Subunit Composition of Lipoprotein(a) Protein†

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ABSTRACT: We determined the molecular weight of four different apo(a) polymorphs by sedimentation equilibrium in 6 M guanidine hydrochloride in order to estimate the molar ratio of apo(a) to apoB in Lp(a). They had molecular weights of 289 000, 310 000, 341 000, and 488 000 and 15, 16, 18, and 27 kringle 4 domains, respectively. Their carbohydrate content was similar (23.2 wt %), as was their partial specific volume (0.682 mL/g). Knowing the mass of apo(a), we estimated the molar ratio of apo(a) to apoB from (1) the molecular weight of the protein moiety of the four respective parent Lp(a) particles as calculated from their mass and percentage composition and the mass of apoB, (2) the mass of apo(a) lost from Lp(a) upon its reduction and carboxymethylation, by determining the difference in mass between Lp(a) and Lp(a-), and (3) from the mass (measured by sedimentation equilibrium in 6 M guanidine hydrochloride) of the lipid-free apoB-apo(a) complex (1.06×10^6 daltons) of the Lp(a) particle with the smallest apo(a) polymorph by subtracting the mass of apoB. Our results obtained with each of the three different physicochemical methods indicated that the protein moiety of each of the four Lp(a) particles that was investigated consisted of a complex of two molecules of apo(a) and one molecule of apoB.

Lp(a)[1] is a LDL-like particle whose protein moiety contains two large disulfide-linked molecular weight glycoproteins, apoB and apo(a) (Fless et al., 1984; Gaubatz et al., 1983; Utermann & Weber, 1983). The latter is a highly unusual protein with many repetitive domains called kringles (McLean et al., 1987), which are triple loop structures of about 80 amino acids, maintained by three disulfide bonds, that are generally found in the noncatalytic regions of regulatory proteases of blood coagulation and fibrinolysis (Patthy, 1985; Sottrup-Jensen et al., 1978). Apo(a) is highly polymorphic in molecular weight (Fless et al., 1984; McLean et al., 1987; Utermann et al., 1987), because it may contain from 12 to 51 domains that resemble the kringle 4 domain of plasminogen (Lackner et al., 1993). Apo(a) also has a kringle 5 and a protease domain with high homology to plasminogen that flank the kringle 4 domains on the C-terminal side of the molecule (McLean et al., 1987).

Much effort has been spent in identifying the cysteine residues involved in linking apo(a) to apoB. Analysis of the cDNA of a 37 kringle-4 apo(a) polymorph demonstrated that the 36th kringle 4 contained seven cysteine residues instead of the usual six (McLean et al., 1987). Since six residues are required to maintain the kringle structure, McLean and colleagues suggested that this extra cysteine (at position 4057) might be involved in the linkage of apo(a) to apoB. This hypothesis was recently confirmed by Koschinsky et al. (1993), who showed that, on conversion of Cys 4057 to Ser by site-directed mutagenesis, apo(a) lost its ability to disulfide-link to apoB of LDL in a recombinant expression system. Using similar techniques, Brunner et al. (1993) came to the same conclusion. Unlike with apo(a), the cysteine residue(s) of apoB involved in the coupling of the two apoproteins have not yet been identified by recombinant techniques. However, in a comparative analysis of Lp(a) and LDL by limited proteolysis Huby et al. (1994) have shown that the C-terminal part of apoB that includes cysteine residues 3734 and 4190 is involved in the linkage of apo(a) to apoB.

The determination of the apoB cysteine residue(s) responsible for linking apo(a) to apoB is important because it relates to the question of whether there are one or two apo(a) molecules attached to apoB. The prevailing view that only one apo(a) is linked to apoB is not consistent with the known physicochemical parameters of Lp(a) and apo(a). In a previous study we had concluded, on the basis of the mass differential between Lp(a) and Lp(a-), that there were two apo(a)s linked to one apoB (Fless et al., 1986). The LDL-like Lp(a-) was produced on reduction of Lp(a) and the removal of apo(a). Knowing the molecular weight of apo(a) from sedimentation equilibrium experiments, the number of apo(a) molecules was determined by dividing the mass lost by Lp(a) on reduction by the mass of apo(a). Since we had studied only one apo(a) polymorph, the possibility existed that this represented an isolated case. In light of the controversy surrounding the stoichiometry of apo(a) to apoB in Lp(a), we reexamined this issue. By determining the molecular weight of four different apo(a) polymorphs, we estimated the molar ratio of apo(a) to apoB from (1) the molecular weight of the protein moiety of the four respective parent Lp(a) particles, (2) the mass of apo(a) lost from Lp(a) upon its reduction and carboxymethylation, and (3) the

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); apoB, apolipoprotein B; LDL, low density lipoprotein; Lp(a-), remnant lipoprotein particle left after the reduction of Lp(a) and removal of apo(a); Na_2EDTA, sodium salt of ethelenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; GuHCl, guanidine hydrochloride; TRIS, 2-ammonio-2-(hydroxymethyl)-1,3-propanediol; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass.

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mass of the lipid-free apoB−apo(a) complex. On the basis of results obtained by this physicochemical analysis, we conclude that each of the Lp(a) species examined contained two molecules of apo(a) and one molecule apoB.

EXPERIMENTAL PROCEDURES

Preparation of Lipoproteins. Autologous Lp(a) and LDL were isolated from the plasma of three subjects, all of whom gave informed consent prior to plasmapheresis. A combination of lysine Sepharose chromatography and density gradient centrifugation was used; these were slightly modified from previously described methods (Fless & Snyder, 1994; Fless et al., 1986; Snyder et al., 1992). Plasma was treated with 0.2 mM phenylmethanesulfonyl fluoride for 20 min, before its density was raised to 1.21 g/mL with solid NaBr. It was subjected to centrifugation in the 50.2 Ti rotor at 45 000 rpm for 16 h at 20 °C. The floating lipoproteins contained in 1 L of plasma were dialyzed against 33 mM phosphate, 1 mM benzamidine, 0.01% Na_2EDTA, and 0.01% NaN_3, pH 7.4, and applied to a lysine-Sepharose column (20 × 2.5 cm) which was equilibrated in the same buffer. Lp(a) from subjects K.B. and L.C. was a mixture containing two different apo(a) isoforms that was eluted from the column with a 200-mL gradient of 0−100 mM *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (tAMCHA). Consecutive 40-mL fractions were collected and made 7.5 wt % with solid CsCl and centrifuged 20 h at 49 000 rpm at 20 °C in the 50.2 Ti rotor using quick seal tubes in order to establish a density gradient. Lp(a) with the lower molecular weight apo(a) isoform floated between $d = 1.050$ and $d = 1.065$ g/mL and was present in the early fractions from the column, whereas the Lp(a) species with the large apo(a) isoform floated between $d = 1.070$ and $d = 1.090$ g/mL and emerged in the later fractions. Lp(a) from donor B.K. was homogeneous and was therefore eluted from the lysine Sepharose column with 100 mM tAMCHA. The pure lipoproteins, in 0.15 M NaCl, pH 7.4, containing 0.01% Na_2EDTA and 0.01% NaN_3, were filter sterilized (0.45 μm) and stored in sterile Sarsted vials filled to allow no air space. Lp(a) with lower molecular weight apo(a) isoforms was kept at 4 °C, whereas Lp(a) with the high molecular weight apo(a) isoform was stored at room temperature, in the dark, because of its tendency to form a gel due to self-association in the cold (Fless & Snyder, 1994). LDL was isolated from the unbound lipoproteins obtained after lysine Sepharose chromatography by density gradient centrifugation in 3.75 wt % CsCl in the Ti50.2 rotor at 49 000 rpm and 20 °C for 20 h. Lipoprotein purity was established by SDS−PAGE on 2.5−16% polyacrylamide gradient gels (Isolab, Akron, OH). Lp(a) particles were phenotyped by electrophoresis using the Phast system (Pharmacia) and the conditions described by Molinari et al. (1990).

Preparation of Lp(a-) and apo(a). Lp(a-) was isolated from Lp(a) by procedures slightly modified from those described previously (Fless et al., 1985, 1986). Briefly, Lp(a) in 0.15 M NaCl, 0.01 M TRIS, 0.01% Na_2EDTA, and 0.01% NaN_3, pH 7.4, was treated with 50 mM dithiothreitol for 1 h at room temperature in the dark. Lp(a) concentration usually varied between 2 and 3 mg/mL protein. Following reduction, the pH of the mixture was brought to 8.0 by elevating the concentration of TRIS to 0.5 M with 2 M TRIS/HCl, pH 8.0. The reduced Lp(a) was alkylated with 150 mM iodoacetic acid at pH 8.0 for approximately 20 min while keeping the pH of the stirred mixture constant with small additions of 1 N NaOH. The modified Lp(a) solution was dialyzed against a deaerated solution of 35 wt % NaBr, 0.01% Na_2EDTA, pH 7.4. Two milliliters of this Lp(a) solution was layered under a linear 7.5−30 wt % NaBr gradient and spun at 20 °C either 4 h at 35 000 rpm or 16 h at 17 000 rpm in the SW 40 rotor. The gradient was monitored at 280 nm by pumping at 1 mL/min through an ISCO UA-5 density gradient monitor. Fractions containing Lp(a-) were pooled and dialyzed against 0.15 M NaCl, 0.01% Na_2EDTA, and NaN_3, pH 7.4.

The reduced and carboxymethylated apo(a) was recovered from the tube bottom and dialyzed against 6 M GuHCl, 10 mM TRIS/HCl, and 0.01% Na_2EDTA, pH 7.4. Apo(a) was further purified by gel filtration on Sepharose 4B-CL (1 × 50 cm) which was equilibrated in the above dialysis buffer. Unlike the small molecular weight apo(a) isoforms, the larger isoforms sometimes showed signs of degradation upon reduction and carboxymethylation such as the appearance of peptide fragments upon gel filtration in 6 M GuHCl or SDS−PAGE. This was true despite the fact that the original starting material was not degraded when analyzed by SDS−PAGE. This problem was eliminated by dialyzing these Lp(a) preparations against 6 M GuHCl, 10 mM TRIS/HCl, and 0.01% Na_2EDTA, pH 8.0. Reduction and carboxymethylation was carried out as described above. The modified Lp(a) in a volume of 2 mL was placed in a 50.3 centrifuge tube (Beckman) and overlayered with 5 M GuHCl. Lp(a-) floated in a thin layer at the top of the tube whereas apo(a) was recovered from the bottom after centrifugation in the 50.3 rotor at 40 000 rpm and 20 °C for 16 h. Apo(a) was then purified by dialysis and gel filtration in GuHCl as described above.

Preparation of the apoB−apo(a) Complex. Lp(a), at a concentration of 2 mg/mL protein, was dialyzed against 0.2 mM *N*-ethylmorpholine, pH 10, and delipidated at 4 °C using mixtures of diethyl ether and ethanol as previously described (Fless et al., 1990). ApoB-apo(a) was dialyzed against 0.2 mM *N*-ethylmorpholine to remove the organic solvents before it was dialyzed against 6 M GuHCl, 10 mM TRIS/HCl, and 0.01% Na_2EDTA, pH 7.4. The complex was further purified by gel filtration on Sepharose 4B-CL which was equilibrated in the above dialysis buffer.

Equilibrium Ultracentrifugation. Sedimentation and flotation equilibrium experiments were performed using a Beckman Optima XLA ultracentrifuge interfaced to an IBM PS/2 model 55SX personal computer, an An-60 Ti four-place rotor, and analytical cells equipped with six-channel charcoal-filled centerpieces. Equilibrium ultracentrifugation of Lp(a), Lp(a-), and LDL was conducted using three different solutions of NaBr that varied between 1.5 and 20 wt % in concentration, and 1.01 and 1.20 g/mL in density as previously described (Fless et al., 1976, 1986). Depending on the particular lipoprotein being analyzed, an intermediate NaBr solution was chosen which avoided conditions where the buoyancy factor $(1 - \bar{v}_2\rho) = 0$. Usually a concentration of NaBr was selected that gave a negative buoyancy factor of magnitude similar to that achieved with 1.5 wt % NaBr. Lipoprotein concentrations were used that gave absorbance values at 280 nm of less than 0.3. Rotor speeds varied between 3000 and 4000 rpm in order to maintain approximately a 4-fold difference in concentration between the meniscus and the cell bottom, which allowed lipoprotein concentrations to be evaluated at every point in the cell. The

cells were scanned every 12 h in order to determine the time required to reach equilibrium (usually less than 48 h). This was done by determining the time necessary for the difference plot of successive scans to reach values approaching zero. Upon completion of the run the rotor was accelerated to 40 000 rpm, held at that speed for 2 h, and returned to the original speed. This was followed immediately with a baseline scan.

Data were fitted to the standard equilibrium equation:

$$c_r = c_m \exp[(M(1 - \bar{v}_2\varrho)\omega^2/2RT)(r^2 - r_m^2)]$$

where c_m and c_r are the equilibrium solute concentrations at the meniscus and at some point r, M is the weight-average molecular weight (Mw), $\bar{v}_2$ is the partial specific volume of the protein, ϱ is the solution density, ω is the angular velocity, R is the universal gas constant, T is the absolute temperature, and r is the radial distance from the center of rotation, using the XLAEQ program (Beckman, Palo Alto, CA) assuming a single ideal solute. M is equivalent to the whole cell weight average molecular weight, $\bar{M}$w, when c_m and c_r are the equilibrium concentrations at the meniscus and cell bottom, respectively. $\bar{M}$z, the Z-average molecular weight was obtained by extrapolating Mw values obtained at each point of the solution column to the cell bottom. In cases where the baseline run indicated significant absorption, this was subtracted from the equilibrium data and reanalyzed using Kaleidagraph software. Molecular weights and partial specific volumes were obtained simultaneously by plotting ϱ against $M(1 - \bar{v}_2\varrho)$ (Fless et al., 1976, 1986).

Sedimentation equilibrium experiments with reduced and carboxymethylated apo(a) obtained by gel filtration in 6 M GuHCl were conducted with the six-channel center piece at speeds ranging between 4000 and 6000 rpm. Before analysis, apo(a) was dialyzed against the gel filtration buffer for 3 days to ensure that equilibrium was attained. Each apo(a) sample was loaded at three different concentrations that had absorbance values at 280 nm varying between 0.1 and 0.3. The time required to reach sedimentation equilibrium ranged between 4 and 5 days.

Equilibrium centrifugation with the apoB−apo(a) complex was carried out as described with apo(a) above, except that nine different sample solutions ranging in concentration from 0.03 to 0.4 absorbance units at 280 nm were loaded. Low-speed sedimentation equilibrium was carried out at 3000 rpm, whereas high-speed experiments were conducted at 10 000 rpm. Seven to eight days were required to reach equilibrium at 3000 rpm. Baseline runs at 40 000 rpm were carried out for 16 h as described above. Sectors loaded with samples having an absorbance at 280 nm lower than 0.1 were scanned at wavelengths ranging between 230 and 240 nm.

Solvent densities were measured with a Precision Density Meter DMA-02-C (Mettler/Paar). The instrument was calibrated with distilled water and dry air at known barometric pressure. The temperature of the vibrating density meter cell was controlled to ±0.01 °C with an external water bath.

The partial specific volumes of the different apo(a) polymorphs were calculated from their amino acid and carbohydrate compositions; partial specific volumes of amino acids were taken from Cohn and Edsall (1943) and those of sugars from Gibbons (1966). When molecular weight determinations were carried out in 6 M GuHCl, the partial specific volume $\bar{v}_2$ was replaced by the apparent specific volume ϕ'_2 which we assumed to be 0.015 mL/g lower in magnitude as suggested by Hade and Tanford (1967).

The partial specific volume of the apoB−apo(a) complex was calculated from the partial specific volumes of apoB, apo(a), and residual phospholipid. A value of 0.724 mL/g was obtained for apoB from its amino acid sequence and carbohydrate content (7 wt %). The partial specific volume of phospholipid was taken to be 0.97 mL/g (Patterson et al., 1987). Correction for preferential binding of GuHCl by apoB−apo(a) was carried out as indicated above for apo(a).

Chemical Analysis. Protein content was determined by the method of Lowry et al. (1951) as modified by Markwell et al. (1978) using bovine serum albumin as standard, as previously described (Fless et al., 1986). We used correction factors to relate the chromogenicity of albumin to that of apoB, apo(a), and apoB−apo(a). A number of investigators recognized that the Lowry method overestimates apoB protein by a factor ranging from 1.07 to 1.10 (Kane et al., 1975; Levy et al., 1967; Muesing & Nishida, 1971). To correct apoB protein values determined by Lowry, we used the factor of 0.926, which is the average reported by the above investigators. The factor for apo(a) was determined by amino acid analysis on two different preparations of each of the four apo(a) polymorphs. These indicated that apo(a) protein is seriously underestimated by Lowry's procedure. The factors obtained for the four different apo(a) polymorphs were not statistically different from each other [1.42 and 1.50 for apo(a) of KB-1 Lp(a); 1.51 and 1.40 for LC; 1.39 and 1.28 for BK; and 1.37 and 1.33 for apo(a) of KB-2 Lp(a)]. Therefore we used the averaged multiplier of 1.40 ± 0.08 to correct the Lowry value of each apo(a) sample to the absolute protein concentration.

Phospholipid (Bartlett, 1959), cholesterol, both free and esterified (Allain et al., 1974; Gallo et al., 1978), and triglyceride analyses (Wahlefeld, 1974) were also performed as described previously (Fless et al., 1986).

Carbohydrate Analysis. Carbohydrate components of apo(a) were quantified by a modification of the method of Reinhold using gas-liquid chromatography (Reinhold, 1972). Samples of apo(a) containing 40−55 μg of protein or standard carbohydrates (5−10 μg each) were subjected to methanolysis at 80 °C in 3 N HCl/methanol (Pierce Chemical Co.) for 18 h. After drying of the samples, re-*N*-acetylation of the amino sugars was accomplished by brief incubation in pyridine/acetic anhydride at room temperature. The dried samples were then de-*O*-acetylated in 0.5 N HCl/methanol at 65 °C for 45 min. The de-*O*-acetylated, dried samples were trimethylsilated with Sylon BFT/pyridine (1:2) (Supelco) at room temperature overnight. Derivatized samples were injected directly from the above solution in volumes of 0.7−1.2 μL onto a capillary column (DB-5, J&W Scientific Co.) and analyzed on a 5890A Hewlett-Packard gas chromatograph, using a temperature program (75 °C for 1 min, then increasing at a rate of 20 °C/min up to 150 °C and holding for 1 min, and finally increasing at a rate of 8 °C/min up to 300 °C). All solvents used were newly purchased reagent grade and stored in a dry chamber prior to use. Samples were subjected to lyophilization and drying over P_2O_5 under vacuum prior to methanolysis.

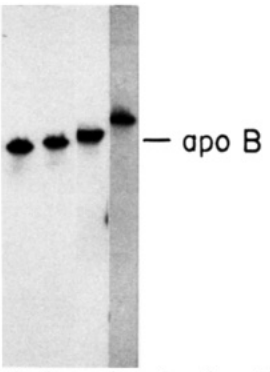

FIGURE 1: SDS gel electrophoresis of purified reduced and carboxymethylated apo(a) on a 4−15% acrylamide Phast gel (Pharmacia). Loading concentration was 5 ng of protein per well. The gel was pressure blotted to Immobilon-P PVDF membrane (Millipore), exposed to anti-apo(a) serum, and visualized by chemiluminescence (Amersham). From left to right, apo(a) derived from the Lp(a) species KB-1, LC, BK, and KB-2. The position of apoB relative to the apo(a) samples is indicated.

RESULTS

Molecular Weight Determination of Apo(a). Four different Lp(a) species were purified from the plasma of three subjects and served as the starting material for the isolation of four different apo(a) polymorphs. This was achieved by the reduction and carboxymethylation of Lp(a) followed by rate-zonal density gradient centrifugation to separate apo(a) from the floating lipoprotein particle. The relative mobility of the purified apo(a) polymorphs after electrophoresis on 4−15% polyacrylamide gels in the presence of SDS is shown in Figure 1. Two apo(a) proteins (KB-1, LC) had a mobility greater, one was slightly smaller (BK) and one had a mobility much smaller (KB-2) than that of apoB.

Initial attempts to determine the molecular weight of apo(a) in 0.15 M NaCl indicated that apo(a) self-associated in this solvent (Figure 2). The molecular weight near the meniscus approached that of the monomer, whereas the molecular weight at the cell bottom was approximately fourfold higher. This is in contrast to recombinant apo(a) which is not reduced and carboxymethylated and which does not self-associate in a similar solvent (Phillips et al., 1993). To eliminate the problem of self-association, we determined the molecular weight of apo(a) in 6 M GuHCl. A representative plot of apo(a) at sedimentation equilibrium is shown in Figure 2 indicating an excellent fit of the raw data points to the standard equilibrium equation for a single solute component. Because of its unfolded state in 6 M GuHCl and the large size of apo(a), almost 5 days were needed to achieve sedimentation equilibrium (see inset in Figure 2). None of the apo(a) samples exhibited signs of heterogeneity, and plots of d ln c/dr^2 were linear.

The partial specific volume of apo(a) was calculated from its amino acid sequence and carbohydrate composition. A monosaccharide analysis of the four apo(a) polymorphs is given in Table 1, which shows that within the experimental error of the method there are no significant differences in their sugar content. The carbohydrate data of the apo(a) polymorphs were therefore averaged, resulting in a mean carbohydrate content of 23.2 ± 1.2 wt %. The mean partial specific volume of the apo(a) carbohydrate moiety was 0.621 ± 0.001 mL/g, and the partial specific volume of apo(a) protein ranged from 0.701 mL/g for the smaller apo(a)'s to 0.700 mL/g for the largest apo(a). The calculated partial specific volumes (i.e., protein plus carbohydrate) of the apo(a)'s also did not vary significantly from each other and were therefore averaged, yielding a mean value of 0.682 ± 0.002. In estimating the molecular weight of apo(a), the partial specific volume was treated according to the suggestions of Hade and Tanford (1967) to account for the preferential binding of GuHCl. Thus ϕ'_2 is assumed to be 0.015 mL/g less than $\bar{v}_2$. The calculated molecular weights of the four apo(a) polymorphs are given in Table 1 and ranged from 289 000 to 488 000, and the corresponding values of their

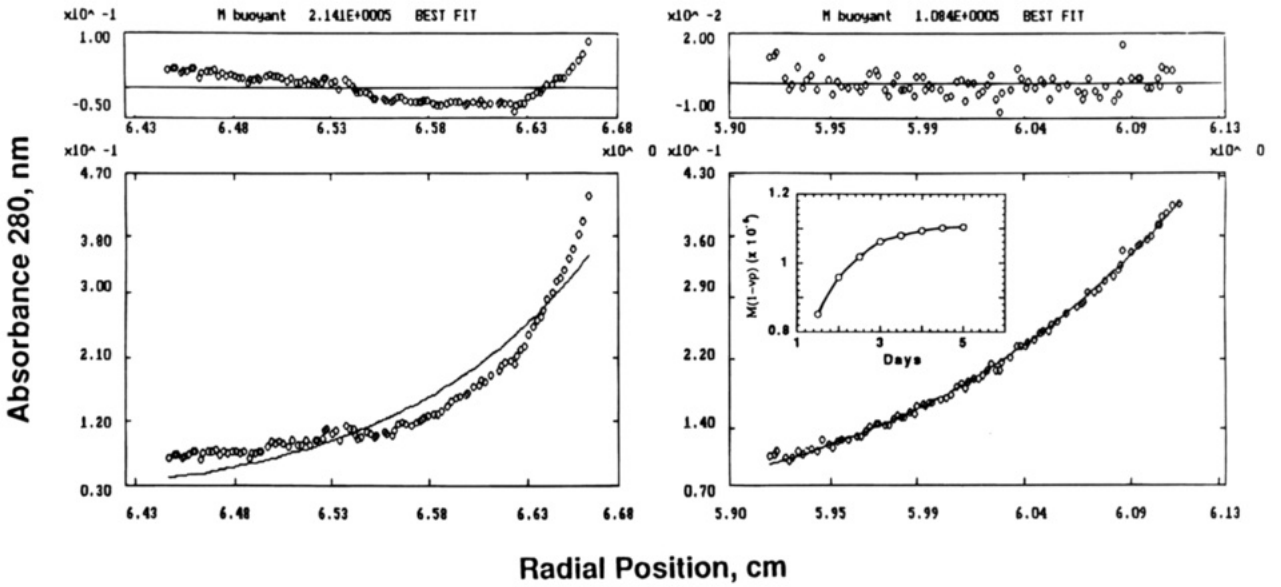

FIGURE 2: Sedimentation equilibrium. (Left panel) Distribution of BK apo(a) dissolved in 10 mM phosphate, 150 mM NaCl, pH 7.4, after 3 days of centrifugation at 4000 rpm and 20 °C. Its absorbance at 280 nm is plotted as a function of radial distance. Apo(a) was loaded at a concentration of 0.33 absorbance units. The experimental points were fitted to the sedimentation equilibrium equation describing a single ideal solute using XLA-DATA analysis software (Beckman). The small panel is a residual error plot. (Right panel) Distribution of KB-2 apo(a) dissolved in 6 M GuHCl after 4 days of centrifugation at 5000 rpm and 20 °C. The initial concentration of apo(a) had a concentration of 0.25 absorbance units at 280 nm. The inset figure is a plot of $M(1 - \bar{v}_2\rho)$ against time that shows the number of days required to reach equilibrium.

Table 1: Physicochemical Properties of Apo(a)

	Lp(a) type				
	KB-1	LC	BK	KB-2	mean apo(a)
Man[a,b] (μg/mg)	25.1 ± 0.9	24.7 ± 0.4	26.2 ± 0.7	26.4 ± 1.0	25.6 ± 1.0
Gal[a,b] (μg/mg)	50.9 ± 1.9	54.3 ± 2.8	51.6 ± 3.7	58.5 ± 1.8	54.1 ± 4.1
GalNAc[a,b] (μg/mg)	51.1 ± 3.2	52.2 ± 3.3	51.3 ± 4.8	55.6 ± 0.8	52.4 ± 4.0
GlcNAc[a,b] (μg/mg)	51.3 ± 0.8	56.0 ± 3.5	56.1 ± 3.0	57.7 ± 1.7	55.4 ± 3.4
NeuAc[a,b] (μg/mg)	117.1 ± 2.7	109.5 ± 6.6	113.6 ± 15	115.6 ± 3.6	114.2 ± 5.1
M_r of apo(a)[c] ($\times 10^{-3}$)	289 ± 14	310 ± 14	341 ± 14	488 ± 26	
M_r of apo(a) protein ($\times 10^{-3}$)	222 ± 11	238 ± 12	262 ± 13	375 ± 21	
number of kringle 4's	15 ± 1	16 ± 1	18 ± 1	27 ± 2	

[a] Values represent the mean ± standard deviation of three independent measurements. [b] Micrograms of monosaccharide per milligram of apo(a) protein. [c] Mean ± standard deviation of two different preparations analyzed in triplicate.

Table 2: Physicochemical Properties of LDL[a,b]

	LDL donor		
	KB	LC	BK
protein (%)	17.2 ± 0.20	20.2 ± 0.33	19.0 ± 0.67
carbohydrate (%)	1.3 ± 0.01	1.5 ± 0.02	1.4 ± 0.04
phospholipid (%)	24.2 ± 1.97	21.4 ± 0.43	21.8 ± 1.42
free cholesterol (%)	7.2 ± 0.43	6.5 ± 0.17	7.2 ± 0.13
cholesteryl ester (%)	46.4 ± 2.77	45.3 ± 1.17	46.0 ± 0.87
triglyceride (%)	3.7 ± 0.07	5.1 ± 0.27	4.6 ± 0.29
M_r or LDL ($\times 10^{-6}$)	2.88 ± 0.11	2.78 ± 0.13	2.51 ± 0.10
density (g/mL)	1.031 ± 0.004	1.036 ± 0.005	1.037 ± 0.005
exp. M_r of apoB ($\times 10^{-3}$)	495	562	477
% difference[c] (%)	−3.3	9.8	−6.8

[a] Compositional values represent the mean ± standard deviation of three analyses done in triplicate. [b] The molecular weight and buoyant density of each LDL type was determined by equilibrium centrifugation at three different densities and three different concentrations. [c] Percent deviation of the experimentally determined molecular weight of apoB from the known molecular weight of 512 000.

protein portions ranged from 222 000 to 375 000. On the basis of their mass, the number of kringle 4 domains in these apo(a) polymorphs ranged from 15 to 27.

Knowing the molecular weight of the four apo(a) polymorphs, the molar ratio of apo(a) to apoB in each of the different Lp(a) particles could be determined from the following physicochemical parameters:

Chemical Composition and Mass of Lp(a). The number of apo(a) molecules present on a Lp(a) particle can be estimated from the percentage chemical composition and molecular weight of Lp(a). The percentage of protein is a measure of both the apoB and apo(a) content of the particle and when applied to the molecular weight of Lp(a) gives the molecular weight of apo(a) protein when that of apoB is subtracted. Because the determination of Lp(a) protein mass requires protein, carbohydrate, and lipid analyses with their accompanying errors, we included the analysis of autologous LDL as a control. Since apoB is the sole protein of LDL, applying the percentage protein to the molecular weight of LDL should result in a value of 512 000 for the molecular weight of apoB. The deviation from this value reflects the accuracy of the method. The data obtained with the LDL of the three subjects are shown in Table 2 and indicate that this method estimates the respective molecular weight of apoB to 3.3, 6.8, and 9.8% of its actual value.

The chemical analyses of the four Lp(a) species and their molecular weight data are shown in Table 3. As expected, the percent protein and carbohydrate was greater in the Lp(a) particles with the larger apo(a) polymorphs. These components are also a major contributor to Lp(a) density and are responsible for the greater density of the larger apo(a) containing Lp(a) particles. The mass of Lp(a) protein ranged from 0.953×10^6 to 1.231×10^6 and increased steadily with increasing apo(a) size. This data is represented graphically in Figure 3 as a plot of the molecular weight of Lp(a) protein against that of apo(a). With the inclusion of apoB as a data point, i.e., (0, 512 000), the slope of the line is 1.97 ± 0.15, with $r = 0.992$, indicating that there are two apo(a)s per Lp(a) particle. The y-intercept is 522 000 ± 36 000 which is not significantly different from the sequence derived molecular weight of apoB. Overall, the mass of Lp(a) protein determined experimentally agrees within 0.5−5.7% of the theoretical value that was calculated assuming that that each of the four different Lp(a) particles has two apo(a)s and one apoB molecule .

Differences in the Masses of Lp(a) and Lp(a-). The ratio of apo(a) to apoB was also determined from the mass of apo(a) lost from Lp(a) upon its reduction and carboxymethylation. Since this method does not involve chemical analysis, it is not subject to the accompanying errors of the preceding method; it is based entirely on analytical centrifugation. Previously it was shown that the removal of apo(a) from Lp(a) results only in the loss of apo(a), because the complete lipid complement remained with apoB in Lp(a-), the LDL-like moiety of Lp(a) (Fless et al., 1986). Apo(a) mass was determined as the difference between the masses of Lp(a) and the corresponding Lp(a-). The results are shown in Table 4, which includes not only the mass of the various lipoproteins but also their buoyant densities. Two different preparations of each lipoprotein were analyzed. Lp(a) particle molecular weights did not vary much and were close to 4×10^6. Although not the sole determinant of Lp(a) density, the size of apo(a) contributed to the density of Lp(a) as is seen in the incremental increases with Lp(a) particles having larger apo(a) proteins. The Lp(a-) particles had buoyant densities and molecular weights that are typical of LDL. However, they were slightly more buoyant, and their masses were generally larger by 10−20% than that of autologous LDL (see Table 2). When the mass of Lp(a-) was subtracted from the mass of the parent Lp(a) particle, a quantity was obtained that is representative of the mass of apo(a) lost from Lp(a) upon its reduction and carboxymethylation. This quantity was much larger than could be accounted by the loss of one apo(a) molecule. When the difference is divided by the mass of one apo(a) molecule, it indicates that two molecules of apo(a) are lost from each Lp(a) particle.

Molecular Weight of the Lipid-free ApoB−apo(a) Complex. In one Lp(a) phenotype (KB-1), the ratio of apo(a) to

Table 3: Determination of the Ratio of Apo(a) and ApoB from the Physicochemical Parameters of Lp(a)[a,b]

	Lp(a) type			
	KB-1	LC	BK	KB-2
protein (%)	25.4 ± 0.36	28.3 ± 0.17	28.5 ± 0.40	36.2 ± 0.47
carbohydrate (%)	7.7 ± 0.15	8.5 ± 0.12	8.7 ± 0.17	10.9 ± 0.20
phospholipid (%)	18.6 ± 0.59	17.1 ± 1.24	17.4 ± 0.80	15.4 ± 1.17
free cholesterol (%)	6.2 ± 0.03	4.3 ± 0.30	5.6 ± 0.07	5.5 ± 0.19
cholesteryl ester (%)	38.2 ± 0.96	33.0 ± 0.22	34.0 ± 0.51	30.2 ± 1.02
triglyceride (%)	4.0 ± 0.23	8.7 ± 0.29	5.8 ± 0.37	1.8 ± 0.07
M_r of Lp(a) ($\times 10^{-6}$)	3.75 ± 0.14	3.45 ± 0.19	3.85 ± 0.13	3.40 ± 0.02
density (g/mL)	1.054 ± 0.003	1.065 ± 0.003	1.069 ± 0.003	1.090 ± 0.000
M_r of Lp(a) protein ($\times 10^{-6}$)	0.953 ± 0.038	0.976 ± 0.054	1.097 ± 0.040	1.231 ± 0.018

[a] Compositional values represent the mean ± SD of three analyses done in triplicate. [b] The molecular weight and buoyant density of each Lp(a) type was determined by equilibrium centrifugation at three different densities and three different concentrations.

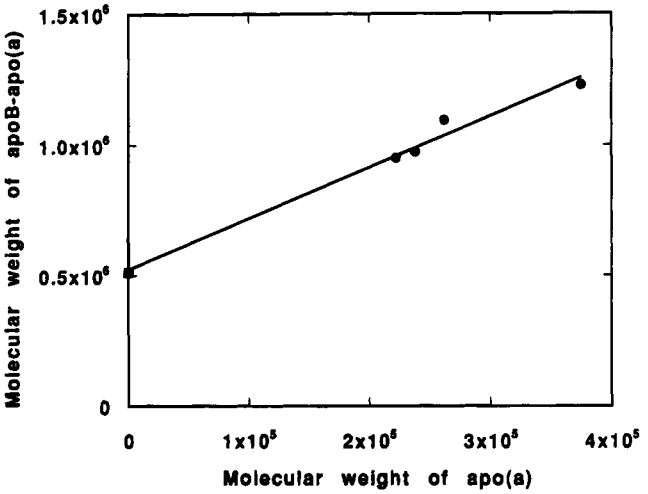

FIGURE 3: Variation of the molecular weight of Lp(a) protein as a function of apo(a) molecular weight. Data for Lp(a) protein are derived from Table 3, and that for apo(a) are from Table 1. A data point for apoB (■) is included, i.e. (0, 512 000). The solid line through the data points was obtained by linear least-squares regression and yielded a slope of 1.97 ± 0.15, a y-intercept of 522 000 ± 36 000, and a regression coefficient of 0.992. A slope of 2 is indicative of two apo(a) molecules per Lp(a) particle.

Table 4: Mass of Apo(a) and Lost upon the Reduction of Lp(a)[a]

	Lp(a) type							
	KB-1		LC		BKn		KB-2	
Lp(a) mass (megadaltons)	3.91	3.94	3.94	4.14	4.03	3.64	3.88	4.01
density (g/mL)	1.056	1.058	1.064	1.066	1.069	1.064	1.083	1.082
Lp(a-) mass (megadaltons)	3.20	3.34	3.25	3.49	3.29	2.89	2.89	3.17
density (g/mL)	1.027	1.030	1.029	1.032	1.030	1.027	1.033	1.034
apo(a) mass (megadaltons)	0.71	0.60	0.69	0.65	0.74	0.75	0.99	0.84
M_r of apo(a) ($\times 10^{-6}$)	0.289		0.310		0.341		0.488	
mol of apo(a) lost	2.5	2.1	2.2	2.1	2.2	2.2	2.0	1.7

[a] Two different preparations of each Lp(a) type were analyzed together with the corresponding Lp(a-) particle.

apoB was determined by delipidating Lp(a) and measuring the molecular weight of the lipid-free apoB−apo(a) complex. Delipidated samples were analyzed for residual lipid, and these assays showed undetectable levels of cholesterol and triglyceride, but 1.5−2.0% phospholipid. Although apoB−apo(a) is soluble in 0.15 M NaCl, previous electrophoretic and immunochemical studies indicated that its solution properties are nonideal and that the complex is probably self-associated (Fless et al., 1990). For this reason, the molecular weight of apoB−apo(a) was determined in 6 M GuHCl where the complex was essentially monomeric as shown by the excellent fit of the experimental data to the equilibrium equation for a single solute component (Figure 4A). Because of the huge size of the protein and its unfolded state in 6 M GuHCl, it took 7−8 days to reach equilibrium at 3000 rpm (see inset in Figure 4A). The apparent molecular weight values corrected for phospholipid content showed a slight concentration dependence and were extrapolated to zero concentration in order to obtain a molecular weight unencumbered by nonideality (Figure 5).

Although the solution was essentially monomeric, there was some heterogeneity present as indicated by the 6% higher value of $\bar{M}z$ relative to the whole cell weight average molecular weight, $\bar{M}w$. This was assumed to be the result of the presence of low molecular peptides that may have been produced by degradation during the delipidation procedure. To reduce the heterogeneity, fresh preparations were passed over Sepharose 4B-CL and only the fraction representing the center of the apoB−apo(a) peak was analyzed. Surprisingly this fraction (Figure 4B), as well as two additional ones, exhibited self-association when analyzed by low-speed sedimentation equilibrium. This was evident from values of $\bar{M}z/\bar{M}w$, shown in Table 5, that deviated significantly from unity. It is not clear why the first preparation was less heterogeneous than the other three, although the mechanics of the delipidation procedure may be a factor. Since the meniscus was not depleted of higher molecular weight components by the low-speed method, the last two preparations were also analyzed at a higher speed. This allowed the estimation of the monomer molecular weight because, unlike at the lower speed (Figure 4A), at the higher speed the experimental points fit the equation for a single solute component very well (Figure 4C). The partial specific volume calculated for a 2:1 apo(a) to apoB complex was 0.703 mL/g, whereas that of a 1:1 complex was 0.710 mL/g. The monomer molecular weight of apoB−apo(a), corrected for concentration and phospholipid content and using the partial specific volume of a 2:1 complex, was 1.06 $\times 10^6$ for both preparation 3 and 4 and is given in Table 5. This value is slightly larger than that of preparation 1 and deviates by 6.3% from the theoretical molecular weight (1.13 $\times 10^6$) of a complex consisting of 1 mol of apoB and 2 mol of apo(a). [ApoB was assumed to have 7 wt % carbohydrate and a mass of 553 000 daltons, whereas the mass of apo(a) was 289 000 daltons.] Because the deviation of the experi-

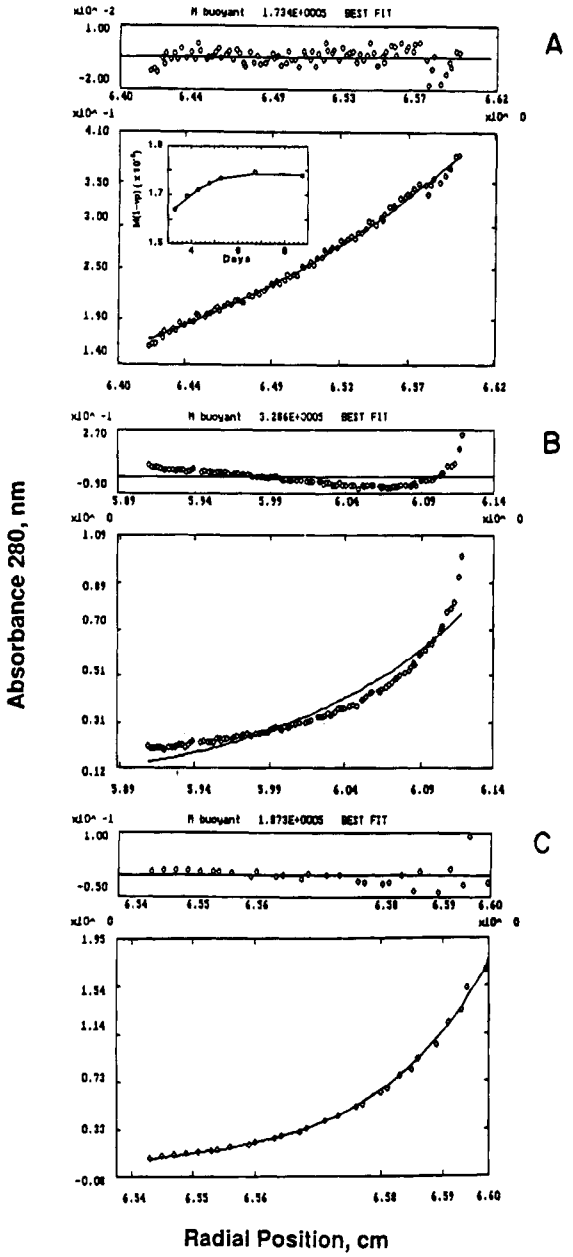

FIGURE 4: Sedimentation equilibrium of the delipidated apoB–apo(a) complex of KB Lp(a)-1 dissolved in 6 M GuHCl. The absorbance at 280 nm is plotted as a function of radial distance. (Panel A) Preparation 1 (see Table 5) after 8.5 days of centrifugation at 3000 rpm and 20 °C. The experimental data points were fitted to the sedimentation equilibrium equation describing a single ideal solute (solid line) using the XLA-DATA analysis software (Beckman). The upper panel is the residual error plot. The inset figure is a plot of $M(1 - \bar{\nu}_2\varrho)$ against time that shows the number of days required to reach equilibrium. (Panel B) Preparation 3 after 7 days of centrifugation at 3000 rpm and 20 °C. (Panel C) Same preparation after 6 additional days at 10 000 rpm.

mental value from a 1:1 apoB–apo(a) complex is greater, being 25.9%, the results of this method also indicate that a ratio of two apo(a)s to one apoB is more consistent with the experimental data than a 1:1 ratio. Had we calculated the molecular weight using the partial specific volume of a 1:1 complex, its experimental molecular weight would have been approximately 4% higher and would have increased the percentage deviation even more.

DISCUSSION

Studies based on the determination of the subunit structure

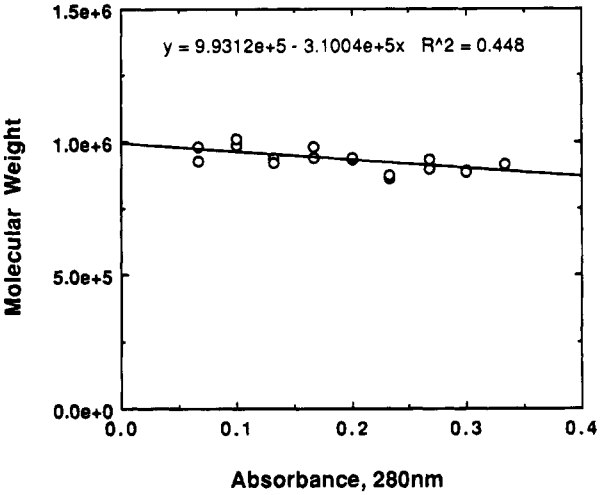

FIGURE 5: Concentration dependence of the molecular weight of the delipidated apoB–apo(a) complex in 6 M GuHCl. Preparation 1 (see Table 5) was placed into three six-channel Yphantis cells at nine different concentrations, loaded into a four-place An 60 Ti rotor and spun to equilibrium at 3000 rpm at 20 °C. Data were fitted to a least-squares straight line.

Table 5: Molecular Weight of the ApoB–Apo(a) Complex of Lp(a)[a]

	method		
	low speed sedimentation equilibrium		meniscus depletion
	3000 rpm, $\bar{M}_w$[b] × 10^{-6}	3000 rpm, $\bar{M}z/\bar{M}w$[b]	10 000 rpm, Mw × 10^{-6}
preparation 1	0.993 ± 0.034	1.06	
preparation 2	1.77 ± 0.072	1.25	
preparation 3	1.87 ± 0.061	1.40	1.06 ± 0.051
preparation 4	1.50 ± 0.063	1.32	1.06 ± 0.088

[a] Molecular weights were corrected for concentration dependence by linear regression of data sets obtained at nine different concentrations. [b] $\bar{M}w$ is the whole cell weight average molecular weight, $\bar{M}z$ is the whole cell Z-average molecular weight, and $\bar{M}z/\bar{M}w$ is essentially a heterogeneity index. A homogeneous component with no contaminants or tendency to self-associate should have a heterogeneity index of 1.

of Lp(a) protein have focused either on the identification of the cysteines involved in the linkage of apo(a) to apoB, or on the molecular weight and composition of Lp(a) and apo(a). Analyses of the secondary structure of apoB have shown that it contains 25 cysteine residues, of which at least 16 are involved in intramolecular disulfide bonds, leaving 9 sulfhydryls unpaired and potentially available for intermolecular disulfide bridges (Yang et al., 1990). The identification of candidate cysteines involved in the linkage of apo(a) to apoB has relied heavily on their reactivity with water soluble fluorescent probes. Two groups, located at positions 3734 and 4190 of the C-terminal end of LDL-apoB, can be labeled with 5-iodoacetamido fluorescein (Coleman et al., 1990). On the other hand, Sommer et al. (1991), using the fluorescent probe acrylodan, labeled three free cysteine residues in LDL of unknown location. Because they were able to modify only two cysteines in apoB of Lp(a), they concluded that a single surface-exposed cysteine of apoB was involved in the intermolecular disulfide linkage to apo(a). From this the authors surmised that there was only one apo(a) molecule bound to apoB. However in a subsequent study, Mims et al. (1993) found that acrylodan was not specific for cysteine sulfhydryl groups but also had the ability

to react with lysine amino groups. This effectively compromised the use of acrylodan in determining the number of apoB cysteine(s) involved in binding apo(a). With the use of another fluorescent dye, Guevara et al. (1993) showed that in contrast to LDL, cysteine 3734 of Lp(a), was not labeled with fluorescein-5-maleimide. On the basis of this evidence, and aided by molecular modeling studies, they concluded that this cysteine was involved in the disulfide linkage of apo(a) to apoB. Similar studies with cysteine 4190 indicated that it had a much lower probability of being part of the disulfide linkage. These findings suggest that at least one apo(a) molecule is linked via one of the two surface exposed cysteines of apoB of Lp(a) but do not rule out the possibility that one of the seven "buried" cysteines might be involved in binding a second apo(a) to apoB.

Using a different approach, based on the ultracentrifugally determined mass differential between Lp(a) and Lp(a-) caused by the loss of apo(a) from reduced Lp(a) and on the mass of apo(a), we had concluded that Lp(a) contained two molecules of apo(a) (Fless et al., 1986). Since only one apo(a) polymorph was characterized in that study, we expanded our investigation to include the analysis of four different apo(a) polymorphs in order to confirm that our prior conclusions were general and not confined to an isolated case. Furthermore we expanded the analysis to include other methods by which the ratio of apo(a) to apoB in Lp(a) can be determined. This more rigorous reexamination of the issue confirms our original conclusion, that at least in these four Lp(a) particles, the ratio of apo(a) to apoB is 2:1.

One of the most important parameters in determining the stoichiometry of the two apoproteins is the correct molecular weight of apo(a). It is reassuring therefore that the molecular weight of LC apo(a) as determined here is only 10% higher than the one determined previously (Fless et al., 1986). A second finding that supports the credibility of the molecular weight determination of apo(a) are the similarities in the physical parameters of a recombinant apo(a) described by Phillips et al. (1993) to those of B.K. apo(a). Both apo(a)s had similar mobilities relative to apoB on SDS−PAGE, with that of the recombinant apo(a) being slightly greater and the one of BK apo(a) being slightly smaller. Both apo(a)s had 23 weight % carbohydrate, a similar partial specific volume [0.684 mL/g vs 0.682 mL/g for BK apo(a)], and a similar molecular weight. The molecular weight of the recombinant apo(a) as determined by sedimentation equilibrium was 325 000, whereas the one of BK apo(a) was slightly larger (341 000). With the carbohydrate portion subtracted, the molecular weight of B.K. apo(a) protein is 262 000, which is equivalent to a protein with 18 kringle 4s; whereas, on the basis of its cDNA sequence, the recombinant apo(a) has 17 kringle 4s and a protein molecular weight of 249 000 (Koschinsky et al., 1991). Hence the similarity in electrophoretic mobility, carbohydrate content, partial specific volume, mass and kringle number of the recombinant apo(a) compared to B.K. apo(a) supports the accuracy of the molecular weight determination of apo(a) by sedimentation equilibrium.

A second important variable in determining the ratio of apo(a) to apoB, as used in methods two and three, is the mass and composition of Lp(a). From the analysis of LDL as an internal control we concluded that the efficacy of the molecular weight and chemical analyses was good, since we were able to estimate the mass of apoB to within 3.3, 6.8, and 9.8% of its true value. These differences are actually somewhat greater than those found between the experimental and theoretical molecular weights of a 2:1 apo(a)−apoB complex that ranged between 0.5 and 5.7% (see Table 3). Since the values of a 1:1 apo(a)−apoB complex deviated much more, e.g., 30−40%, it is more probable that the actual ratio is 2:1. It can be argued that the molecular weight analysis of Lp(a) may be more complicated than that of LDL because of the presence of apo(a). However, our previous analysis of Lp(a) and Lp(a-) suggests that this is not the case (Fless et al., 1986). In that study we determined both the mass and chemical composition of Lp(a) and Lp(a-) in two different subjects. Once apo(a) is removed from Lp(a), the resulting Lp(a-) particle is virtually identical to LDL, both in terms of its structural and functional properties (Armstrong et al., 1985; Fless et al., 1986). Because our results indicated that the number of lipid molecules in Lp(a-) were essentially the same as those in the parent Lp(a) particle, they also provide evidence that the Lp(a) molecular weights are correct.

Our final approach was to determine the ratio of apo(a) to apoB from the molecular weight of the apoB−apo(a) complex of KB Lp(a)-1. Because previous electrophoretic and immunological analyses indicated that the complex was self-associated at neutral pH in a low ionic strength buffer (Fless et al., 1990), the molecular weight determination was carried out in 6 M GuHCl. Although it had been shown that apoB resists complete unfolding and retains some residual structure in 6 M GuHCl (Patterson et al., 1987), we anticipated that the solution properties of apoB would not be problematic because it was complexed to the hydrophilic, highly glycosylated apo(a). This was found to be true for the first preparation; however, the other three were self-associated. One reason for this nonideal behavior may have been the absence of a reducing agent that prevented complete unfolding and denaturation of either protein by GuHCl and thereby promoted self-association. Although preparations 3 and 4 were self-associated, we were able to determine the weight average molecular weight of the monomer by using the high-speed meniscus depletion method. The value of 1.06×10^6 did not differ significantly from the whole cell weight average molecular weight of apoB−apo(a) of preparation 1 (see Table 5) and was only 6.3% lower than the theoretical molecular weight of a complex containing two molecules apo(a) and one molecule apoB.

The question of whether there are one or two molecules of apo(a) linked to apoB is of great importance because it relates directly to the problem of how and where Lp(a) is synthesized. It should be pointed out, that none of the methods used in this study provides any insight into the structural organization of the apoB-apo(a) complex, only that the stoichiometry of apo(a) to apoB is 2:1. Recent studies on the synthesis of Lp(a) by baboon hepatocytes (White et al., 1993), and by transgenic mice expressing human apo(a) infused with human LDL (Chisea, 1993), suggest that Lp(a) formation occurs in plasma. If Lp(a) is formed in plasma, this implies that the linkage of apo(a) to apoB is a random process. Thus if there are two molecules of apo(a) to one apoB, one should expect in heterozygotes Lp(a) particles with two different apo(a) polymorphs, in addition to particles having only one apo(a) polymorph. However, in none of our studies (Fless et al., 1984; Fless & Snyder, 1994) or those by others (Kraft et al., 1992) has the existence of Lp(a)

particles with more than one kind of apo(a) been demonstrated. It appears, therefore, that the linkage of apo(a) to apoB of LDL may be more complicated than previously thought, if the formation of Lp(a) particles with heterogeneous apo(a) polymorphs is to be avoided. A hypothetical model of Lp(a) synthesis might have to invoke a mechanism that segregates the different apo(a) polymorphs in order to avoid the formation of mixed apo(a)−Lp(a) particles.

The presence of two molecules of apo(a) on an Lp(a) particle may be of profound functional importance, because every constitutive functional property, of which the presence of lysine binding sites is one, would be doubled. The lysine binding site is probably located on kringle 4_{37} which, because of its proximity to kringle 4_{36} and Cys 4057 [thought to be involved in the linkage of apo(a) to apoB] would place it close to the lipoprotein surface. It is not clear whether one apo(a) might interfere sterically with the ligand binding activity of another as only one linkage cysteine on apoB has been identified with some certainty and it is unclear how distant the second cysteine is in the lipoprotein surface. On the other hand, kringle 4_2, which is the domain that is repeated many times in apo(a), may be located some distance from the particle surface. For example, Phillips et al. (1993) estimated from electron microscopy that the extended length of the 17 kringle recombinant was 800 Å. Apo(a) is believed to make minimal contact with the lipoprotein surface and, except for its attachment to apoB, is thought to extend into the aqueous medium (Fless et al., 1984, 1986; Phillips et al., 1993; Zawadzki et al., 1988). Phillips et al. (1993) determined that recombinant apo(a) was flexible and complexed to LDL via one or two kringles. If the structure of Lp(a) is similar, a second apo(a) molecule would essentially provide two arms and thus greatly extend the reach of Lp(a). Although Lp(a) already has the potential of forming crosslinks because apo(a) is made up of repetitive kringle domains, its magnitude would be increased with two apo(a) subunits. Therefore interactions involving the kringle 4_2 domains might be especially suited for cross-linking purposes. In a study of the binding of Lp(a) to fibrin that was generated by the treatment of plastic bound fibrinogen with thrombin we found that there are two types of binding sites on Lp(a) (Fless & Snyder, 1994). One was lysine specific; the other was not; however, both had comparable affinities for fibrin. Interestingly, the percentage of nonlysine binding sites was greatly increased in large molecular weight apo(a) containing Lp(a), which also has more of the repetitive kringle 4_2 domains. Although we have not proven that kringle 4_2 is involved in binding to fibrin, one could envision bifunctional Lp(a) particles crosslinking newly generated fibrin with the kringle 4_2 and kringle 4_{37} domains of their apo(a) side chains during clot formation, thereby increasing clot strength. If this hypothesis is correct, Lp(a) would not only be antifibrinolytic but also prothrombotic.

In conclusion, the results shown here represent strong physical evidence for the presence of two apo(a) molecules on each Lp(a) particle. This finding is not incompatible with the fact that there is only one type of apo(a) bound to an Lp(a) particle but does imply that the assembly process for these particles is somehow more ordered or restricted than the random self-association that might occur if this event took place in plasma. The presence of two apo(a)s per Lp(a) particle may have functional consequences whose importance remain to be determined.

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