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The Linkage with Apolipoprotein (a) in Lipoprotein (a) Modifies the Immunochemical and Functional Properties of Apolipoprotein B†

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ABSTRACT: Lipoprotein (a) [Lp(a)] was isolated from several donors and its apolipoprotein (a) [apo(a)] dissociated by a reductive treatment, generating the apo(a)-free form of Lp(a) [Lp(a-)] that contains apolipoprotein B (apo B) as its sole protein. Using anti-apo B monoclonal antibodies, the properties of apo B in Lp(a), Lp(a-), and autologous low-density lipoprotein (LDL) were compared. Marked differences in apo B immunoreactivity were found between these lipoproteins, due to the presence of apo(a) in Lp(a). Apo(a) enhanced the expression of two epitopes in the amino-terminal part of apo B while it diminished the immunoreactivity of three other epitopes in the LDL receptor binding domain. Accordingly, the binding of the lipoproteins to the LDL receptor was also decreased in the presence of apo(a). In a different experimental system, the incubation of antibodies that react with 27 distinct epitopes distributed along the whole length of apo B sequence with plastic-bound Lp(a) and Lp(a-) failed to reveal any epitope of apo B that is sterically hindered by the presence of apo(a). Our results demonstrate that the presence of apo(a) modified the organization and function of apo B in Lp(a) particles. The data presented indicate that most likely the modification is not due to a steric hindrance but that some more profound conformational changes are involved. We suggest that the formation of the disulfide bridge between apo B and apo(a) in Lp(a) alters the system of disulfide bonds present in apo B and thereby modifies apo B structure.

Discovered by Berg in 1963, Lp(a)[1] was long considered to be a genetic variant of LDL, but it was later found to be present in most human subjects (Albers et al., 1977; Albers & Hazzard, 1974). Recently the Lp(a) apolipoprotein was shown to be protein of about 1.2 million daltons which upon reduction yielded two subunits with molecular weights of about 645 000 and 490 000 that have been respectively identified as apo(a) and apo B (Gaubatz et al., 1983). The apo B component was identified on the basis of its apparent molecular weight and its cross-reactivity with an antiserum to LDL (Mondola & Reichl, 1982; Utermann & Weber, 1982; Fless et al., 1984). Subsequently, it was also demonstrated that Lp(a) can show both inter- and intraindividual heterogeneity with respect to particle size, and this heterogeneity was found to be related at least in part to the size of apo(a) (Seman & Breckenridge, 1986; Utermann et al., 1987).

Several methods have been reported recently that allow the selective removal of apo(a) from Lp(a) (Fless et al., 1985; Armstrong et al., 1985; Seman & Breckenridge, 1986) and which demonstrate that apo(a) is linked to Lp(a) apo B via disulfide bonds and that it has little avidity for the lipids. Particles remaining after the selective removal of apo(a) from Lp(a) [apo(a)-free Lp(a), Lp(a-)] have been found to be

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[1] Abbreviations: Lp(a) lipoprotein (a); apo(a), apolipoprotein (a); Lp(a-), apo(a)-free Lp(a); apo B, apolipoprotein B; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Na_2EDTA, sodium salt of ethylene diaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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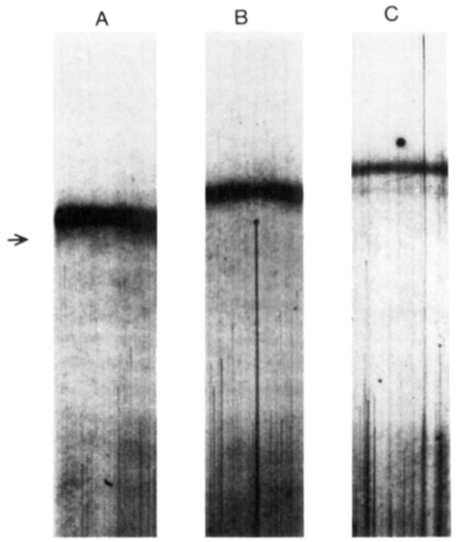

FIGURE 1: Characterization of apo(a) isoforms. Lp(a) was subjected to treatment with dithiothreitol and ultracentrifugation as described in the text. The apo(a) protein pellet was subjected to SDS–PAGE gradient gel electrophoresis in a 3.3–7% gel, and protein was detected with silver stain or immunoblot. Approximately 3–5 μg of apo(a) was applied to each well. The identification is as follows: (A) apo(a)$_i$; (B) apo(a)$_{ii}$; (C) apo(a)$_{iii}$. The position of the arrow indicates the relative position of apo B to the apo(a) isomorphs.

similar to, albeit not identical with, autologous LDL (Fless et al., 1986; Groener & Kostner, 1987). The extent of similarity of apo B organization in Lp(a), apo(a)-free Lp(a), and LDL remains to be established. For instance, conflicting evidence has accumulated regarding the interaction of apo B present in Lp(a) with LDL receptor (Martmann-Moe & Berg, 1981; Havekes et al., 1981; Floren et al., 1981). Using a procedure based on the reduction of Lp(a) with DTT and ultracentrifugation of the reduced lipoprotein for the preparation of apo(a)-free Lp(a), we have studied the immunoreactivity of apo B in the native Lp(a) and in its apo(a)-free form with a series of monoclonal antibodies directed against human apo B, which have been characterized in terms of their specificity and position of the different epitopes identified on the apo B molecule (Marcel et al., 1982, 1984, 1987; Milne et al., 1983).

All antigenic determinants of LDL apo B that were tested have been found to be expressed on both native and denatured Lp(a) apo B, but the apo B present in the native Lp(a) exhibits specific immunological differences compared to the apo B in LDL, some of which are attributable to the interaction between apo B and apo(a). We also report the results of LDL receptor competitive binding studies which demonstrate that the presence of apo(a) in Lp(a) interferes with the receptor binding capability of apo B. These results indicate that apo(a) contributes to a modification of apo B structure in Lp(a).

Materials and Methods

Isolation of Lipoproteins. The donors used in this study for the preparation of Lp(a) have been characterized earlier (Seman & Breckenridge, 1986) and were found to have by SDS–polyacrylamide electrophoresis a single-band apo(a) phenotype identified as apo(a)$_i$, -(a)$_{ii}$, and -(a)$_{iii}$ (Figure 1). These isoforms are very similar to S1, S2, and S3 in the nomenclature of Utermann et al. (1987). Isolation of Lp(a) from human plasma containing aprotinin (0.05 mg/mL) was performed by density gradient ultracentrifugation as described previously (Lee & Downs, 1985; Seman & Breckenridge, 1986) with minor modifications to improve the separation of Lp(a) from LDL and HDL. The plasma (27 mL) was adjusted to a density of 1.040 g/mL with KBr and added to each ultracentrifuge tube for the Beckman 50.2 rotor. This solution was then underlayered with a KBr solution (10.5 mL) at a density of 1.151 g/mL. After ultracentrifugation for 30 h at 10 °C and 49 000 rpm, two dense yellow bands could be seen that corresponded to LDL (approximately one-fourth from the top of the tube) and HDL (about three-fourths from the top of the tube). The clear region between the two bands contained Lp(a) and occasionally revealed a discrete band when Lp(a) concentrations were particularly high. The Lp(a)-enriched fraction was collected, concentrated, and purified by gel filtration on a Bio-Gel A-5M column equilibrated and eluted with a Tris–saline buffer, pH 8.2, containing 0.1 M Tris, 0.158 M NaCl, 0.02% NaN_3, 1 mM Na_2EDTA, and 0.65 mM phenylmethanesulfonyl fluoride (PMSF) Only those fractions displaying a single band with pre-β mobility on agarose gel electrophoresis (Maguire & Breckenridge, 1975) were pooled to yield pure Lp(a).

Low-density lipoprotein (LDL) was isolated by ultracentrifugation (40 000 rpm for 20 h in a Beckman 40.3 rotor) between the densities of 1.025 and 1.050 g/mL. Autologous control LDL were isolated as described for Lp(a) and represented the fractions that upon gel filtration did not contain Lp(a).

Dissociation of Apo(a) from Lp(a). The procedure involves the reduction of disulfide bonds in Lp(a) and ultracentrifugation to separate apo B and lipids of Lp(a) by flotation from apo(a), as previously described (Seman & Breckenridge, 1986). Control LDL was treated in an identical manner as Lp(a), and after ultracentrifugation in the presence of dithiothreitol, the reisolated lipoprotein was dialyzed against PBS, 1 mM EDTA, and 0.02% NaN_3 (pH 7.4).

Monoclonal Antibodies and Antisera. We have previously described the production and characterization of monoclonal antibodies against human apo B (Marcel et al., 1982, 1987; Milne et al., 1983). Affinity-purified rabbit anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). To detect Lp(a) we used a sheep polyclonal antibody against human Lp(a) from Immuno Diagnostika (Vienna, Austria) and a rabbit anti-sheep IgG from Cedarlane Laboratories Limited (Hornby, Ontario, Canada).

Electrophoretic Analysis. The apolipoprotein composition of the different LDL and Lp(a) fractions was assessed by SDS–PAGE (Laemmli, 1970) on 3.3% acrylamide slab gels. The samples were heated for 5 min at 100 °C in 3% SDS, 50 mM Tris, pH 6.8, and 20% glycerol, with or without 10% 2-mercaptoethanol. The identification of isomorphic forms of apo(a) was accomplished by using 3.5–7% gradient SDS–PAGE on apo(a) isolated as a pellet, following dissociation of apo(a) from Lp(a) as described above. The migrated proteins were stained with either Coomassie brilliant blue G-250 or silver stain or were transferred electrophoretically to nitrocellulose paper as described below. Lipoproteins were also separated by nondenaturing polyacrylamide gradient gel electrophoresis on a linear 2%–16% linear gradient polyacrylamide slab gel (Pharmacia, PAA 2/16, Sweden) (Gambert et al., 1982). LDL and Lp(a) fractions or plasma samples were prestained 30 min at 4 °C with one-third volume of filtered Sudan Black B solution in ethylene glycol (5 g/L). If the lipoproteins had to be transferred to nitrocellulose paper, the Sudan Black was replaced by sucrose to a final concentration of 30 g/L. After preelectrophoresis in 14 mM Tris, 100 mM glycine, and 0.02% NaN_3, pH 8.3, samples (15 μL) were applied to the gels and the migration was performed at 4 °C, 30 V, for 1 h and then at 80 V for 18 h.

Electrophoretic transfer to nitrocellulose paper (0.45-μm pore size, Millipore) from either SDS–PAGE or nondenaturing gradient PAGE was done according to the method of Towbin et al. (1979) in a Trans-blot cell (Bio-Rad Laboratories). Samples from SDS–PAGE were transferred in 20 mM Tris, 150 mM glycine, pH 8.3, and 20% methanol (v/v) overnight at room temperature; those from nondenaturing gradient gel electrophoresis were transferred in the same buffer as used for the migration (14 mM Tris, 110 mM glycine, pH 8.3, and NaN_3 0.02%) at 100 mA for 48 h and at 4 °C.

The nitrocellulose replica were treated for immunochemical detection with any of six anti-apo B monoclonal antibodies (4G3, 5E11, 2D8, 1D1, 3F5, 3A10) or anti-human Lp(a) as described earlier (Marcel et al., 1982). All incubations were made at 37 °C in 10 mM Tris, 150 mM NaCl, and 0.01% NaN_3, pH 7.4, containing 3% BSA. ^{125}I-Labeled anti-sheep IgG for anti-Lp(a) were added subsequently, and autoradiographies were performed on XAR-5 Kodak films with an intensifier screen (Cronex, Du Pont).

Solid-Phase Radioimmunoassay of Apo B. The immunoassay of apo B was based on competition between normal LDL immobilized on polystyrene wells (Removawells, Dynatech Laboratories Inc., Alexandria, VA) and different dilutions of the sample to be tested for each antibody at its predetermined optimal dilution, as described earlier (Marcel et al., 1984). Maximum binding was determined in wells in which no competing soluble antigen was added. The background binding was determined in wells in which both soluble antigen and monoclonal antibody were omitted. The results are expressed as the ratio of B/B_0. Each point is the mean of two values with a coefficient of variation less than 10%. Dilutions of normal LDL corresponding to apo B concentrations between 20 and 0.020 μg/mL were included in each assay to prepare a standard curve. Proteins were measured according to the method of Lowry et al. (1951) using BSA as a standard. Apo B concentration in Lp(a) was calculated by assuming that it constitutes 66% of Lp(a) protein, as documented earlier (Seman & Breckenridge, 1986).

LDL Receptor Binding Studies. Human skin fibroblasts, strain GM0036 (Human Genetic Mutant Cell Repository, Camden, NJ), were used. The binding experiments were performed according to the procedure of Innerarity et al. (1980) as adapted by Milne et al. (1983). The maximum binding was determined by using cells to which no other lipoprotein than ^{125}I-labeled LDL was added. The results are expressed as the ratio of specific binding in the presence of competing lipoprotein to the maximum specific binding.

Anti-Apo B Antibodies Binding to Solid-Phase Bound Lipoproteins. Fifty microliters of lipoprotein solution (10 μg/mL of protein in glycine buffer) was incubated overnight in polystyrene wells at room temperature. After the wells were rinsed with 0.9% NaCl and 0.025% Tween solution, 100 μL of PBS–0.1% BSA was added for 1 h to saturate the wells. Fifty microliters of 1:500 dilutions of 27 anti-apo B monoclonal antibodies was added in duplicates. After an overnight incubation, the wells were rinsed and 50 μL of ^{125}I-labeled anti-mouse rabbit IgG was added for 5 h. The wells were rinsed, and their radioactivity was counted. Specific binding was calculated by subtracting the amount of radioactivity bound to wells in which PBS–BSA was added instead of monoclonal antibody.

Results and Discussion

Lp(a) was isolated in high purity by a combination of density gradient centrifugation and gel filtration as described above, and analysis of the resulting preparation by agarose gel electrophoresis revealed a single component with pre-β mobility (not illustrated). The characterization of the Lp(a) thus isolated, in terms of its lipid and protein composition, has been described in detail elsewhere (Seman & Breckenridge, 1986).

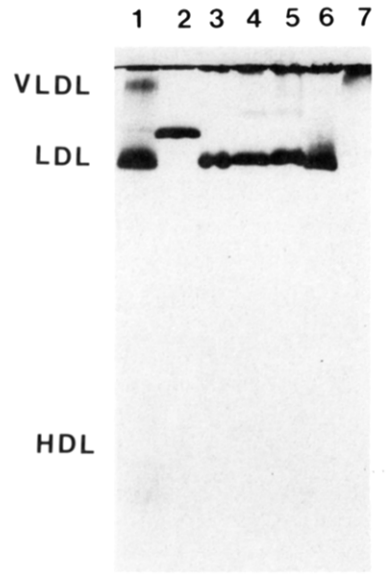

FIGURE 2: Nondenaturing gradient gel electrophoresis (2–16% polyacrylamide); the samples were prestained with Sudan Black: (1) Plasma (10 μL); (2) nontreated Lp(a) (5 μg of protein); (3) top fraction of DTT-treated Lp(a) (2 μg); (4) nontreated LDL (4.3 μg); (5) supernatant of centrifugation of DTT-treated LDL (3.6 μg); (6) nontreated hyper-apo B LDL (4 μg); (7) bottom fraction of DTT-treated Lp(a) (4.5 μg).

Upon electrophoresis on nondenaturing polyacrylamide gel gradients, isolated native Lp(a) migrated characteristically above LDL (Figure 2, lanes 2 and 4, respectively) and to a position corresponding to that of the Lp(a) band in plasma (Figure 2, lane 1). When the same isolated Lp(a) was treated with DTT and reisolated by ultracentrifugation, the resulting lipoprotein presented a size similar to that of LDL (Figure 2, lane 3). This major decrease in the size of the particle is not related to an artifact of reduction and centrifugation since a control LDL submitted to the same treatment exhibited the same size before and after treatment (Figure 2, lanes 4 and 5). A native LDL from a hyperapo-β-lipoproteinemic patient is included to demonstrate that the resolution of the gradient gel electrophoresis is sufficient to detect the slightly smaller size of these particles (Teng et al., 1983) compared to normal LDL (Figure 2, lanes 6 and 4). Finally, the absence of a lipid-stained band in the same electrophoretogram of the infranatant from the centrifugation of the DTT-treated Lp(a) which has been shown to contain apo(a) indicated that little or no lipid remained associated with that fraction (Figure 2, lane 7). This is in agreement with the results of lipid analyses (Seman & Breckenridge, 1986).

The lipoprotein fractions (but without lipid prestaining) were also electrophoresed on the same gradient gel, transferred to nitrocellulose paper, and immunoblotted with an antibody against apo B(4G3). In both native and DTT-treated LDL, only one band immunoreactive with anti-apo B is seen at the position corresponding to the lipid stain, which indicated the lack of effect of DTT and centrifugation on LDL size and on the immunoreactivity of LDL apo B (Figure 3, lanes 1 and 2). In contrast, DTT treatment and centrifugation of Lp(a) resulted in the shift of the anti-apo B positive band from the native Lp(a) position to that of an LDL-size particle (Figure 3, lanes 4 and 3, respectively). Similar results were obtained

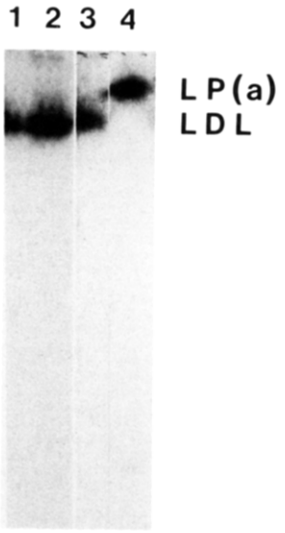

FIGURE 3: Autoradiogaph of Lp(a) and LDL particles separated by gradient gel electrophoresis, transferred on nitrocellulose paper, and incubated with anti-apo B(4G3) and ^{125}I-labeled anti-mouse IgG: (1) DTT-treated LDL (10 μg); (2) nontreated LDL (10 μg); (3) supernatant of ultracentrifugation of DTT-treated Lp(a) (2 μg); (4) nontreated Lp(a) (2.0 μg).

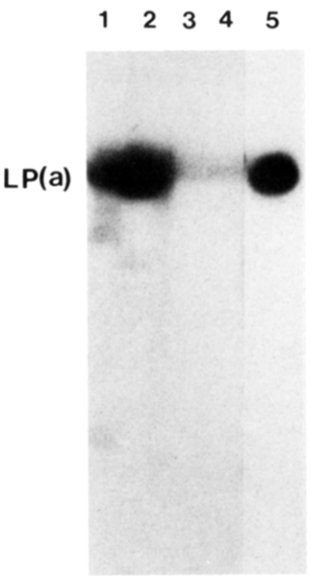

FIGURE 4: Autoradiograph of Lp(a) and LDL particles separated by gradient gel electrophoresis, transferred on nitrocellulose paper, and incubated with anti-Lp(a) (lanes 1–4) or with anti-apo B(4G3) (lane 5); (1) Plasma (10 μL); (2) nontreated Lp(a) (5 μg of protein); (3) supernatant of ultracentrifugation of DTT-treated Lp(a) (5 μg of protein); (4, 5) LDL and nontreated Lp(a) (4 μg of protein).

with other previously described (Marcel et al., 1982; Milne et al., 1983) monoclonal antibodies against LDL (not illustrated). Thus, upon reduction of Lp(a) and centrifugation the lipoprotein collected in the supernatant fraction retains an immunoreactive apo B that reacts with each of the monoclonal antibodies against LDL apo B that was tested. The decrease in size that characterizes the Lp(a) after reduction and centrifugation is related to the loss of the apo(a). Immunoblotting with an antiserum specific for apo(a) of the lipoproteins described above and electrophoresed on nondenaturing gradient gels shows that plasma or native Lp(a) reacts with the antiserum, whereas the Lp(a) reduced and centrifuged does not (Figure 4, lanes 1–3). The infranatant of the reduced and centrifuged Lp(a) fraction contains the immunoreactive apo(a) but no immunoreactive apo B (not illustrated) and no lipids (Seman & Breckenridge, 1986). These results have been further substantiated by SDS gel electrophoresis of the apolipoproteins of LDL and Lp(a) before and after reduction and centrifugation (not illustrated).

The experiments described above corroborate the results of others (Fless et al., 1985; Armstrong et al., 1985; Seman & Breckenridge, 1986) and show that the native isolated Lp(a) contains two major apolipoproteins, apo B and apo(a), which are linked by a disulfide bridge(s) and that upon reduction and centrifugation the apo(a) is lost from the Lp(a) without any concomitant loss of lipids. This apo(a)- free Lp(a) contains an apo B that is identical with LDL apo B in terms of amino acid composition (Seman & Breckenridge, 1986; Fless et al., 1986) and that reacts with all the tested anti-LDL monoclonal antibodies in immunoblots after SDS gel electrophoresis. Each Lp(a) preparation used in these studies contained only one of three different isomorphic forms of apo(a) as indicated in Figure 1. On the basis of the molecular weight (514K) of apo B (Knott et al., 1986) and the relative mobilities of the isomorphs to apo B, the apparent molecular weights of the isomorphs range from about 550K to 650K. These values may be an overestimate due to the large amount of carbohydrate associated with apo(a) (Seman & Breckenridge, 1986). Other studies have reported relatively rare isomorphic forms of apo(a) with molecular weights lower than or equal to that of apo B as well as the present forms (Fless et al., 1984; Gaubatz et al., 1987; Utermann et al., 1987). Having characterized these forms of Lp(a) with or without apo(a), we set out to study the immunoreactivity of apo B in these lipoprotein fractions.

It could be expected that the attachment of a large molecular weight protein like apo(a) at certain sites in apo B molecule would create, apart from other effects, a steric hindrance, affecting mostly the binding of some anti-apo B antibodies that react with epitopes close to the site(s) of apo(a) attachment. To test this possibility we performed an experiment in which plastic-bound Lp(a), Lp(a−), LDL, and DTT-treated LDL were reacted with various anti-apo B antibodies directed against epitopes mapped to different regions of the apo B molecule. We reasoned that differences between the amounts of antibodies fixed on Lp(a) and Lp(a−) would help us determine which regions of apo B, if any, are most susceptible to the steric hindrance exerted by apo(a), i.e., those which are closest to the cysteine bridge(s) linking apo B and apo(a). The antibodies used in this experiment react with epitopes distributed throughout the length of the apo B molecule (Table I). Surpisingly, with none of the antibodies studied was there any significant difference found between the amounts bound to native and reduced Lp(a) (Table I) (as well as LDL). Antibodies MB2 and MB19 seem to bind slightly better and antibodies B2, Bsol 15, and Bsol 20 slightly less to native than to reduced Lp(a) in this system. However, the same was found for control native and reduced LDL and, therefore, these differences probably do not reflect structural differences between apo B in Lp(a) and Lp(a−) due specifically to the presence or absence of apo(a), but result from the effect of reduction on apo B conformation whether in Lp(a) or LDL. The kinetic parameters of the interactions of the antibodies with apo B epitopes were not determined in this study.

The lack of influence of apo(a) presence on binding of the antibodies throughout the length of the apo B molecule, as found in this experiment, was rather unexpected. In our view, the results shown in Table I are most readily explained when one assumes that only one disulfide bond links apo(a) to apo B and that there exists, at least under our experimental conditions, a considerable freedom of rotation around that bond and that, in addition, apo(a) assumes the conformation of an

Table I: Relative Immunoreactivity of Monoclonal Antibodies with Solid Phase-Bound Lp(a), Lp(a−), LDL, and Reduced LDL

		Lp(a)/Lp(a−)[a]					LDL/LDL-red.[b]		
antibody	epitope location[c]	I, a_i[d]	II, a_{ii}[d]	III, a_{ii}[d]	IV, a_{iii}[d]	mean	I, a_{ii}[d]	II, a_i[d]	mean
Bsol 12	1–60	1.08	0.86	0.98	0.93	0.96	0.95	0.94	0.95
1D1	401–582	1.06	0.95	0.97	0.95	0.98	1.02	0.90	0.96
Bsol3	97–689	0.99	0.90	0.99	1.03	0.98	1.04	0.73	0.89
MB24	97–689	1.15	0.95	0.96	1.01	1.01	1.17	0.90	1.03
MB2	1–1297	1.41	1.18	1.31	1.26	1.29	1.22	0.77	0.99
MB19	1–1297	1.17	1.17	1.26	1.18	1.20	1.08	0.80	0.94
MB11	995–1082	1.21	0.94	0.99	0.99	1.03	1.01	0.90	0.96
2D8	1297–1328, 1403–1480	1.11	0.87	0.94	0.97	0.97	1.02	0.88	0.95
B4	1693–2240	1.03	1.07	0.82	0.92	0.96	1.07	0.77	0.92
B5	1693–2240	0.99	0.92	0.96	0.95	0.95	1.07	0.83	0.95
B2	2240–2375	0.77		0.85	0.86	0.87	0.80	0.83	0.82
B3	2240–2375	0.86	0.79	0.98	0.93	0.89	0.93	0.97	0.85
B6	2240–2375	0.95	1.05	0.83	1.13	0.99	1.17		1.17
MB44	2488–2658	0.74	0.74	1.39	0.95	0.95	1.03	0.84	0.94
Bsol6	2488–2658	1.09	1.00	0.98	1.01	1.02	0.98	0.96	0.97
3F5	2658–2816	1.06	0.94	0.92	0.97	0.97	1.02	0.97	0.99
4G3	2980–3080	1.15	0.95	0.94	0.93	0.99	1.03	0.96	0.99
3A10	3441–3568	1.19	0.96	1.19	0.89	1.06	0.96	0.96	0.96
5E11	3441–3568	1.25	0.93	0.93	0.93	1.01	1.00	0.97	0.99
MB47	3441–3568	1.09	0.99	0.97	0.98	1.01	0.99	1.00	0.99
MB43	3925–4005	1.25	0.93	1.07	0.95	1.05	1.02	0.87	0.94
Bsol15	3506–4082	0.88	0.82	0.84	0.83	0.84	0.72	0.83	0.78
Bsol20	3506–4082	0.86	0.97	0.84	0.86	0.88	0.99	0.76	0.88
Bsol16	4154–4189	1.09	0.98	0.96	1.05	1.02	1.04	0.90	0.97
BL3	4082–4525	0.97	0.88	1.03	0.93	0.95	1.02	0.96	0.99
Bsol7	4517–4536	0.97	0.90	1.06	0.91	0.96	1.00	0.93	0.96
Bsol22	4517–4536	1.02	0.96	0.89	0.93	0.95	1.05	1.05	1.05
BSA		0.83	1.02	1.03	1.07	0.99	0.99	1.00	0.96

[a] Radioactivity bound to Lp(a) over radioactivity bound to Lp(a−). [b] Radioactivity bound to LDL over radioactivity bound to LDL-red. [c] From Knott et al. (1986), Marcel et al. (1987), P. F. Chen, Y. L. Marcel, C. Y. Yang, A. M. Gotto, R. W. Milne, J. T. Sparrow, and L. Chan (unpublished results), and R. J. Pease, J. Scott, Y. L. Marcel, and R. W. Milne (unpublished results). [d] Experiment number, apo(a) isoform.

extended and flexible coil as suggested by Fless et al. (1986). On the basis of the lack of separation of apo(a) and Lp(a−) by gel filtration under reducing conditions, other interactions of noncovalent nature between apo(a) and the Lp(a) lipoproteins had been proposed by Fless et al. (1985); however, this claim is not supported by the separation of apo(a) and Lp(a−) by heparin–Sepharose (Armstrong et al., 1985) or by the experiments reported here.

While the direct binding of antibodies to the immobilized antigen as described above would measure primarily the accessibility of epitopes, competitive RIA provides a more sensitive assessment of immunoreactivity and reflects both accessibility of the epitope and its affinity with the antibody. Indeed, the competitive RIA detected substantial differences between apo B immunoreactivity in Lp(a) compared to that of apo B in Lp(a−) and control LDL. These differences varied between epitopes, and Figure 5 summarizes the results obtained with the different antibodies tested and with Lp(a), Lp(a−), and autologous control LDL isolated from a single representative donor.

First, it is strikingly evident that with all antibodies the displacement curves for treated and control LDL, as well as for Lp(a−), are almost identical. This demonstrates that the organization of apo B in LDL and in particles resulting from removal of apo(a) from Lp(a) is very similar. In contrast, Lp(a) apo B could be less, equally, or more immunoreactive than apo B of Lp(a−) or LDL depending on the antibody considered (Figure 5). It should also be emphasized that the reductive treatment and subsequent isolation procedure applied to Lp(a) cannot account for the observed changes, as indicated by the inspection of the curves for DTT-treated and untreated LDL (Figure 5).

Native Lp(a) was a poorer competitor than Lp(a−) and LDL for antibodies 3A10, 5E11, and 4G3 (Figure 5C–E). The epitopes for these antibodies are likely situated near the receptor binding domain of apo B (Milne et al., 1983), as their binding to apo B prevents the apoprotein from being recognized by the LDL receptor. Therefore, the presence of apo(a) seems to markedly decrease the immunoreactivity of this region of the apo B molecule. With antibody 3F5 we observed only nonsignificant differences between immunoreactivity of native and reduced Lp(a) (Figure 5F). Interestingly, the epitope for 3F5 appears to be located on the N-terminal boundary of the receptor binding domain; its binding to apo B only partially blocks the recognition by the receptor. At the other extreme, antibodies 1D1 and 2D8 that react with epitopes located in the portion of the molecule common for B48 and B100 (or more precisely between residues 400 and 481 and 1297 and 1600, respectively) reacted more with Lp(a) apo B than with either Lp(a−) or LDL (Figure 5A,B).

The competitive RIA using the same antibodies was repeated with the Lp(a), Lp(a−), and autologous LDL from six different blood donors who presented three different Lp(a) phenotypes, and the results are summarized in Table II. There was no apparent difference among the three types of isomorphs used in these studies. The pattern of Lp(a) immunoreactivity changes upon reduction was similar in all donors with the exception of two outlying observations: donor 3 with 1D1 and donor 6 with 3F5. As a result, the percentages of change of ED_{50} for 1D1 are not statistically significant, but the mean percentages of change of ED_{50} for 2D8, 4G3, 3A10, and 5E11 (and not for 3F5) antibodies follow the trend observed in Figure 5 and are significant. To test whether the effects documented in Figure 5 and Table II represent a constant and reproducible feature of a given donor's Lp(a), we repeated the experiments with two donors' lipoproteins isolated from blood samples obtained on another occasion. We were able to reproduce the same results as in the original experiment (not illustrated).

Taken together with the binding of antibodies to immobi-

Table II: ED_{50}[a] of Apo B in Lp(a) and Lp(a−) for Different Antibodies

donor	apo(a) isoform	lipoprotein	1D1	2D8	3F5	4G3	3A10	5E11
1	a_i	Lp(a)	263[a]	177	91	1951	2342	1592
		Lp(a−)	400	456	72	629	1322	850
		Δ[b]	+57%	+158%	−20%	−68%	−44%	−47%
2	a_{iii}	Lp(a)	740	153	127	2113	3493	2365
		Lp(a−)	1269	453	120	1119	1864	1208
		Δ[b]	+71%	+196%	−6%	−47%	−47%	−49%
3	a_{ii}	Lp(a)	810	406	178	1643	5901	1442
		Lp(a−)	622	625	165	923	2749	1280
		Δ[b]	−23%	+54%	−7%	−44%	−53%	−11%
4	a_{ii}	Lp(a)	354	158	96	1108	3025	1201
		Lp(a−)	794	430	124	511	1782	1002
		Δ[b]	+124%	+172%	+29%	−54%	−41%	−16%
5	a_{ii}	Lp(a)	530	206	160	2398	5998	2326
		Lp(a−)	1072	430	199	910	1835	1337
		Δ[b]	+102%	+117%	+24%	−62%	−69%	−43%
6	a_i	Lp(a)	787	314	115	2681	4155	5234
		Lp(a−)	2365	660	210	504	2746	2473
		Δ[b]	+200%	+110%	+82%	−81%	−34%	−53%
mean Δ	±SD		+96 ± 74	134 ± 51[c]	+19 ± 37	−59 ± 14[c]	48 ± 12[c]	37 ± 18[c]

[a] Apo B concentration at which B/B_0 = 50% (ng/mL). [b] Percentage of change of ED_{50} upon reduction of Lp(a): Δ = 100[[ED_{50}Lp(a−) − ED_{50}Lp(a)]/ED_{50}Lp(a)]. [c] Significant results based on a difference from control greater than 2 × SD.

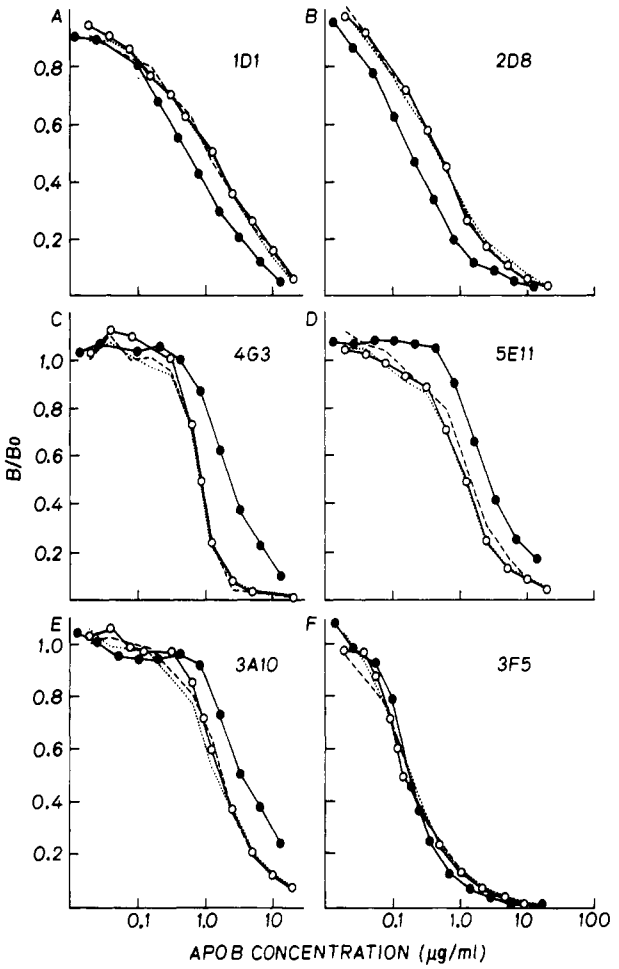

FIGURE 5: Competitive radioimmunoassay studies of Lp(a) (●), Lp(a−) (○), LDL (- - -), and DTT-treated LDL (···). Six different monoclonal antibodies were used: (A) 1D1; (B) 2D8; (C) 4G3; (D) 3A10; (E) 5E11; (F) 3F5.

lized Lp(a) and Lp(a−) (Table I), the RIA results (Table II) shed some new light on the interactions between apo(a) and apo B in Lp(a). The similar binding of antibodies to the immobilized Lp(a) and Lp(a−) does demonstrate that there are no large regions of apo B of Lp(a) masked by apo(a). The decreased immunoreactivity of Lp(a) apo B with 4G3, 3A10, and 5E11 cannot very likely therefore be ascribed only to a simple masking effect of apo(a) on the region of the apo B molecule that contains these epitopes. Bearing in mind that the immunoassays of Table I are carried out with a solid-phase antigen and those of Table II with a soluble antigen, conditions that can always be suspected of generating different conformations of Lp(a), we could conclude from these results (Tables I and II) that the decreased immunoreactivity with 4G3, 3A10, and 5E11 is caused by a lower affinity for Lp(a) apo B, probably related to a change in apo B conformation affecting these epitopes. This notion is further reinforced by the increased immunoreactivity of 1D1 and 2D8 with Lp(a) apo B compared to that of apo B from either Lp(a−) or LDL (Table II). Indeed, the latter observation implies that apo(a), rather than being simply a steric hindrance, introduces more profound modifications of the way apo B is organized in the particle, specifically in the apo B100/B48 common region. Considering that this portion of the molecule is characterized by a very high number of intrachain disulfide bridges, important for stabilization of apo B conformation in LDL, it is possible that apo(a) disulfide linkage to apo B prevents the formation of the normal network of disulfide bonds in the N-terminal half of apo B.

The increased immunoreactivity, upon dissociation of apo(a), of the epitopes mapped to the receptor binding domain of apo B was consistent with the notion that the removal of apo(a) improves the expression of that whole region of the apo B molecule, including the receptor binding site. Therefore, we studied the ability of Lp(a), Lp(a−), and autologous LDL from several donors to compete with ^{125}I-labeled LDL for LDL receptors on human fibroblasts (Table III). Lp(a) of all donors studied was a much poorer competitor of labeled LDL than LDL at the same concentration of apo B. The DTT treatment resulted in an increase of competing ability, making the Lp(a−) an equally efficient ligand of LDL receptor as LDL. This was not due to the effect of the reductive treatment itself on apo B, since the same treatment applied to control LDL obtained from the same donor did not induce any significant changes in receptor binding (Table III, donors 3 and 5′). Experiments in which higher concentrations of apo B were applied gave the same results (not illustrated). Our results are consistent with the conclusion reached by most other authors investigating the Lp(a) uptake by LDL receptor (Arm-

Table III: LDL Receptor Binding of Native and Reduced Lp(a)

donor	apo(a) isoform	lipoprotein	% maximum binding[a]
1	a_i	Lp(a)	84
		Lp(a−)	59
2	a_{iii}	Lp(a)	79
		Lp(a−)	46
3	a_{ii}	Lp(a)	80
		Lp(a−)	42
		LDL	40
		LDL + DTT	45
4	a_{ii}	Lp(a)	65
		Lp(a−)	42
5	a_{ii}	Lp(a)	86
		Lp(a−)	43
5′[b]	a_{ii}	Lp(a)	71
		Lp(a−)	33
		LDL	40
		LDL + DTT	50
6	a_i	Lp(a)	79
		Lp(a−)	46

[a] Maximum binding: ^{125}I-labeled LDL binding with no competing ligand in the medium. [b] 5 and 5′ denote samples from the same donor isolated from blood obtained on different occasions.

strong et al., 1985; Floren et al., 1981; Krempler et al., 1983); some studies, however, seem to indicate that Lp(a) and LDL bind the receptor with equal affinity (Havekes et al., 1981). The data in Table III do not indicate the mechanism of apo(a) interference with receptor recognition of apo B in Lp(a); they confirm the RIA results, pointing to the decreased expression of the apo B receptor binding region in Lp(a). The decrease is not affected by the apo(a) phenotype of Lp(a).

The results of our experiments suggest that it is not simply via a steric hindrance that apo(a) presence modifies the interactions of apo B with antibodies in RIA and with receptors but that probably some conformational changes are involved. Previous results from ourselves and others (Teng et al., 1985; Tikkanen et al., 1983) had demonstrated that the immunoreactivity of most of apo B epitopes could be directly influenced by the lipid composition and relative size of the lipoproteins containing apo B. However, the lipid composition of Lp(a) and apo (a)-free Lp(a) has been found to be similar to that of LDL (Seman & Breckenridge, 1986; Fless et al., 1986), and the increased protein to lipid ratio in Lp(a) appears to be related to the presence of apo(a) on an otherwise normal LDL-like particle. This would lead us to conclude that the changed immunoreactivity of Lp(a) apo B is not related to an effect of the lipid moiety but rather to a general alteration of apo B conformation in Lp(a) caused by its linkage to apo(a).

One can hypothesize that the disulfide bridge(s) linking apo(a) and apo B in Lp(a) involve apo B cysteine residue(s), which in LDL normally participate in the formation of intramolecular disulfide bonds responsible for apo B conformation (Cardin et al., 1982). The formation of such a disulfide bond would imply an intracellular site of synthesis for Lp(a), perhaps at the early stage of apo B synthesis when the disulfide bridges are not formed.

Alternative mechanisms of apo B structure modification by apo(a) [e.g., hydrophobicity/hydrophilicity changes caused by apo(a)] cannot be ruled out.

As indicated above, we believe that the lack of steric interference of apo(a) at any of the 27 epitopes of apo B that we tested is indicative of an extended and free rotating apo(a) and points to the presence of only one disulfide bridge between apo B and apo(a), possibly involving the unpaired cysteine in kringle repeat 36 of apo(a) (McLean et al., 1987). Further experiments will be needed to identify the position of the cysteines involved in both apo B and apo(a) and to evaluate whether this mixed dimer is affected by apo(a) polymorphism.

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Time-Resolved Solution X-ray Scattering of Tobacco Mosaic Virus Coat Protein: Kinetics and Structure of Intermediates†

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ABSTRACT: The kinetics of assembly and disassembly of tobacco mosaic virus coat protein (TMVP) following temperature jumps have been studied by small-angle X-ray scattering and turbidimetry. The structures of the principal aggregates of TMVP oligomers (A protein), intermediate size (helix I) and large size helical rods (helix II), have been characterized by their average radii of gyration of thickness, cross section, and shape obtained from the corresponding regimes of the small-angle scattering pattern. This structural information was obtained within seconds after the temperature-induced initiation of either polymerization or depolymerization and allowed us to detect transient intermediates. This methodology made it possible to observe and characterize the structure of a principal intermediate. Taken together with other kinetic information, these data suggest that polymerization of TMVP under virus self-assembly conditions may proceed via a single-layered helical nucleus that contains about 20 subunits. Previous studies have shown that overshoot polymerization of TMVP can occur and results in metastable long helical viruslike rods which subsequently depolymerize and then form short helical rods, depending on the conditions of the final equilibrium state. The longer rods (helix II) are overshoot polymers which form within seconds and contain $17^1/_3$ subunits per turn (helix IIB), in contrast to the subunit packing arrangement of $16^1/_3$ subunits per turn found in the shorter helical rods (helix IA). The latter packing arrangement is the one found in TMV. An overall polymerization scheme is proposed for the formation of these two helical forms of TMVP.

The assembly of tobacco mosaic virus (TMV)[1] has attracted continued interest for more than 30 years. One remarkable aspect of the properties of the components of this nucleoprotein complex is the fact that the coat protein, TMVP, alone can be made to self-assemble into viruslike rods. Although the assembly of TMV and of TMVP has been extensively studied, resulting in abundant data, and some models have been proposed, many central aspects of the assembly of the virus and of the coat protein remain unclear. As background, we provide only a brief summary of the more recent advances. Comprehensive accounts of the general subject have been given by Caspar (1963), Hirth and Richards (1981), Butler (1984), Stubbs (1984), Bloomer and Butler (1986), and Okada (1986) and in previous reviews cited therein.

Since TMVP can form viruslike rods in the absence of RNA, it is believed that many of the protein interactions in the virus are preserved in RNA-free helical structures. Depending on pH, temperature, protein concentration, and ionic strength, four major types of protein assemblies have been observed in solution: namely, at 100 mM ionic strength, oligomers (A protein), intermediate size polymers, and large helical polymers; at >0.4 M ionic strength, stacked disks (Klug & Durham, 1972). The structure of the long RNA-free helices has been studied by static fiber X-ray diffraction (Mandelkow et al., 1981). It was found that the helical RNA-free protein rods exist in two forms, one of which is the same as the native virus having $16^1/_3$ subunits per turn. The other is a polymorphic variant and has $17^1/_3$ subunits per helical turn. However, it was not known exactly which conditions of polymerization gave rise to each packing arrangement since all diffraction specimens were apparently prepared in the same manner. Originally, it was believed that all the long rodlike helical polymers found in weakly acidic conditions were structurally related to the virus (Holmes et al., 1975; Stubbs et al., 1977) and that the intermediate sized polymers in solution at pH 7, 20 °C, 100 mM ionic strength (20S polymers)

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[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus coat protein; SAXS, small-angle X-ray scattering; R_{G3}, R_{G2}, and R_{G1}, radius of gyration of shape, cross section, and thickness, respectively.

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