of the monomer during ultracentrifugation. This has also been reported for apoE (51) and may be due to the reduced lipophilicity that occurs when lipid-binding units are reduced.

More than 60% of plasma apoD was found with lipoproteins containing both apoA-I and apoA-II (47). These data suggest that most if not all apoD in particles containing both apoA-I and apoA-II is linked by disulfide bonds to apoA-II. As the molar plasma concentration of apoA-II is four times that of apoD (52), and both proteins are mainly in HDL particles, about 16% of apoA-II mass is involved in the formation of this heterodimer.

Existence of cross-reactant proteins to antibodies to apoD

The molecular weights of some of the bands found in nonreduced HDL and VHDL using antibodies to apoD were similar to the apoD cross-reactant proteins previously found in HDL and VHDL (26, 27). However, our results indicate that the broad immunoreactivity is due to disulfide bonds between apoD and other apolipoproteins rather than cross-reactivity with epitopes that are not in apoD. We did not find any additional cause of cross-reactivity during the studies of other lipoproteins and plasma. Therefore, our data do not support the existence of such cross-reactant proteins in human plasma. Because

of the possibility of homo and heterodimers containing apoD, tests to verify the monospecificity of antibodies to human apoD that are to be used in immunoassays should be verified by Western blots of SDS-PAGE under reducing conditions.

ApoD dimer formation in human plasma lipoproteins: a phenomenon common to other apolipoproteins

In LDL, apoB-100 contains two cysteines (residues 3734 and 4190) that face the exterior of the particle (53). We speculate that at least one of these forms a disulfide bond with apoD. The single cysteine at residue-6 of human apoA-II (54) must be involved in the apoD–apoA-II heterodimer formation. The identity of the cysteine residue of apoD that is involved in the disulfide bonds with apoA-II and apoB-100 is unknown. However, cysteines involved in intramolecular disulfide bonds are highly conserved during evolution (55); rat and rabbit apoD contains only four cysteines (14, 16). Thus, it is likely that the additional cysteine in human apoD, located at residue 116, is the one involved in disulfide formation. This hypothesis is currently under study.

Like human apoE, apoA-II, apoB-100, and apoLP(a) (29, 45, 54, 53, 56), apoD cross-link with other apolipoproteins via a disulfide bond. It has been reported that the apoE–apoA-II heterodimer and the apoE homodimer, both of which are composed of hepatically derived proteins, are formed in plasma (45). Thus, it is likely that the apoD–apoA-II heterodimer is formed in plasma because, unlike apoA-II, apoB-100, and apoE, the liver is not the major production site of apoD (13, 14, 17).

Most of the free cysteines of soluble human apolipoproteins involved in dimer formation do not occur in the apolipoproteins of other animal species (6, 14, 16, 57, 58). The presence of multiple disulfide complexes between soluble apolipoproteins is a structural characteristic of human lipoproteins that has so far not been fully recognized.

ApoD function and heterodimer formation

The physiological relevance of the homo and heterodimers formed by cysteine-containing apolipoproteins is not clear. However, in vitro studies suggest that they affect lipoprotein metabolism (45, 51, 59–61). In the case of apoD, the existence of a major plasma fraction complexed with apoA-II could influence apoD function and metabolism at different levels. An in vitro test for apoD function and the complete isolation of the apoD–apoA-II heterodimer will be needed before one can infer what effect, if any, has the heterodimer formation in the ligand properties of apoD.

ApoD belongs to a superfamily of proteins that do not associate with lipoproteins. The question arises whether the heterodimer formation is important or necessary for apoD binding to lipoproteins. The fact that there is monomeric apoD in all lipoprotein fractions, and that

rabbit apoD that contains no free cysteine is also bound to HDL (14), argue against the notion that heterodimer formation is necessary to the lipoprotein binding of apoD. However, the affinity of a dimeric form for the lipoprotein surface is probably greater than that of the monomer.

Dimerization mechanism

Three factors regulate the occurrence of intermolecular disulfide bonds formed among apolipoproteins: *1)* the presence of at least one free sulfhydryl group; *2)* a structure around the sulfhydryl groups that does not hinder disulfide bonding; and *3)* concentrations of cysteine-containing proteins high enough to lead to diffusion-controlled collision between the monomeric species on a physiologically relevant time scale. Apparently these criteria are satisfied by human apoD and other cysteine-containing apolipoproteins. However, how they satisfied the third point is not obvious. The respective plasma concentrations of apoD, apoE, apoB-100, and apoA-II are 5 μM, 2 μM, 5 μM, and 23 μM (52). Considering that the collision frequency between these species is the product of their respective plasma concentrations and the diffusion-controlled rate constant (about 10^{+8} $M^{-1}sec^{-1}$), the number of disulfide-linked species would be expected to be rather small even in the case of apoA-II which is the most abundant of cysteine-positive apolipoproteins. Furthermore, only one out of a large number of amino acids can form disulfide bonds; therefore, it is surprising that they occur at all. In fact, we were unable to observe dimers of any of these isolated proteins using concentrations that are similar to those found in plasma. However, all of these apolipoprotein species are bound to a common surface where there is a much higher local concentration. The resulting increase in the rate of collision between protein molecules could increase the probability of disulfide bridge formation. This mechanism, however, remains to be proved. ■

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