# Determinants of Lipoprotein(a) Assembly: A Study of Wild-Type and Mutant Apolipoprotein(a) Phenotypes Isolated from Human and Rhesus Monkey Lipoprotein(a) under Mild Reductive Conditions†

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*Received July 20, 1995; Revised Manuscript Received October 17, 1995*⊗

ABSTRACT: We previously observed that rhesus monkey lipoprotein(a) [Lp(a)], is lysine-binding defective ($Lys^-$) and attributed this deficiency to the presence of Arg72 in the lysine-binding site (LBS) of kringle IV-10 of apolipoprotein(a) [apo(a)] [Scanu, A. M., Miles, L. A., Fless, G. M., Pfaffinger, D., Eisenbart, J., Jackson, E., Hoover-Plow, J. L., Brunck, T., & Plow, E. F. (1993) *J. Clin. Invest. 91*, 283−291]. We also identified human mutants having Arg72 instead of Trp72 (wild type) in the LBS of kringle IV-10 [Scanu, A. M., Pfaffinger, D., Lee, J. C., & Hinman, J. (1994) *Biochim. Biophys. Acta 1227*, 41−45]. Unique to the human mutant phenotype were the very low levels of plasma Lp(a), suggesting structural differences between human and rhesus apo(a) and a possible divergent mode of Lp(a) assembly. In order to explore the possibility of a relationship between apo(a) LBS and Lp(a) assembly, we developed a novel method for isolating wild-type and mutant apo(a) phenotypes in a free form by subjecting each parent Lp(a) to mild reductive conditions using 2 mM dithioerythritol (DTE) and 100 mM of the lysine analogue, $\epsilon$-aminocaproic acid (EACA). The application of this method to the study of wild-type and mutant apo(a) species showed that regardless of the source of Lp(a), i.e., positive lysine binding ($Lys^+$) or negative lysine binding ($Lys^-$), all of the isolated free apo(a)s were $Lys^+$. Moreover, incubation of free apo(a)s with their autologous human or rhesus low-density lipoproteins (LDL) generated Lp(a) complexes which were structurally and functionally indistinguishable from their parent native Lp(a). In each instance, the reassembly process was inhibited by the presence of either EACA or proline. These two reagents had a minimal effect on either Lp(a) or reassembled Lp(a) [RLp(a)]. Free apo(a) bound to apoB100 of very low density lipoproteins (VLDL) to form a triglyceride-rich Lp(a). These results show that (1) both human and rhesus Lp(a) are amenable to dissassembly and reassembly, (2) the presence of Arg72 in the LBS of kringle IV-10 is not involved, at least directly, in this process, (3) its cleavage from apoB100 opens up in apo(a) a domain that is both EACA and proline sensitive and involved in Lp(a) assembly, and (4) the apoB100 of VLDL is also competent to bind apo(a). Our observations also suggest that the difference in plasma Lp(a) levels between the rhesus and the human mutant, both having Arg72 in the LBS of apo(a) kringle IV-10, is not related to the assembly process, but more likely to a divergence in production/secretion rates between the two apo(a) phenotypes.

Lipoprotein (a) [Lp(a)][1] refers to a class of lipoprotein particles in which apoB100 is covalently linked to apolipoprotein (a) [apo(a)] by a disulfide bond (Koschinsky et al., 1993; Brunner et al., 1993; Van Der Hoek et al., 1994; Scanu & Fless, 1990). Apo(a) is a large glycoprotein, polymorphic in size (250−800 kDa), containing kringles highly homologous to kringles 4 and 5 of plasminogen (Scanu & Edelstein, 1995). Some of the kringles in apo(a) have been predicted to have a functional lysine-binding site (LBS), in particular kringle IV-10, comprising two anionic (Asp55 and Asp57), two cationic (Arg35 and Arg71), and three nonpolar (Trp62, Trp72, and Phe64) amino acids (Guevara et al., 1992, 1993). LBS is also present in plasminogen kringle 4 except that Lys35 has been replaced by Arg. Studies on apo(a) kringle IV-10 cloned and expressed in *Escherichia coli* showed that this kringle binds avidly to lysine and its analogues (LoGrasso et al., 1994).

The information regarding the site(s) of the Lp(a) assembly process remains unclear. An intracellular assembly has been observed in primary human hepatocyte cultures (Edelstein et al., 1994), whereas an extracellular event has been reported in cultures of baboon hepatocytes (White et al., 1993; White & Lanford, 1994), HepG2 cells (Koschinsky et al., 1991) and by infusing human low-density lipoprotein (LDL) into

† This work was supported by the National Institutes of Health Grant HL-18577.

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: Lp(a), lipoprotein(a); RLp(a), reassembled Lp(a); apo(a), apolipoprotein(a); Lp(a−), Lp(a) devoid of apo(a); VLDL, very low density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; CE, cholesteryl esters; FC, free cholesterol; PL, phospholipids; PAGE, polyacrylamide gel electrophoresis; GGE, native gradient gel electrophoresis; PFGE, pulsed-field gel electrophoresis; EACA, $\epsilon$-aminocaproic acid; DTE, dithioerythritol; PMSF, phenylmethylsulfonyl fluoride; BHT, $\beta$-hydroxytoluene; KI, Kallikrein inactivator; EDTA, ethylenediaminetetraacetic acid; $\beta$-ME, $\beta$-mercaptoethanol; LBS, lysine-binding site; $Lys^-$, deficient lysine binding; $Lys^+$, positive lysine binding; BD, binding domain; ELISA, enzyme-linked immunosorbent assay.

0006-2960/95/0434-16483$09.00/0 

mice transgenic for a human 17-kringle apo(a) construct (Chiesa et al., 1992). The assembly of Lp(a) was also shown to occur in mice transgenic for human apoB100 and a 17-kringle apo(a) construct (Linton et al., 1993; Callow et al., 1994). In all cases, the reassembled lipoprotein was reported to have an apo(a) disulfide linked to apoB100. In fact, the requirement for this disulfide has been documented by Brunner et al. (1993).

Of note, Phillips et al. (1993), working with an apo(a) 17-kringle construct, demonstrated an important role for non-covalent interactions in Lp(a) assembly. Subsequently, several studies (Ernst et al., 1995; Frank et al., 1994a,b; Trieu & McConathy, 1995) have provided evidence that a lysine dependent site(s) in kringle IV-10 and in kringles IV-5 through IV-9 may be important in the early assembly step-(s) preceding the stabilization of the Lp(a) complex by a disulfide linkage. Of interest, Trieu et al. (1991) demonstrated that like $\epsilon$-aminocaproic acid (EACA) proline inhibits the binding of recombinant apo(a) to LDL and concluded that these reagents may bind to the same site on apo(a).

In our own laboratory we previously showed (Scanu et al., 1993) that rhesus monkey Lp(a) has an impaired lysine-binding capacity, which we attributed to the presence of Arg72 in the apo(a) kringle IV-10 LBS. Subsequently, we identified human subjects in whom the Lp(a) is lysine binding defective ($Lys^-$) (Scanu et al., 1994) having in the LBS of kringle IV-10, Arg72 instead of the Trp72 present in wild-type positive lysine binding ($Lys^+$) subjects. The $Lys^-$ human subjects have plasma levels of Lp(a) that are too low to unequivocally define whether they represent either an Lp-(a) complex, free apo(a), or both. On the other hand, the rhesus monkeys that we studied, in spite of their $Lys^-$ Lp-(a), have plasma levels which are about 3-fold higher than those of control $Lys^+$ human subjects (Scanu et al., 1993). Prompted by these observations we wanted to examine whether there might be a relationship between apo(a) LBS function and Lp(a) assembly. To this end, we devised a novel method for the disassembly and reassembly of both human and rhesus Lp(a) and applied this technology to the analysis of some of the determinants of Lp(a) assembly taking advantage of the $Lys^+$ and $Lys^-$ Lp(a) models.

## EXPERIMENTAL PROCEDURES

*Chemicals and Reagents.* Materials were purchased from various sources as follows: Cyanogen bromide (CNBr)-Sepharose 4B, EACA, Tween 20, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), L-lysine, L-proline, dithioerythritol (DTE), $\beta$-mercaptoethanol ($\beta$-ME), phosphate-buffered saline packets (PBS), and cross-linked phosphorylase molecular weight standards for SDS−polyacrylamide gel electrophoresis (SDS−PAGE) from Sigma Chemical Company (St. Louis, MO); Kallikrein inactivator (KI) from Calbiochem, (San Diego, CA); molecular weight standards for native gradient gels from Pharmacia-LKB (Alameda, CA); $\lambda$ DNA size standards and polyacrylamide from Bio-Rad (Richmond, CA); Immobilon-P membranes from Millipore (Bedford, MA); and an enhanced chemiluminescent kit (ECL Western Blotting Detection kit) from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade.

Antisera to purified preparations of Lp(a) and LDL were raised in the rabbit, and affinity-purified antibodies to apo-(a), Lp(a), and LDL were prepared as previously described (Fless et al., 1989). Anti-Lp(a) were shown to be devoid of immunoreactivity to LDL and plasminogen, and anti-LDL were unreactive to Lp(a) and apo(a).

Table 1: Lp(a) Levels, Phenotypes, and Genotypes of Wild-Type and Mutant Subjects

| subject | Lp(a) protein (mg/dL)[a] | phenotype (kDa)[b] | Genotype (kb)[c] |
|---|---|---|---|
| K. B. | 32 | 289; 488 | 58; 113 |
| D. G. | 15 | 379; 401 | 74; 103 |
| B. K. | 43 | 333; 341 | 69; 74 |
| P. T. | 35 | 330; 379 | 69; 73 |
| T. T. | 0.16 | 279 | 56 |

[a] Measured by ELISA as Lp(a) protein. [b] The molecular weights of each of the two phenotypes from K. B. and one of the phenotypes from B. K. (341 kDa) were measured in the analytical ultracentrifuge (Fless et al., 1994) and included the weight of the carbohydrates. The size of the phenotypes from D. G. and P. T. was estimated on Western blots of reduced SDS−PAGE. [c] Allele size was estimated by PFGE in 1% agarose gels using the mobilities of $\lambda$ DNA standards in a 48.5 kb ladder encompassing a size range of 0.05−1 mb.

*Buffers.* Buffer A was 10 mM phosphate containing 1 mM EDTA, 0.02% $NaN_3$, pH 7.5. Buffer B was 10 mM phosphate containing 1 mM EDTA, 0.02% $NaN_3$ and 100 mM EACA, pH 7.5. All other buffers were prepared as described in the text.

*Human Subjects Used in the Study.* The four wild-type subjects were two males (one Afro-American, one Caucasian) and two females (Caucasians), all healthy with Lp(a) protein levels in the range 15−43 mg/dL (Table 1). All of them were heterozygous for apo(a) size isoforms on the basis of protein and genomic analyses. One healthy subject (male, Caucasian) with the Trp72→Arg mutation in apo(a) kringle IV-10 had plasma Lp(a) protein levels of 0.16 mg/dL (Scanu et al., 1994) (Table 1) and had a single allele and a single apo(a) isoform. The plasma from all subjects was obtained by plasmapheresis performed in the Blood Bank of the University of Chicago. The steps for Lp(a) and LDL isolation were carried out immediately after blood drawing. The plasma samples used for the isolation of VLDL were obtained from either normolipidemic healthy human donors or dyslipidemic subjects (type IV) before receiving treatment at the University of Chicago Lipid Clinic and had plasma levels of Lp(a) protein below 1 mg/dL. The blood was drawn in EDTA-containing tubes and the plasma, prepared as previously described (Edelstein & Scanu, 1986), and was utilized within 1−12 h from the time of blood withdrawal. All of the subjects used in the study gave their written informed consent.

*Phenotyping and Genotyping of Apo(a).* Apo(a) phenotyping was performed on reduced plasma, isolated apo(a), or Lp(a) samples by SDS−PAGE followed by immunoblotting using anti-Lp(a). The mobility of the individual apo-(a) bands was compared with isolated apo(a) isoforms of known molecular weights (Fless et al., 1994). For apo(a) genotyping, DNA plugs were prepared from blood mononuclear cells and subsequently fractionated by pulsed-field electrophoresis, and the blots were probed with an apo(a) specific probe essentially as described earlier (Lackner et al., 1991).

*Preparation of VLDL and LDL.* For the isolation of VLDL, the plasma samples from wild-type and mutant subjects and from rhesus monkeys were adjusted with 0.01% $NaN_3$, 10 000 units of KI/L, and 1 mM PMSF and centrifuged at $d$ = 1.006 g/mL at 412 160$g$ in a Beckman

TLA100.3 rotor for 3 h at 15 °C. The floating fraction was placed under an equal volume of saline and respun under the same conditions. The final floating VLDL was tested for the presence of apo(a) by Western blots of SDS−PAGE gels, stored at 4 °C in saline containing 2 mM PMSF and the same inhibitors, under nitrogen, and utilized within 24 h. LDL were isolated at $d$ = 1.030−1.050 g/mL by sequential flotation as previously described (Schumaker & Puppione, 1986).

*Preparation of Human Wild-Type and Mutant Lp(a).* To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% $NaN_3$, 10 000 units of KI/L and 1 mM PMSF. Wild-type Lp(a) was isolated by sequential ultracentrifugation and lysine-Sepharose chromatography as previously described (Fless et al., 1994). For the isolation of mutant Lp(a), the plasma was brought to $d$ = 1.050 g/mL with solid NaBr, and proline at a final concentration of 100 mM was added to prevent the interaction of LDL with the small amounts of Lp(a) present in these subjects. The solution was ultracentrifuged at 302 120$g$ for 20 h at 15 °C, the floating fraction was removed, and the density of the bottom fraction was adjusted to $d$ = 1.10 g/mL and recentrifuged under the same conditions. Subsequently, the floating fraction was dialyzed against 10 mM Tris-HCL, pH 7.5, and subjected to FPLC ion-exchange chromatography as described previously (Scanu et al., 1993). The purity of the product was assessed by mobility on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA) and Western blots of SDS−PAGE, utilizing anti-Lp(a) and anti-apoB. The purified Lp(a) were filter sterilized and stored at 4 °C. An aliquot of both blood and plasma from each subject was utilized for apo(a) genotyping and phenotyping, respectively. In three of the subjects (D. G., B. K., and P. T), the two apo(a) phenotypes were close in size (Table 1); thus Lp(a) species containing a single phenotype could not be separated by the procedure used. On the other hand, in the case of K. B., the high differential in apo(a) size and thus Lp(a) density permitted the separation of two Lp(a)s, one containing the 289 kDa and the other the 488 kDa phenotype. The technique used was an adaptation of that described by Fless et al., (1994).

*Preparation of Rhesus Monkey Lp(a).* The four rhesus monkeys studied were from the same pedigree previously described (Scanu et al., 1993). All had high plasma Lp(a) protein levels varying between 20 and 40 mg/dL, exhibited a single band phenotype, and were housed at the Southwest Foundation for Biomedical Research in San Antonio, TX. The monkeys were fasted overnight before 20 mL of venous blood was collected in tubes containing 0.01% EDTA. The isolation procedure was essentially as described previously (Scanu et al., 1993). Since rhesus apo(a) is $Lys^-$, we could not utilize lysine-Sepharose chromatography for the isolation of Lp(a). In brief, the plasma was spun at 302 120$g$ at 10 °C for 20 h at $d$ = 1.050 g/mL. After removal of the top layer, the infranatant was adjusted to $d$ = 1.070 g/mL with solid NaBr and spun under the same conditions. The top layer containing mainly Lp(a) was removed, dialyzed against 10 mM Tris-HCl, 0.01% EDTA, 0.01% $NaN_3$, and subjected to FPLC ion-exchange chromatography as fully described by Scanu et al. (1993). The isolated Lp(a) were filter sterilized and stored refrigerated in 33 mM phosphate buffer containing 2 mM PMSF, 0.15% EDTA, and 0.01% $NaN_3$, pH 7.5, under nitrogen.

*Dissociation and Isolation of Apo(a) from Lp(a).* Lp(a), 1 mg of protein/mL (2 mL), was incubated with DTE at a final concentration of 2 mM in buffer A for 10 min under argon gas at room temperature. EACA to a final concentration of 100 mM was then added in small increments, and the reaction mixture was protected from light with aluminum foil and rotated slowly (7 rpm) in a general purpose rotator at room temperature for 1 h. Subsequently, the incubated mixture was dialyzed for 2 h at room temperature against two changes of 4 L each of buffer B purged with nitrogen gas. After dialysis, an equal volume of 60% sucrose in buffer B was added, and the resulting mixture was distributed into Beckman polycarbonate tubes so that about 0.5 mg of free apo(a) was contained in each tube, placed into a TLA 100.3 titanium rotor, and spun in a tabletop TL100 ultracentrifuge at 10 °C, 412 160$g$ for 18 h at acceleration and deceleration settings of 6. After centrifugation, the top 0.5 mL fraction contained LDL without apo(a) [Lp(a−)] and unreacted Lp(a), and the bottom 1.0 mL fraction contained free apo(a) in pure form. The latter was stored in the sucrose solution at −80 °C. The yield of free apo(a) was 90%−100%. When we used 0.5 and 1 mM DTE, while maintaining the other experimental conditions unchanged, the resulting apo(a) behaved in a similar way but the yields, on the basis of the ELISA of the Lp(a) remaining in the floating fraction, were significantly lower than those obtained with 2 mM DTE: about 55% and 70% with 0.5 and 1 mM DTE, respectively, vs 90% with 2 mM DTE. The disassembly process was the same, whether starting from Lp(a) preparations containing one or two phenotypes.

*Reassembly of Lp(a) from Apo(a) and LDL.* Apo(a) (1 $\mu$g) of a defined phenotype obtained from human Lp(a) by the procedure outlined above was incubated with an homologous preparation of LDL in buffer A at different apoB100:apo(a) molar ratios in a total volume of 175 $\mu$L [final apo(a) concentration was 5.7 $\mu$g/mL] in a shaking water bath at 37 °C for various time intervals in the presence of 50 $\mu$M BHT, KI (10 000 KI units/mL) and 1 mM PMSF under nitrogen. Aliquots of the reaction mixture were then analyzed on Western blots of SDS−PAGE gels under nonreducing and reducing (3% $\beta$-ME for 5 min at 95 °C) conditions. In some experiments, the apo(a) was incubated with 100 mM EACA for 60 min at 37 °C before the addition of LDL and the mixture was then incubated for the desired time. To quantitate the amount of Lp(a) assembled, an aliquot (125 $\mu$L) of the reaction mixture was diluted with an equal volume of 60% sucrose in buffer A containing 200 mM EACA and spun in a TLA100 rotor (tube capacity, 250 $\mu$L) at 412 160$g$ at 15 °C for 18 h. The top fraction (105 $\mu$L) was removed and quantitated by ELISA designed to measure the apoB100:apo(a) complex (Fless et al., 1989). The bottom 100 $\mu$L containing free apo(a) was also quantitated by a sandwich ELISA that was specific for apo(a) and used anti-Lp(a) for coating and alkaline phosphatase-conjugated anti-Lp(a) for detection.

In the studies directed at defining the properties of RLp(a), 1−2 mg of apo(a) was incubated with LDL in buffer A containing 2 mM PMSF at an apoB100:apo(a) molar ratio of 25:1 at 37 °C for 24 h under nitrogen. The reaction mixture was centrifuged overnight in 30% sucrose in buffer B, $d$ = 1.127 g/mL, the floating fraction was dialyzed against buffer A, and RLp(a) was isolated from unreacted LDL by lysine-Sepharose chromatography or, in the case of the human mutant and rhesus monkeys, by FPLC. Alternatively,

the reassembly reaction mixture, after dialysis against buffer B, was subjected to density gradient ultracentrifugation (Nilsson et al., 1981) which effects an efficient separation of LDL from Lp(a) and apo(a). The fractions containing Lp(a) were dialyzed against buffer A and further purified to homogeneity by affinity chromatography with lysine-Sepharose or FPLC.

*Reassembly of Lp(a) from Apo(a) and VLDL.* The procedure was the same as that described for the reassembly with LDL. The molar ratio of apoB100 to apo(a) was based on the concentration of apoB100 in VLDL as measured by ELISA.

*Preparation of Fully Reduced and Alkylated Apo(a).* To reduce all the disulfide bonds, apo(a) was incubated in buffer A containing 50 mM DTE and 6 M guanidine hydrochloride for 2 h at room temperature. The mixture was made 150 mM with respect to iodoacetamide, and the incubation continued for an additional hour. Before use, the reduced and alkylated apo(a) was dialyzed against buffer A.

*Lysine-Sepharose Chromatography.* CNBr-activated Sepharose 4B was coupled to the α-amino group of lysine essentially according to the instructions supplied by Pharmacia-LKB. The amount of lysine cross-linked to the beads was assessed according to Wilkie and Landry (1988) and ranged between 16 and 21 μmol of lysine per milliliter of bead suspension. Chromatography was performed at room temperature on a Bio-Rad Econo chromatography system. Columns were packed with lysine-Sepharose at a ratio of 5 mL of packing material to 1 mg of Lp(a) protein and equilibrated with PBS containing 1 mM EDTA and 0.02% $NaN_3$. After loading, the column was washed with at least 3 column volumes of equilibrating buffer at the same flow rate. Fractions containing apo(a) or Lp(a) were pooled and dialyzed against buffer A.

*Electrophoretic Methods.* SDS−PAGE (3.5% separating gel, 2.75% stacking gel) was performed on a Novex system (Novex, San Diego, CA) for 1.5 h at constant voltage (120 V) at room temperature. The samples were prepared by heating at 95 °C for 5 min in sample buffer which consisted of 94 mM phosphate buffer, pH 7.0, 1% SDS, and 2 M urea with or without 3% β-ME. Immediately after electrophoresis, the gels were placed onto Immobilon-P sheets which were previously wetted with a buffer containing 48 mM Tris and 39 mM glycine, pH 8.9. Blotting was performed on a horizontal semidry electroblot apparatus (Pharmacia-LKB) at 0.8−1 mA/cm$^2$ for 45 min at room temperature. To assess the size and integrity of LDL, Lp(a), and RLp(a), nondenaturing PAGE (GGE) was performed on precast 2.5%−16% polyacrylamide slab gels (Isolab, Akron, OH) as described by Nichols et al. (1986).

*Immunoblotting.* After electroblotting, the Immobilon-P blots were blocked in PBS containing 5% nonfat dry powdered milk and 0.3% Tween 20 followed by incubation with anti-Lp(a) or anti-apoB antibody. The blots were washed and incubated with anti-rabbit horseradish peroxidase-labeled IgG. Subsequently, the blots were developed with the ECL Western Detection Reagent according to the manufacturer's instructions.

*Amino Acid Analyses.* Amino acid analyses were performed at the University of Kentucky Macromolecular Structure Analysis Facility. The mildly (2 mM DTE) and fully reduced and alkylated apo(a) were dialyzed against a 10 mM solution of 4-ethylmorpholine acetate, pH 8.0, and lyophilized for shipment. The moles of free sulfhydyls in apo(a) that were alkylated were determined as carboxymethylcysteine.

*Electron Microscopy.* Solutions containing lipoproteins at 0.05 mg/mL protein in 10 mM $NH_4HCO_3$ were transferred to Formvar carbon-coated copper grids. Lipoproteins were allowed to adhere, and the excess was removed by touching the edge of the grids with filter paper. After the grids were washed twice with deionized water, each grid was coated with one drop of 1% phosphotungstic acid. The excess phosphotungstic acid was removed, and the grids were air-dried and examined in a Philips CM 10 electron microscope at an accelerating voltage of 100 kV.

*Circular Dichroic Measurements.* CD spectra were measured on a Jasco J-600 spectropolarimeter (Jasco, Japan) and analyzed using the J-700 software after conversion of the data using the Softsec File conversion program (Softwood Co., CT). Spectra were recorded at protein concentrations ranging from about 0.1 to 2 mg/mL in cuvettes of 0.01−0.1 cm path length. Mean residue ellipticities were calculated using the following mean residue weights: 112.8 for apo(a), and 113.3 for Lp(a) and RLp(a). The secondary structure content was calculated by two methods, using the program VARSLC1 starting with a set of 33 reference proteins (Manavalan & Johnson, 1987) and the program CONTIN (Provencher & Glockner, 1981). All samples were previously dialyzed against buffer A but without PMSF since this reagent interfered with CD absorption in the far-ultraviolet region of the spectrum.

*Lipid, Lipoprotein, and Apolipoprotein Analyses.* Total cholesterol and free cholesterol were measured with enzymatic kits from Boehringer Mannheim (Indianapolis, IN). The mass of cholesteryl esters was the difference between total and free cholesterol multiplied by the factor 1.68. Triglycerides were determined by a test kit from Sigma (TG INT 336), and phospholipids were determined as inorganic phosphorous using the Fiske and Subbarow reagent (Fisher, USA) following the method of Bartlett (1959) and using the factor 25 to convert inorganic phosphate to phospholipid mass. Lp(a) and LDL protein were quantitated by a sandwich ELISA essentially as previously described (Fless et al., 1989) except that anti-Lp(a) IgG was used as the capture antibody and anti-apoB IgG conjugated to alkaline phosphatase as the detection antibody. For the ELISA quantitation of apo(a), anti-apo(a) IgG conjugated to alkaline phosphatase was used as the detection antibody. Protein determinations were performed by the Bio-Rad DC protein assay.

## RESULTS

*Studies with Human Wild-Type Lp(a). Properties of Isolated Apo(a).* The Western blots (Figure 1) of the products dissociated from Lp(a) by the action of DTE show that only free apo(a) was present in the sedimenting fraction (panel A, lanes 2 and 5) but not in the floating fraction (panel A, lanes 3 and 6) which contained only apoB100 and barely detectable quantities of unreacted Lp(a) (panel B, lane 3). Reduction with 3% β-ME caused the apo(a) bands of the two phenotypes, 488 and 289 kDa, to shift to a position in the gel corresponding to that of the parent-reduced Lp(a) (panel A, lanes 7−10). In terms of isoform number, the pattern of the isolated free apo(a) resembled that of the parent Lp(a) as exemplified in panel C.

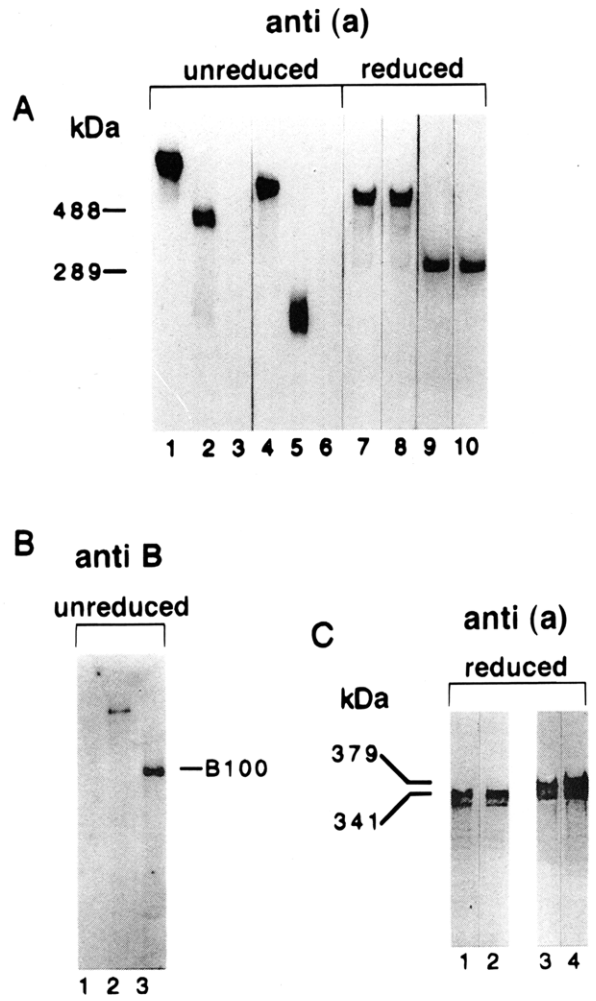


FIGURE 1: Western blots of parent Lp(a)s, apo(a)s, and LDLs from various phenotypes before and after mild reduction of Lp(a). The Lp(a) from subject K. B. containing two apo(a) phenotypes (488 and 289 kDa) was separated into two Lp(a) species, each containing one of the two phenotypes (see Experimental Procedures), and then incubated with 2 mM DTE in the presence of 100 mM EACA. The resulting products were isolated at $d = 1.127$ g/mL in 30% sucrose and analyzed on Immobilon-P blots of 3.5% polyacrylamide SDS−PAGE gels followed by immunoblotting with monospecific anti-Lp(a) or anti-apoB100 antibodies and visualized by chemiluminescence. Panel A, anti-Lp(a) immunoblot: lane 1, parent Lp(a) containing the 488 kDa apo(a) phenotype complexed to apoB100; lane 2, sedimenting free apo(a) (488 kDa phenotype); lane 3, floating Lp(a−); lane 4, parent Lp(a) containing the 289 kDa apo(a) phenotype complexed to apoB100; lane 5, sedimenting free apo(a) (289 kDa phenotype); lane 6, floating Lp(a−); lanes 7 and 8, samples as in lanes 1 and 2 after reduction with 3% $\beta$-ME; lanes 9 and 10, samples as in lanes 4 and 5 after reduction with 3% $\beta$-ME. The positions of the reduced apo(a) isoforms are shown on the left. Panel B, anti-apoB100 immunoblot: lane 1, sedimenting free apo(a) as in lane 5 of panel A; lane 2, Lp(a) as in lane 4 of panel A; lane 3, floating Lp(a−) as in lane 6 of panel A. The position of apoB100 is shown on the right. Panel C, reducing gels (3% $\beta$-ME) of anti-Lp(a) immunoblots: lane 1, Lp(a) of subject B. K. with two apo(a) isoforms; lane 2, sedimenting apo(a) from subject B. K.; lane 3, Lp(a) of subject P. T. with two apo(a) isoforms; lane 4, sedimenting apo(a) from subject P. T. The positions of the 341 and 379 kDa isoforms from subjects B. K and P. T., respectively, are shown on the left.

The isolated apo(a) could be stored for at least 2 months at −80 °C in a 30% sucrose solution containing 100 mM EACA, without apparent changes in properties on the basis of CD analyses and lysine binding (see below). However, lysine binding decreased significantly (up to 40% in 2 weeks) when the isolated apo(a) was stored only in buffer A at −80 °C. On the basis of amino acid analyses, apo(a) contained

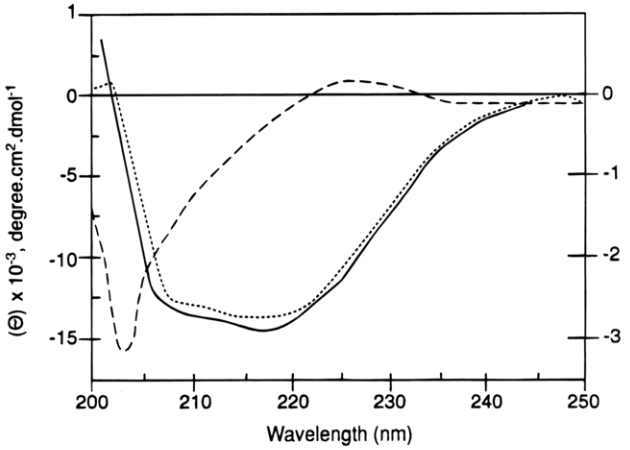


FIGURE 2: Far-ultraviolet circular dichroic spectra of apo(a), parent Lp(a) and RLp(a). Free apo(a) (− − −); parent Lp(a), (—) isolated from subject K. B. containing the 289 kDa apo(a) isoform; RLp-(a), (- - -) containing the 289 kDa apo(a) isoform and LDL from subject K. B. The right ordinate refers to the molar ellipticity of apo(a). All samples were equilibrated in 10 mM phosphate buffer, pH 7.5, before the spectra were recorded at 25 °C.

$3 \pm 2$ ($n = 7$) mol of cysteine per mol of protein (289 kDa isoform) as compared to $100 \pm 7$ ($n = 7$) for the fully reduced and alkylated product. The theoretical number of fully reduced cysteines was calculated to be 106. The isolated apo(a) examined by far-ultraviolet CD spectroscopy gave a spectrum (Figure 2) characterized by a strong negative band at 203 nm and a positive band at 222−232 nm. The analysis of the spectrum by the CONTIN and VARSLC1 methods indicated 0%−2% α-helix, 66% $\beta$ structure, and the remainder 32% in mainly random conformation. Although the CD deconvolution programs are mainly suited to globular proteins, our values agreed with those predicted for apo(a) by Guevara et al. (1992). Free apo(a) was bound to a lysine-Sepharose column and could be eluted from it with 200 mM EACA but not with PBS (Figure 3C), a behavior similar to that of the parent Lp(a) (Figure 3A).

*Reassembly Studies. i. Reassembly of Lp(a) from Free Apo(a) and LDL.* In order to limit the number of variables, we used LDL preparations that were homogeneous on non-denaturing GGE and banded in the density range 1.030−1.050 g/mL. The apo(a) size isoforms studied (Table 1) gave comparable results in terms of forming RLp(a). The yields were comparable among all the apo(a) isoforms except for the high molecular weight 488 kDa phenotype from subject K. B. which, on a molar basis, associated comparatively less efficiently with LDL (see below).

Western blots probed with anti-apo(a) and anti-apoB100 (Figure 4, lanes 3 and 5, respectively) showed that the band corresponding to RLp(a) contained both protein components even though the sample had been boiled in SDS prior to gel electrophoresis, suggesting a covalent association between apoB100 and apo(a). This was corroborated by the finding that samples reduced with 3% $\beta$-ME generated two bands corresponding to free apo(a) and apoB100 (Figure 4, lanes 9 and 11, respectively). The percent of apo(a) that was recovered in the RLp(a) was a function of both time of incubation and the initial apoB100:apo(a) molar ratios in the mixture. At a molar ratio of 50:1, 50% of the initial apo(a) mass became RLp(a) in the first hour (Figure 5A). The progressive increase in the reassembly process reached a plateau after 5 h, at which time 75% of apo(a) was associated with apoB100. At a molar ratio of 25:1 it took a longer

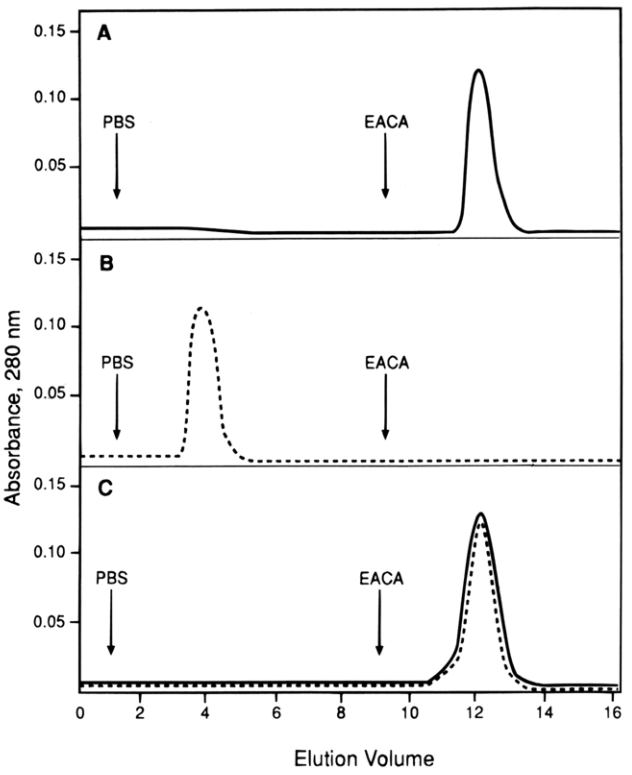


FIGURE 3: Lysine-Sepharose affinity chromatography of human wild-type and rhesus Lp(a) and their respective apo(a)s isolated from Lp(a) by reduction with 2 mM DTE. The dissociated apo(a), 0.3 mg, was isolated by sedimentation at $d = 1.127$ g/mL. (A) Human Lp(a) from subject P. T. (B) Rhesus Lp(a). (C) Human and rhesus apo(a) chromatographed separately on the same column but displayed here on the one graph. All the samples were dialyzed against buffer A and loaded onto 2 mL columns. The sample was applied at a flow rate of 7.8 mL/h. After being washed with four column volumes of PBS, the bound components were eluted with 200 mM EACA at a flow rate of 15 mL/h.

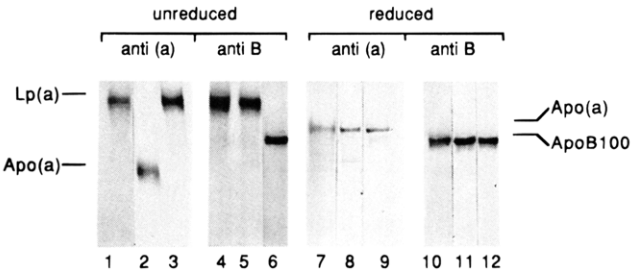


FIGURE 4: Western blots of Lp(a) reassembled from apo(a) and LDL. Apo(a) from subject D. G. was incubated with LDL (subject D. G.) at an apoB100:apo(a) molar ratio of 25:1 for 24 h at 37 °C, and the reassembled Lp(a) was separated by lysine-Sepharose chromatography and analyzed on Western blots of 3.5% polyacrylamide slab SDS−PAGE gels with antibodies to Lp(a) and apoB100. Lanes 1−3, anti-Lp(a) blots of unreduced gels of parent Lp(a), free apo(a), and RLp(a), respectively; lanes 4−6, anti-apoB100 blots of unreduced gels of parent Lp(a), RLp(a), and control LDL respectively; lanes 7−9, blots of reduced samples as in lanes 1−3; lanes 10−12, blots of reduced samples as in lanes 4−6. Markers for Lp(a) and apo(a) are shown on the left of the unreduced gels, and apo(a) and apoB100 are on the right of the reduced gels.

time to reach similar levels of reassembly (data not shown). Varying the initial apo(a) concentration (5.7, 11.4, 28.6, and 45.9 $\mu$g/mL) in a system with a 50:1 apoB100:apo(a) molar stoichiometry had no significant effect on Lp(a) reassembly. In terms of size polymorphism, the high molecular weight isoform (488 kDa) gave an Lp(a) reassembly efficiency that was 2-fold lower than that with the 289 kDa isoform.

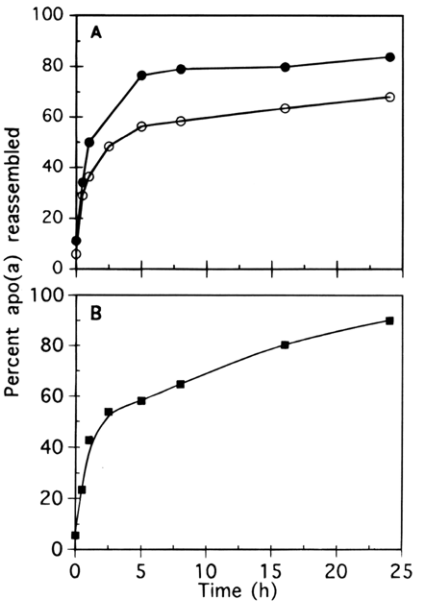


FIGURE 5: Effect of time of incubation on the reassembly of Lp(a) from apo(a) and LDL. Panel A, apo(a) from subject B. K. (●) or from rhesus (○) was incubated with LDL (subject B. K.) at an apoB100:apo(a) molar ratio of 50:1 for the designated time intervals at 37 °C. The reassembled products were separated by flotation at $d = 1.127$ g/mL and quantitated by ELISA. Panel B, apo(a) from subject B. K. incubated with VLDL (■) from a dyslipidemic subject under the same conditions. The results are presented as the percent of the total apo(a) mass in the reaction mixture that underwent reassembly.

Lysine and proline have been previously reported to inhibit the assembly of Lp(a) (Chiesa et al., 1992; Phillips et al., 1993; Trieu et al., 1991). In our system, incubation of apo(a) with either of the two reagents prior to incubation with LDL significantly inhibited Lp(a) reassembly. In each case, at 500 mM, a maximum inhibition of 90% was attained (data not shown). At concentrations equal to or below 200 mM, EACA appeared to be a relatively more potent inhibitor than proline (Table 2). On the premise that EACA and proline interact at different sites on apo(a) (Trieu et al., 1991), we incubated apo(a) with 100 mM each of EACA and proline prior to incubation with LDL. Under these conditions we obtained a 2-fold higher inhibition of the Lp(a) reassembly, as compared to the incubation with a single inhibitor (Table 2). Moreover, when apo(a) was fully reduced with 50 mM DTE, dialyzed, and incubated with LDL, less than 5% of the total apo(a) was reassembled. In addition, when the fully reduced apo(a) was alkylated and applied to a lysine-Sepharose column, all of the apo(a) was recovered in the unbound flow-through fraction (data not shown).

*ii. Properties of Lp(a) Reassembled from Apo(a) and LDL.* When apo(a) was incubated with LDL at an apoB100:apo(a) molar ratio of 25:1 for 6 h, and the mixture was fractionated by isopycnic density gradient ultracentrifugation, we found that RLp(a) banded in the same density position as the parent Lp(a). Regardless of the procedure of isolation, RLp(a) bound to lysine-Sepharose and was eluted from it with 200 mM EACA in a manner comparable to native Lp(a). By electron microscopy (Figure 6), the RLp(a) particles were slightly heterogeneous with an average diameter of 27.9 ± 4.0 nm ($n = 115$), which is very similar to that of their native Lp(a) counterparts (average diameter, 28.4 ± 4.1 nm ($n = 115$). The LDL utilized for the reassembly showed a similar heterogeneity with an average diameter of 27.5 ± 3.5 nm ($n = 130$). On GGE, RLp(a) had the same mobility as control

Table 2: Effect of EACA and Proline on the *in Vitro* Reassembly of Lp(a) from Human Wild-Type and Rhesus Monkey Apo(a) and Human LDL[a]

| | |
|---|---|
| human apo(a) + LDL | 100[b] |
| human apo(a) + LDL + EACA | [% ± x% (SD)] |
| preincubation[c] | 18.8 ± 5.4 |
| postincubation[d] | 88.5 ± 15.8 |
| human apo(a) + LDL + proline | |
| preincubation | 28.7 ± 6.3 |
| postincubation | 93.5 ± 10.3 |
| human apo(a) + LDL + proline + EACA[e] | 10.0 ± 2.0 |
| rhesus apo(a) + LDL | 100[b] |
| rhesus apo(a) + LDL + EACA | [% ± x% (SD)] |
| preincubation[c] | 15.0 ± 5.3 |
| postincubation[d] | 86.2 ± 16.2 |
| rhesus apo(a) + LDL + proline | |
| preincubation | 30.3 ± 7.1 |
| postincubation | 94.7 ± 9.3 |
| rhesus apo(a) + LDL + proline + EACA[e] | 10.2 ± 3.1 |

[a] All incubations were performed using human or rhesus apo(a) and human LDL at an apoB100:apo(a) molar ratio of 50:1 for 5 h at 37 °C. After centrifugation overnight in sucrose at $d = 1.127$ g/mL, the floating fraction was analyzed for the presence of apoB100:apo(a) complexes by ELISA as described in Experimental Procedures. [b] For ease of comparison, the fraction of the total apo(a) covalently linked to apoB100 was given a value of 100. All of the percentages were calculated relative to this value and were the results of five experiments. [c] EACA or proline (100 mM) was preincubated with apo(a) for 1 h at 37 °C before incubation with LDL. [d] EACA or proline (100 mM) was added after the complex was formed and incubated for 1 h. [e] Both EACA and proline, each at 100 mM, were preincubated with apo(a) for 1 h at 37 °C before interaction with LDL.

Lp(a) indicating that both had a similar size or Stokes's radius. Moreover, as measured by circular dichroism, the conformation of RLp(a) closely resembled that of native Lp(a) (Figure 2). The spectra of both lipoproteins were characterized by negative bands at 218 and 210 nm. Secondary structure calculations performed using the programs VARSLC1 and CONTIN gave 24% α-helix, 29% β-sheet, and 47% random structure for RLp(a), and 23% α-helix, 31% β-sheet, and 46% random structure for Lp(a). The chemical composition of the RLp(a) was also comparable to that of the parent Lp(a) and had the same lipid composition as the LDL preparation used in the reassembly system (Table 3). On the basis of ELISA analysis of apoB100 and the apo(a):apoB100 complex in RLp(a), the molar stoichiometry of apoB100:apo(a) was calculated to be 1:1.

We also assessed the effect of EACA and proline on the stability of RLp(a). After RLp(a) was formed, it was incubated with 100 mM of either EACA or proline for 1 h at room temperature. The mixture was then centrifuged at $d = 1.127$ g/mL for 18 h, and the floating fraction containing the apoB100:apo(a) quantified by ELISA. Addition of either EACA or proline caused some apo(a) to dissociate [10%–12% of the total RLp(a)] (see Table 2), indicating that a small portion of apo(a) complexed with apoB100 through noncovalent interactions and copurified with the stable disulfide-linked complex. On the other hand, when 2 mM DTE was added to RLp(a) there was an almost complete dissociation of apo(a) from apoB100.

*Reassembly of Lp(a) from Apo(a) and VLDL.* We have previously shown that apo(a) linked to apoB100 can be found in triglyceride-rich particles isolated from hyperlipidemic plasma (Scanu et al., 1992). In the current study we examined the *in vitro* interaction of apo(a) isolated from an Lp(a) having a single apo(a) isoform (289 kDa) with preparations of VLDL isolated from the plasma of two hypertriglyceridemic subjects (R. W. and R. Z.) with type IV dyslipoproteinemia and very low plasma levels of Lp(a) protein, i.e., 0.1 and 0.3 mg/dL, respectively. The experimental conditions for the reassembly were as described for the LDL experiments using a 50:1 apoB100:apo(a) molar ratio. In the early phase (up to 2.5 h) the reassembly process between apo(a) and VLDL followed a course (Figure 5B) that was similar to that observed between apo(a) and LDL (Figure 5A). However, VLDL required 13 h to reach the 75% reassembly level, which in the case of LDL was achieved in 5 h. As in the case of LDL, the reassembly of apo(a) with VLDL was inhibited by 100 mM EACA or proline [14% and 23% of apo(a) reassembled, respectively; compare with data in Table 2]. Moreover, like the reassembled product from apo(a) and LDL, the one obtained from the interaction between apo(a) and VLDL was only dissociable by 2 mM DTE, indicating that a disulfide linkage had been formed.

*Properties of Human Mutant Apo(a) and RLp(a).* In spite of the fact that subject T. T. had very low plasma levels of Lp(a) (0.16 mg/dL, Table 1), we were able to obtain by plasmapheresis sufficient amounts of plasma to isolate Lp(a) and prepare apo(a) in a 200 μg yield. As with the human wild-type, the mutant free apo(a) was water-soluble and stable when stored in 30% sucrose and 100 mM EACA at −80 °C. In terms of binding to lysine-Sepharose, parent Lp(a) was $Lys^-$, (Scanu et al., 1993) and thus eluted in the flow-through volume. In contrast, free apo(a) bound effectively to the column and was eluted specifically with EACA (data not shown) similar to Rhesus apo(a) (see below). Incubation of apo(a) with either autologous or wild-type LDL at an apoB100:apo(a) molar ratio of 50:1 for 5 h at 37 °C caused 60% of the initial apo(a) to become complexed to LDL, as assessed by ELISA. Western blot analyses of the fraction floating in sucrose at $d = 1.127$ g/mL showed that the apoB100:apo(a) complex was sensitive to reduction by β-ME (Figure 7, lanes 2 and 5). As with the wild-type, both EACA and proline prevented reassembly between mutant apo(a) and LDL.

*Properties of Rhesus Apo(a) and RLp(a).* Rhesus Lp(a), after reduction with 2 mM DTE yielded an apo(a) that was water-soluble and exhibited properties that were very similar to the human mutant apo(a). By chromatography on lysine-Sepharose, rhesus Lp(a), due to the Trp72→Arg substitution in apo(a) kringle IV-10, was incompetent to bind and was eluted in the flow-through PBS buffer (Figure 3B). On the other hand, apo(a), once freed of its LDL moiety, bound tightly to the column and could only be eluted with EACA (Figure 3C). Rhesus apo(a), at an apoB100:apo(a) molar ratio of 50:1, also bound equally to either autologous LDL or rhesus Lp(a−) or human wild-type LDL. The reaction between rhesus apo(a) and human LDL followed a time course of reassembly (Figure 5A) similar to that of the wild-type human apo(a), reaching a maximum of about 60% in 5 h. Western blot analyses of the reassembled fraction which was contained in the $d = 1.127$ g/mL floating fraction, showed that an apoB100:apo(a) complex was formed and only dissociated in the presence of β-ME (Figure 7, lanes 3 and 6). EACA and proline inhibited the reassembly, although inhibition by either of these reagents was slightly more efficient than in the human model (Table 2).

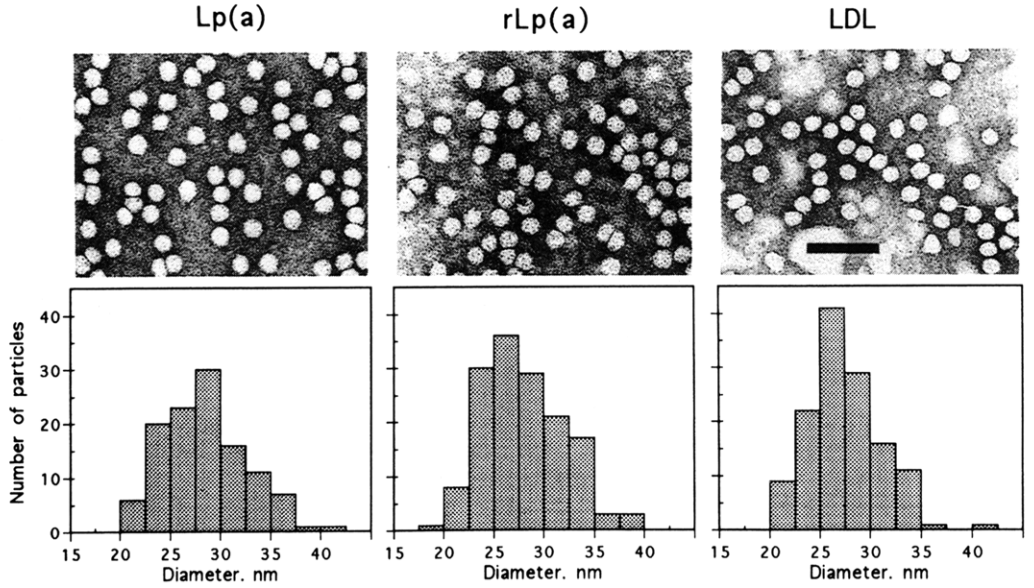


FIGURE 6: Electron micrographs of human Lp(a), RLp(a), and LDL. All samples were obtained from subject K. B. with the 289 kDa apo(a) phenotype at a concentration of 50 $\mu$g/mL in 10 mM $NH_4HCO_3$. Top panel, control Lp(a), RLp(a) isolated by lysine-Sepharose chromatography, and control LDL, respectively. The electron micrographs were taken at an instrumental magnification of 46 000. The bar indicates 100 nm. Bottom panel, bar graph of the size distribution of particles for each corresponding sample.

Table 3: Chemical Composition of RLp(a) and Control Lipoproteins[a,b]

| sample | protein | PL | CE | FC | TG |
|---|---|---|---|---|---|
| RLp(a) | 26.0 ± 1.5 | 17.2 ± 2.0 (23.2) | 41.6 ± 2.3 (56.2) | 8.8 ± 1.4 (12.0) | 6.4 ± 1.3 (8.6) |
| Lp(a) | 26.9 ± 1.7 | 14.6 ± 1.6 (20.1) | 42.9 ± 2.0 (58.6) | 11.0 ± 1.2 (15.0) | 4.6 ± 0.8 (6.3) |
| LDL | 23.0 ± 1.3 | 17.6 ± 1.7 (22.9) | 44.5 ± 1.8 (57.7) | 10.3 ± 1.1 (13.3) | 4.6 ± 0.5 (6.1) |

[a] Lp(a) was isolated from plasma and utilized for the preparation of apo(a). The latter and LDL were used to prepare RLp(a). Apo(a), Lp(a), and LDL were obtained from subject K. B. with the 289 kDa phenotype. The percent was calculated from four experiments. [b] Results shown as $\%x \pm \%y$ (SD). Values in parentheses correspond to the lipid distribution only.

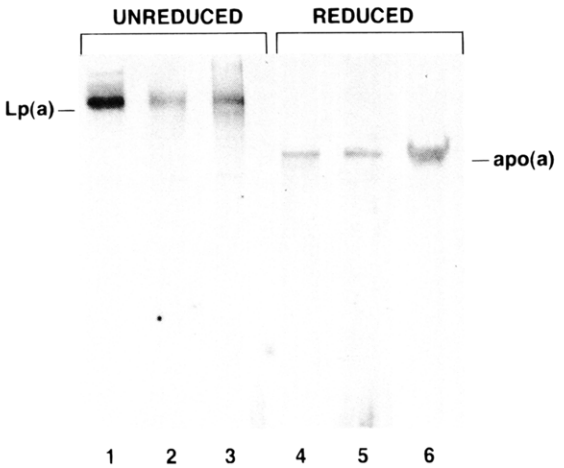


FIGURE 7: Western blots of human mutant and rhesus Lp(a) reassembled from their respective apo(a)s and human LDL. Human mutant apo(a) from subject T. T. or rhesus apo(a) was incubated with human LDL (subject K. B.) at an apoB100:apo(a) molar ratio of 50:1 for 5 h at 37 °C, and the reassembled Lp(a) was separated by ultracentrifugal flotation in 30% sucrose in the presence of 100 mM EACA, $d = 1.127$ g/mL. The floating fractions were analyzed on Western blots of 3.5% polyacrylamide slab SDS−PAGE gels and probed with Lp(a) antibodies. Lanes 1−3, unreduced gels of control human wild-type Lp(a) from subject K. B., human mutant RLp(a), and rhesus RLp(a), respectively; lanes 4−6, reduced gels as in lanes 1−3. Markers for Lp(a) are shown on the left of the unreduced gels and for apo(a) on the right of the reduced gels.

## DISCUSSION

The main goal of our study was to explore the possible relationship between the LBS function of apo(a) kringle IV-10 and Lp(a) assembly. For this purpose we developed a novel method for isolating wild-type and mutant apo(a) phenotypes in a free form by subjecting each parent Lp(a) to mild reductive conditions using 2 mM DTE and 100 mM of the lysine analogue, EACA. This procedure caused the cleavage of the interchain disulfide between one of the unpaired cysteines (Cys-3734, -4190, -4326) of apoB100 and the unpaired Cys-4057 in apo(a) kringle IV-9 without an apparent disruption of the intrachain disulfides in the kringles of apo(a). By amino acid analyses, the apo(a) freed from Lp(a) had three cysteine residues instead of the single one which would have been expected if the mild reductive conditions had acted only on the interchain disulfide between apo(a) and apoB100. We believe that the "extra" two cysteines, rather than from kringles, might have derived from one of the six disulfides located in the protease region of apo(a), on the assumption that this region represents a comparatively more open structure than that comprising the kringles (Scanu & Edelstein, 1995) and, thus, is potentially more amenable to disulfide cleavage.

The isolation of a water-soluble, functionally competent apo(a) is an important development particularly when one considers that essentially all apo(a) in the plasma is covalently linked to apoB100 and that much of the current information on apo(a) has been derived from results on recombinant products which may not necessarily reflect the properties of "native" apo(a). This development was dependent on various factors: (1) the use of very low concentrations of DTE; (2) the presence in the reaction mixture of EACA for the purpose of inhibiting the reassociation between apo(a) and LDL, and (3) at the end of the reaction, the use of sucrose in order to achieve the necessary medium density for separating apo(a) from the LDL moiety by ultracentrifugal flotation. Sucrose had to be used because in its absence apo(a) came out of solution at the NaCl

concentrations required to float LDL and any unreacted Lp(a). Moreover, sucrose proved to be a good stabilizing factor in storing free apo(a) at −80 °C. The ability to prepare relatively large amounts of free apo(a) of a defined phenotype provides a powerful tool for gaining a broader knowledge on the structural properties of this unique glycoprotein and for defining, on a physiological level, its role in the process of Lp(a) assembly.

An important finding in our studies was that regardless of the functional state of the LBS of kringle IV-10 (i.e., $Lys^+$ and $Lys^-$) of the parent Lp(a), all free apo(a)s bound specifically to lysine-Sepharose in that they could only be eluted by the lysine analogue, EACA. This indicates that the detachment from apoB100, opened in apo(a) a second domain which contained sites for both lysine and proline normally buried in native Lp(a). These sites may either be independent from each other or, more likely, act in a cooperative way via the interkringle linkers. This hypothesis is based on the finding that preincubation of the free apo(a)−LDL mixture in the presence of equimolar concentrations (100 mM each) of EACA and proline resulted in a 2-fold decrease in Lp(a) reassembly compared to the experiments in which each inhibitor was used alone.

The effect of proline on the interaction between apo(a) and apoB100 was first recognized by Trieu et al. (1991) and in a baboon cell system by White and Lanford, (1994). From the modeling studies of Guevara et al. (1993), we may suggest that kringle IV-4 and/or kringle IV-9, containing a hydrophobic pocket may be able to accommodate bulky hydrophobic amino acid residues such as proline. On the other hand, Ernst et al. (1995), by studying human apo(a) mutant constructs expressed in HepG2 cells, identified a site spanning kringle IV-5 through IV-9 which was believed to be involved in interaction between apo(a) and apoB100. The assignment of this site, however, must be viewed as tentative in that the constructs used in the reassembly experiments were not purified from the cell medium, the yields of reassembled Lp(a) were based solely on immunoblot data, and the properties of the reassembled products were not provided. Similarly, by incubating LDL with the media from cos-7 cells transfected with apo(a) constructs, Frank et al. (1994a,b) concluded that apo(a) kringle IV-6 is required for Lp(a) reassembly. Moreover, Trieu and McConathy, (1995), utilizing apo(a) constructs partially purified from the media of transfected CHO cells, suggested that both kringle IV-6 and IV-7 can sustain Lp(a) reassembly. It should be noted that none of the cell studies listed above ruled out the possibility that other factor(s) in the cell medium might have influenced the interaction between each apo(a) construct and LDL. On the other hand, our current studies, which were based on naturally occurring products from human and rhesus monkey sources, are the first ones to demonstrate that, in their pure form, apo(a) and LDL are competent by themselves to sustain the formation of Lp(a). However, the reformation of the disulfide bond between apo(a) and apoB100 appeared to be relatively inefficient, in that the process required a large molar excess of LDL over apo(a). At present, we cannot determine whether our experimental conditions were optimal for the reassembly process or whether there are factors *in vivo* that may facilitate a rapid reassembly of Lp(a).

Our studies have established that a functional LBS in kringle IV-10 is not directly responsible for Lp(a) reassembly, in that apo(a)s obtained from either the human Lp(a) mutant or rhesus monkey Lp(a), both $Lys^-$, were as efficient as wild-type human apo(a) in forming an Lp(a) complex. Moreover, the disassembly behavior of the two $Lys^-$ species was comparable to that of native $Lys^+$ Lp(a). We wish to note that the lack of participation of apo(a) kringle IV-10 in Lp(a) reassembly has been suggested by Ernst et al. (1995) from the study of apo(a) recombinants. Thus, the lys/pro binding domain appears to play a dominant role in Lp(a) assembly (see Figure 8). This would explain why human subjects and rhesus monkeys with a functionally defective LBS in kringle IV-10 are competent to form Lp(a). At this time we have little knowledge about the structural properties of the lys/pro domain except that the collective evidence suggests that it probably spans the apo(a) region between kringle IV-4 and kringle IV-9 including the interkringle linkers. This would be in keeping with the finding that the kringle IV-2 repeats have no affinity for lysine (Li et al., 1992) and are unable to sustain Lp(a) assembly (Ernst et al., 1995; Frank et al., 1994a,b).

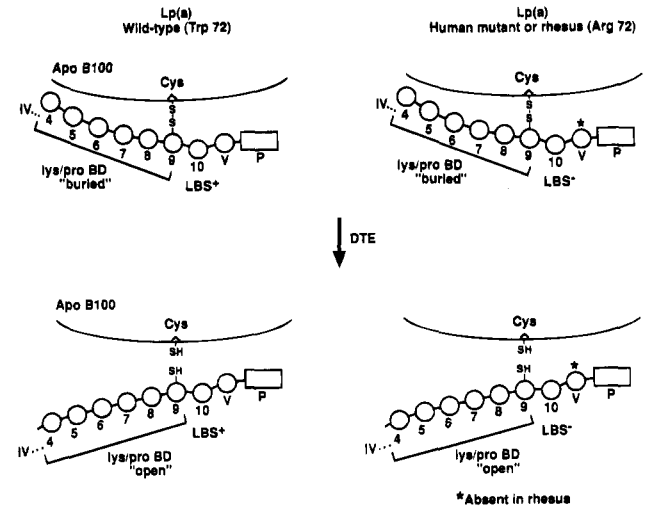


FIGURE 8: Models of Lp(a) assembly for Trp72 and Arg72 apo(a). Kringles IV-4 to IV-10 and kringle V representing the COOH terminal domain of apo(a) are drawn as circles, and the protease region (P) is drawn as a rectangle. The interchain disulfide between kringle IV-9 and apoB100 is depicted as present in both the wild-type and mutant apo(a). When the disulfide bridge is intact, lysine binding of Lp(a) occurs via the LBS of kringle IV-10. Once the disulfide is cleaved by the action of DTE, the apo(a) domain between kringles IV-4 and IV-10 interacts with proline and lysine, a property not exhibited by apo(a) bound to apoB100.

Our current *in vitro* studies have also shown that the apoB100 of VLDL readily affiliates with apo(a) to form a stable complex that can only be dissociated under reducing conditions. This observation establishes that VLDL is competent to form a covalent complex with apo(a) and corroborates the results of previous studies demonstrating the occurrence in human plasma of apo(a) linked to triglyceride-rich lipoprotein particles (Bersot et al., 1986; Scanu et al., 1992; Selinger et al., 1993). These observations also support the notion (Scanu, 1990) that Lp(a) represents a broad class of lipoprotein particles, both cholesteryl ester and triglyceride-rich having as a protein moiety apoB100 linked to apo(a).

One of the attempts of the current studies was to determine whether a reassembly defect could account for the very low plasma levels of Lp(a) present in the human mutant with kringle IV-10 Arg72 in contrast to the normal levels exhibited by the rhesus monkeys with the same substitution. Behind this hypothesis was the assumption that free apo(a) incompetent to affiliate with the apoB100 of LDL, would be cleared

from the plasma at a comparatively higher rate than Lp(a). However, this hypothesis was not supported by the experimental findings. First, the apo(a) present in the plasma of the human mutant was in the form of Lp(a). Second, the free apo(a), obtained by the mild reduction of Lp(a) from either the human mutant or the rhesus monkey, when mixed with either human or rhesus LDL formed an Lp(a) complex with comparable kinetics. Third, the LDL obtained by the reduction of either the human mutant or rhesus Lp(a) was able to restore an Lp(a) complex when mixed with wild-type free apo(a). These results suggest that the low plasma levels of Lp(a) observed in the human mutant may depend not on a reassembly process but on defective production and/or secretion of apo(a) and that the difference in plasma levels of Lp(a) between human mutant and rhesus monkeys may be attributable to the divergent structural properties demonstrated to exist between the two apo(a) species (Tomlinson et al., 1989).

In summary, we have shown that apo(a) can be readily dissociated from Lp(a) *in vitro* and that the resulting native apo(a) has the capacity to covalently link again with apoB100 to reconstitute either CE-rich or TG-rich lipoproteins. The domain in apo(a) involved in the reassembly process is spatially removed from kringle IV-10 which is responsible for the binding of Lp(a) to lysine-Sepharose. These *in vitro* findings, which are based on naturally occurring products, clearly support and extend the notion that Lp(a) particles can be formed extracellularly although do not rule out an intracellular event. Moreover, the readiness whereby apo(a) is released from Lp(a) suggests that the production of apo(a) can occur *in vivo* at sites where reductive conditions prevail. At this time, we do not know how the functional properties of Lp(a) (i.e., fibrin binding, plasminogen receptor interactions, etc.) compare to those of apo(a). However, we believe that acquisition of such knowledge is important for the understanding of the molecular basis for the atherothrombotic potential of Lp(a).

## ACKNOWLEDGMENT

We gratefully acknowledge Ms. Yimei Chen and Dr. Robert Josephs of the Electron Microscopy and Image Processing Laboratory of the Department of Biochemistry and Molecular Biology, University of Chicago, IL, for helping in the electron microscopy analyses, Janet Hinman for the ELISA assays and Core B of Program Project HL 18577 for the preparation of Lp(a). We also thank Dr. Olga Klezovich for constructive comments during the course of this work.

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BI951669Z