Isolation of apolipoprotein(a) from lipoprotein(a)

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Abstract An easy method was developed for the rapid and selective isolation of apo(a) from human plasma Lp(a). This procedure was applied to a "low density" Lp(a) subspecies (usually found in the density interval 1.050 to 1.070 g/ml) from a single individual whose apo(a) was of a size smaller than apoB-100. After reduction with 0.01 M dithiothreitol, apo(a) was separated from the Lp(a) particle by rate zonal centrifugation on a 7.5–30% NaBr density gradient. Two completely water-soluble products were recovered: apo(a), which contained less than 1% each of phospholipid and cholesterol, remained at the bottom of the gradient, and a lipid-rich floating LDL-like particle which contained apoB but not apo(a) and which we referred to as Lp(a−). The separation of these two components was also achieved by subjecting reduced Lp(a) to electrophoresis on 2.5–16% polyacrylamide gradient gels. However, dissociation of reduced Lp(a) could not be achieved by gel filtration in either low or high salt solutions. These observations indicate that apo(a) is associated to Lp(a) by non-covalent interactions in addition to its disulfide linkage to apoB. The latter is sensitive to chemical reduction whereas the former are broken through the action of a gravitational or electrical field. —**Fless, G. M., M. E. ZumMallen, and A. M. Scanu.** Isolation of apolipoprotein(a) from lipoprotein(a). *J. Lipid Res.* 1985. **26:** 1224–1229.

Supplementary key words Lp(a) • apo(a) • rate zonal density gradient centrifugation • gradient gel electrophoresis • "Western" immunoblotting • gel filtration • self-association • chemical reduction

Lipoprotein(a) is a particle of unusual structure consisting of apoprotein(a) which is linked to apoB of an LDL-like particle through disulfide bonds (1–3). We previously determined that the surface arrangement of the protein moiety of Lp(a) is different from that of LDL and postulated that apoB alone may be sufficient to stabilize the hydrophobic lipid constituents of the Lp(a) particle, whereas apo(a) may interact more with apoB and the aqueous environment than with the lipid surface (3). This suggested that, with the exception of its covalent attachment through disulfide bonds, apo(a) may be loosely bound to Lp(a). We also reported that apo(a) may be dissociated from Lp(a) by centrifugation after reduction with 2-mercaptoethanol in the presence of 6 M guanidine hydrochloride, whereas apoB remained with the lipoprotein particle (3). Based on these findings and the attending assumptions, we wanted to devise a method for isolating apo(a) from the Lp(a) complex as a starting point in the investigation of the structural organization of Lp(a). The description of such a procedure is the purpose of this report. A preliminary communication of this work has been presented (4).

METHODS

Isolation of Lp(a)

Lp(a) was prepared from the plasma of a female donor (LC) as previously described (3). This subject exhibited a "low density" Lp(a) whose apo(a) was of a size smaller than apoB-100 and usually located in the density interval of 1.050–1.070 g/ml (3). Lipoproteins were floated by adjusting plasma that was 1 mM in DFP, 0.15% Na_2 EDTA, and 0.01% NaN_3 to d 1.21 g/ml with solid NaBr and spinning it at 49,000 rpm for 20 hr at 15°C in the 50.2 Ti rotor (Beckman, Palo Alto, CA). The floated lipoproteins were aspirated and adjusted with solid NaBr to d 1.4 g/ml before rate zonal centrifugation. Typically, 5 ml of the material was layered under a linear 0–30% NaBr gradient and spun at 59,000 rpm for 1.75 hr at 20°C in a 60 Ti rotor (Beckman). After fractionation, the pooled Lp(a) and LDL were dialyzed against 100–200 volumes of 0.01% Na_2 EDTA and NaN_3, pH 7.0. The Lp(a)-containing fraction was then adjusted with solid CsCl to obtain a 7.5% (w/w) solution and subjected to density gradient centrifugation in a 50.2 Ti rotor at 49,000 rpm for 20 hr at 20°C. Samples of Lp(a) obtained from fractionation were dialyzed against 0.15 M NaCl, 0.01% Na_2 EDTA, and NaN_3, and checked for purity by polyacrylamide gradient gel electrophoresis (3). Lp(a) contained less than

Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a) which is crosslinked to apoB of Lp(a) through disulfide bonds; Lp(a−), remnant lipoprotein particle obtained upon the reduction and subsequent removal of apo(a) from Lp(a); VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VHDL, very high density lipoprotein; DTT, dithiothreitol; DFP, diisopropyl fluorophosphate; Na_2 EDTA, sodium salt of ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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1% LDL as determined by gel scanning at 590 nm using the Varian DMS-90 spectrophotometer (Varian, Palo Alto, CA) and was not further purified by chromatofocusing. Whenever possible throughout the isolation procedure, all sample solutions were kept under a nitrogen atmosphere and only freshly prepared Lp(a) was used for preparing apo(a).

Isolation of apo(a)

Lp(a) obtained as described, usually at a concentration of approximately 2 mg/ml protein, was made 0.01 M with respect to DTT by adding 1 M DTT directly to the sample. Complete reduction of Lp(a) by 0.01 M DTT was achieved by incubation either at 37°C for 15 min, or at room temperature for 1 hr. Lp(a) was then dialyzed against a deaerated solution consisting of 35% NaBr, 0.01 M DTT (dialysate adjusted w/solid NaBr to account for sample volume). Dialysis was continued for 3–6 hr at room temperature, resulting in a twofold concentration of the original sample. Two ml or less of this adjusted Lp(a) solution was layered under a linear 7.5–30% NaBr gradient and spun for 16 hr at 17,000 rpm and 20°C in an SW-40 rotor (Beckman). The gradient was fractionated and monitored by pumping out at 1 ml/min through an ISCO UA-5 density gradient monitor set at 280 nm. Fractions containing apo(a) were pooled and dialyzed against 0.20 mM N-ethylmorpholine, pH 7.0, while fractions containing Lp(a−) were pooled and dialyzed against 0.15 M NaCl, 0.01% Na_2 EDTA, and NaN_3, pH 7.0.

Electrophoretic methods

Gradient gel electrophoresis either in the presence or absence of SDS on 2.5–16% polyacrylamide gels (Isolab) was carried out according to the methods outlined by Pharmacia and described by us previously (3). It should be noted that, when reducing Lp(a) prior to examination by SDS-PAGE, it is important that the sample be incubated with reducing agent prior to addition of SDS. Using either 2-mercaptoethanol or DTT, the time required for complete reduction increased fourfold, from 15 min to 1 hr, in the presence of SDS. Gels were scanned at 590 nm when stained with Coomassie blue G-250 using the Varian DMS-90 spectrophotometer (Varian, Palo Alto, CA). High molecular weight protein standards for SDS-PAGE were purchased from Bio-Rad (Richmond, CA). Thyroglobulin, catalase, ferritin, and bovine serum albumin were purchased from Sigma (St. Louis, MO).

Immunological methods

Lipoproteins were transferred from gradient gels to nitrocellulose film using the Bio-Rad transblot cell according to the method of Towbin, Staehelin, and Gordon (5). Transfer was done at 50 V, 1.5 A, overnight with cooling. Antigens were identified with a double antibody technique involving IgG coupled to horseradish peroxidase (Miles) using 4-chloronaphthol as the chromogen (6). For immunodot assay of LDL- and Lp(a)-containing fractions, 1-μl aliquots were applied directly to nitrocellulose and identified with a double antibody technique as above.

Antisera

Antisera to Lp(a) or LDL were prepared as previously described (3).

Chemical analyses

Protein content was determined by the method of Lowry et al. using bovine serum albumin as standard (7). Phosphorus was measured according to Bartlett (8). The phospholipid concentration (as lecithin) was obtained by multiplying the phosphorus concentration by a factor of 25. Total cholesterol was determined as previously described (3).

RESULTS

Reduction of Lp(a) with 0.01 M DTT produced a modified particle that was stable in solution and did not undergo visible aggregation. Upon rate zonal centrifugation in the SW-40 rotor (17,000 rpm, 16 hr, 20°C), the reduced Lp(a) dissociated into two water soluble products: apo(a), which was found in the d 1.35 mg/ml bottom fraction and a floating apoB-containing lipoprotein that we call Lp(a−). Phosphorus analysis indicated that only 0.92% phospholipid (two determinations) and 0.7% cholesterol (one determination) were associated with the purified apo(a) which indicated that apo(a) was almost lipid free. When unreduced Lp(a) was subjected to rate zonal centrifugation under conditions identical to those for reduced Lp(a), only a single band was observed whose position on the gradient was different from that of Lp(a−) because of a slower flotation rate (**Fig. 1a**). LDL similarly treated showed no change in flotation rate upon reduction, but its position on the gradient was intermediate between Lp(a) and Lp(a−) (Fig. 1b). Unreduced Lp(a) and reduced and unreduced LDL retained the full complement of protein following ultracentrifugation. Protein analysis of the fractions obtained from these three gradients indicated that of the total protein recovered less than 1% was present in the bottom of the tubes (fractions 20–24). The increased absorbance toward the bottom of the gradient that contained reduced LDL was caused solely by the presence of DTT.

The speed and time of centrifugation that we chose in the experiment to separate apo(a) from Lp(a) were dictated by the convenience of an overnight run (Fig. 1). However, we achieved equally good separations by using the same density gradient system with other combinations at 20°C such as 35,000 rpm for 3.5 hr in the SW-40 rotor, or 60 min at 59,000 rpm in the 60 Ti rotor. The latter

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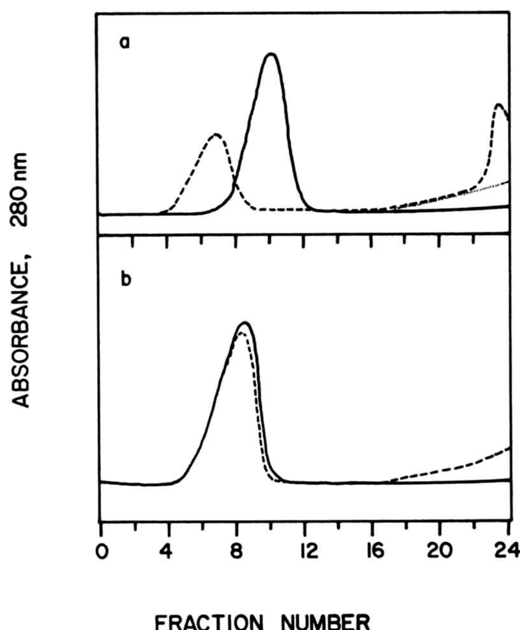

Fig. 1. Dissociation of apo(a) from reduced Lp(a) by rate zonal density gradient centrifugation. (a) Native unreduced Lp(a) (—); reduced Lp(a) (---); dithiothreitol control (· · · ·). (b) Native unreduced LDL (—); reduced LDL (---). Reduced or unreduced lipoprotein samples in 35% NaBr, 0.01% Na_2 EDTA were layered under a linear 7.5-30% NaBr gradient and centrifuged in an SW-40 rotor at 17,000 rpm for 16 hr at 20°C. Each gradient was pumped out at 1 ml/min and monitored at 280 nm.

rotor is more convenient for large batches because a 5-ml sample per tube can be used as opposed to a 2-ml sample in the SW-40 rotor. Another alternative is to use a fixed density such as 1.08 g/ml which is intermediate to the density of apo(a) and Lp(a–) in combination with a fixed-angle rotor. We chose not to use the latter method because of the tendency of all components to pile up either at the top or the bottom of the centrifuge tube. A major advantage of rate zonal density gradient centrifugation was its capacity to separate as well as identify Lp(a), Lp(a–), LDL, and apo(a) by their positions on the gradient (Fig. 1).

Lp(a), Lp(a–), and apo(a) obtained by rate zonal centrifugation were examined by gradient gel electrophoresis on 2.5–16% polyacrylamide gels in the absence of SDS and subsequently by Western immunoblotting (**Fig. 2**). Lp(a–) migrated further into the gel than Lp(a) indicating that, with the loss of apo(a), Lp(a) had decreased in size. Two or more bands were observed with apo(a) depending on the preparation (Figs. 2 and 4) which upon Western immunoblotting reacted with anti-Lp(a) but not with anti-LDL (Fig. 2, lane 6). The Lp(a–) particles generated from the reduction of Lp(a) reacted only with anti-LDL, indicating that the loss of apo(a) was complete. In turn, unreduced Lp(a) reacted with both anti-Lp(a) and anti-LDL (Fig. 2).

By SDS gradient gel (2.5–16%) electrophoresis, isolated Lp(a–) and apo(a) each exhibited a single band whose respective positions coincided with the two bands observed with reduced Lp(a) (**Fig. 3**). These two bands were shown previously to correspond to apoB-100 and apo(a) (3). In order to assess whether apo(a) could be removed from reduced Lp(a) by a force other than gravitation, we subjected native, non-delipidated Lp(a) treated with 0.01 M DTT to gradient gel electrophoresis in the absence of SDS. In **Fig. 4** the comparison between native and reduced Lp(a) clearly shows the transformation of Lp(a) into Lp(a–) and apo(a). The latter had a size approximately equal to that of thyroglobulin [85.0 Å diameter (9)]. Lp(a–) had a mobility similar to LDL.

When a sample of Lp(a) reduced with 0.01 M DTT was applied to a Bio-Gel A-5 m column equilibrated with 0.001 M DTT in 0.15 M NaCl, only a single peak was obtained whose elution volume, as compared to that of Lp(a–) or apo(a), indicated a particle of larger size (**Fig. 5**). All fractions across this peak when checked by immunodot assay reacted with both anti-Lp(a) and anti-LDL. This indicated that, in spite of the cleavage of the disulfide bonds by reduction, apo(a) was still associated with Lp(a). To assess the possible role of high concentrations of salt in the dissociation, gel filtration was also carried out in 35% NaBr. Only one peak was obtained which had a K_d similar to Lp(a) run in the absence of salt. Again, all fractions across the peak reacted with both anti-Lp(a) and anti-LDL.

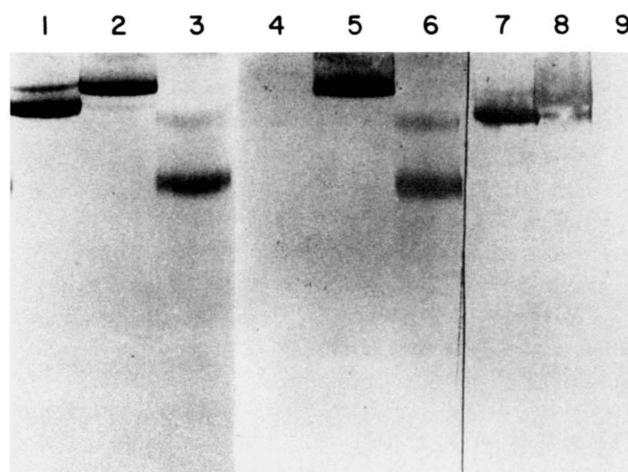

Fig. 2. Characterization of Lp(a), Lp(a–), and apo(a) by gradient gel electrophoresis on 2.5–16% gels and subsequent "Western" immunoblotting. The gel was electrophoresed in the absence of SDS at a constant voltage of 150 volts for 16 hr at 15°C. All three samples were obtained from the rate zonal centrifugation shown in Fig. 1a. Lanes 1, 4, and 7 contain Lp(a–); lanes 2, 5, and 8, Lp(a), and lanes 3, 6, and 9, apo(a). The gradient gel after staining with Coomassie blue G-250 is pictured in lanes 1–3. Gels identical to lanes 1–3 were subjected to "Western" blotting using either anti Lp(a) (lanes 4–6) or anti LDL (lanes 7–9). Sample load per lane was 20 μg of protein.

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In contrast to Lp(a) and Lp(a−), the elution profile of apo(a) indicated heterogeneity (Fig. 5). We therefore examined three fractions, corresponding to the elution volumes of 55, 60, and 65 ml, by gradient gel electrophoresis in the absence of SDS and Western blotting against anti-Lp(a). These results indicated that the first fraction consisted completely of higher molecular weight forms of apo(a). The intermediate fraction contained approximately equal quantities of the low and high molecular weight forms, whereas the third fraction consisted primarily of low molecular weight forms with a size similar to that of thyroglobulin. As already shown above, apo(a) exhibited only one band by SDS-PAGE (Fig. 3) but three to five bands in the absence of SDS (Figs. 2, 4); all of the bands reacted with anti Lp(a) but not anti apoB. This suggested to us that apo(a) had undergone self-association. The molecular weight of apo(a) was estimated to be 260,000 to 280,000 by SDS-PAGE but gave an apparent molecular weight of 670,000 in the absence of SDS. Considering that in the latter method the migration of proteins is affected by their shape, and that apo(a) is mostly random in structure (Fless, G. M., M. E. ZumMallen, and A. M. Scanu, unpublished results), unlike the globular protein standards used, the apparent molecular weight of apo(a) was probably grossly overestimated. Thus, we were not able to establish with the evidence at hand whether the lowest molecular weight band seen by electrophoresis in the absence of SDS represented the apo(a) monomer. We do not feel that the self-association of apo(a) was related to the formation of intermolecular disulfide bonds because SDS gel electrophoresis of apo(a) carried out in the absence of a reducing agent, mercaptoethanol, gave only one band. Furthermore, the results of gel filtration (Fig. 5) indicated that apo(a) was self-associated under reducing conditions.

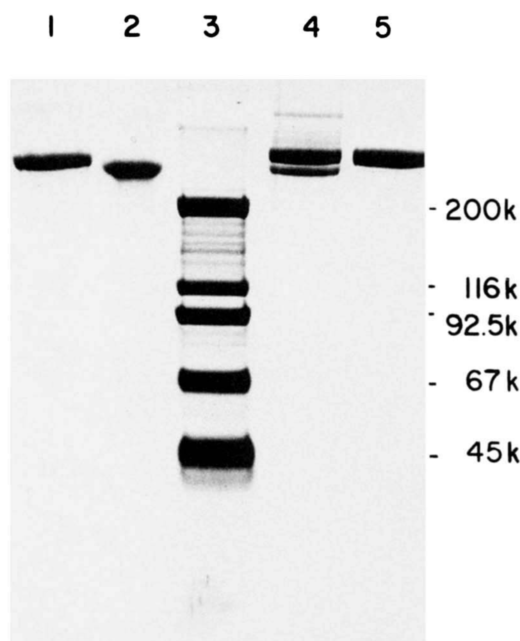

Fig. 3. Characterization of purified apo(a) by SDS gradient gel electrophoresis on 2.5–16% gels. Lane 1, Lp(a−); lane 2, apo(a); lane 3, molecular weight standards myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (67,000), and ovalbumin (45,000); lane 4, reduced Lp(a); lane 5, LDL control. The molecular weight standards and the Lp(a) sample were reduced with 5% 2-mercaptoethanol before application to the gel. Electrophoresis was carried out at 150 V constant voltage for 3 hr. Each sample and molecular weight marker represents 20 μg of protein.

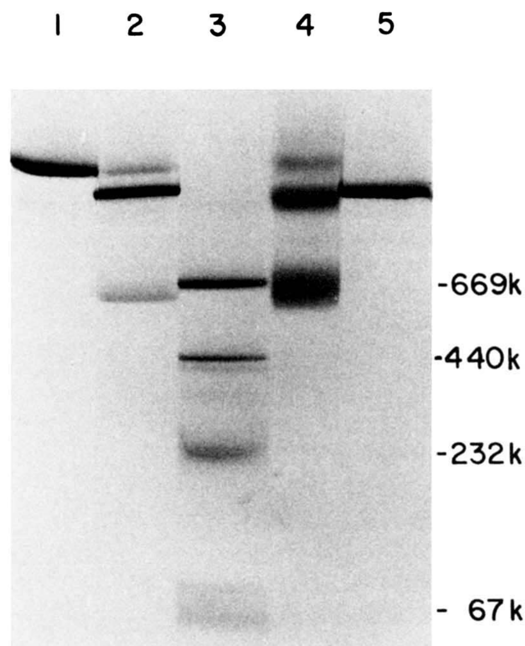

Fig. 4. Dissociation of apo(a) from reduced Lp(a) by gradient gel electrophoresis on 2.5–16% polyacrylamide gel in the absence of SDS. Lane 1, native unreduced Lp(a); lane 2, reduced Lp(a); lane 3, molecular weight standards, thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and bovine serum albumin (67,000); lane 4, apo(a); lane 5, LDL. Electrophoresis conditions were identical to those of Fig. 2. Each sample and molecular weight marker represents 20 μg of protein.

DISCUSSION

Previous experiments suggested that apo(a), although attached by covalent disulfide bond(s) to apoB, appears to be loosely bound to Lp(a) and interact more with the aqueous environment, or apoB, than with the lipid surface (3). Based on this premise we developed a fast and easy method for preparing apo(a) in almost lipid-free form from Lp(a). Thus, reduction of the disulfide bonds

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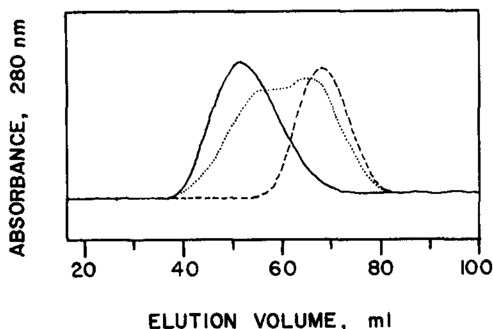

Fig. 5. Gel filtration of Lp(a) (——), Lp(a−) (- - -), and apo(a) (· · · ·) on Bio-Gel A-5m. The column (1.27 × 90 cm) was equilibrated with 0.15 M NaCl, 0.001 M DTT, 0.01% Na_2 EDTA, and 0.01% NaN_3 at a flow rate of 5 ml/hr. Samples (2 ml) were applied to the column in the same eluant but contained 0.01 M DTT.

of Lp(a) and subsequent rate zonal centrifugation readily separated apo(a) from the floating parent lipoprotein particle. This procedure involves neither delipidation of Lp(a) by organic solvents to obtain its lipid-free apoproteins, nor their exposure to detergents, and avoids chromatography of the apoproteins which would be difficult to carry out owing to the poor solubility of apoB in aqueous solvents. The method relied on the fact that apoB, due to its tight association with the lipoprotein particle, does not dissociate from Lp(a) upon reduction. Although the results reported here were obtained with Lp(a) of one individual, we have successfully prepared apo(a) from the Lp(a) of three other persons. Our procedure ensures the recovery of two water-soluble products, namely apo(a), which carries the epitopes that constitute the (a) antigen, and an Lp(a) particle free of apo(a) which we have called Lp(a−). This lipoprotein contains apoB-100 and all the lipid, and has characteristics similar to those of LDL. However, Lp(a−) is not identical to homologous LDL in that it is larger in size, higher in lipid content, and its density is slightly lower than LDL isolated from the same individual (Fless, G. M., M. E. ZumMallen, and A. M. Scanu, unpublished observations). These differences account for the faster flotation rate of Lp(a−) in comparison to LDL.

Other investigators have attempted the purification of apo(a) in the absence of reducing agents; however, their preparations were not homogenous by polyacrylamide gel electrophoresis (10–12). Multiple bands of sizes smaller than apo(a) were observed upon aging of Lp(a) preparations, or following repeated freezing and thawing, or treatment with urea and detergents (11, 12). Fragmentation of apo(a) may have been the result of uncontrolled degradative processes such as proteolysis or oxidation. It is of interest to note that, with the dissociation of the apo(a) peptides from Lp(a), an LDL-like particle was produced (10, 12). By including protease inhibitors and EDTA during the isolation of Lp(a) from plasma, and by maintaining antiseptic conditions and a nitrogen atmosphere whenever possible, we found that the degradation of apo(a) was largely prevented.

Our working assumption that apo(a) is loosely bound to Lp(a) proved to be correct. However, we found it surprising that, upon gel filtration in either low or high salt concentration, apo(a) failed to separate from the reduced Lp(a). This observation appears to indicate that, in addition to its disulfide linkage to apoB, some portions of apo(a) are bound to the lipoprotein surface by non-covalent interactions instead of loosely extending into the aqueous environment. According to our results, this surface interaction can be overcome either by a gravitational or electrical field but the mechanism remains to be established. We noted that when apo(a) is set free from Lp(a) it tends to self-associate as suggested by the results in Figs. 2, 4, and 5. We speculate that the same domains of apo(a) that are normally involved in the interaction(s) with the Lp(a) surface are also the ones responsible for the self-association of the free apo(a) in aqueous solution. Alternatively, self-association could be promoted by the few lipid molecules that are bound to apo(a). Lipid analysis of apo(a) indicated that approximately three phospholipid and five cholesterol molecules are associated to one apo(a) molecule (M_r 280,000). Further work is in progress to define the molecular weight of the apo(a) monomer and to determine its mode of self-association in aqueous buffers.

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