Structural Domains of Apolipoprotein(a) and Its Interaction with Apolipoprotein B-100 in the Lipoprotein(a) Particle†

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ABSTRACT: The structural domains of human apolipoprotein(a) [apo(a)] and its interaction with apolipoprotein B-100 (apo B-100) in the lipoprotein(a) [Lp(a)] particle were investigated by limited proteolysis with thermolysin and cathepsin D. We characterized the proteolytic products by sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis, followed by immunoblotting using different antibodies. For apo B-100 in Lp(a), the digestion patterns were found to be identical to those previously described [Chen et al. (1989) *J. Biol. Chem. 264*, 14369–14375; Chen et al. (1991) *J. Biol. Chem. 266*, 12581–12587] for apo B-100 in LDL. Thus, we compared the digestion patterns of apo B-100 in Lp(a) resolved under reducing and nonreducing migrating conditions. Using an antibody specific for a synthetic peptide of apo B-100 (residues 4004–4021), we confirmed that apo B-100 was linked to apo(a) by its C-terminal end. Various Lp(a)s isolated from several donors, and containing different isoforms, were used to study the structural domains of apo(a). Using the same procedure as for apo B-100, several common features were found for the different isoforms. (1) Apo(a) can be cleaved into two structural domains: one was of constant size (170 kDa) and was linked to apo B-100. Using an antibody specifically directed against kringle V, we demonstrated that this fragment corresponded to the C-terminal part of apo(a). (2) The other domain, whose size varied according to the digested apo(a) isoform, was not linked to apo B-100. Finally, when a recombinant apo(a) was used instead of Lp(a), it was also cleaved into two domains. This result could indicate that the structure of apo(a) exists independently of the Lp(a) particle and is not due to interactions of apo(a) with apo B-100 or with lipids.

Lipoprotein(a) [Lp(a)][1] was first described in 1963 by Berg (Berg, 1963), and has since been demonstrable in almost every human individual (Boerwinkle *et al.*, 1992). Lp(a) has gained considerable clinical interest since a strong positive correlation between its plasma concentration and cardiovascular disease was discovered (Utermann, 1990). Although Lp(a) may play an important role in the development of atherosclerosis, little is known about its structural and functional aspects. The Lp(a) particle closely resembles low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoprotein B-100 (apo B-100). However, the presence of apo(a) in this particle differentiates Lp(a) from LDL. The primary structure of apo(a) has been deduced from the sequence of a cloned apo(a) cDNA (McLean *et al.*, 1987). The apo(a) cDNA contained multiple copies of a sequence that encodes a protein motif called a kringle. The repeated kringle in apo(a) is designated kringle IV because it closely resembles the fourth kringle of plasminogen. The apo(a) sequence also comprises one copy of kringle V and a serine protease domain (both similar to the corresponding domains in plasminogen). Consequently, part of the secondary structure of apo(a) has been established since it is mainly constituted of kringles, structures in which the amino acid chain is interconnected by three disulfide bonds. Kringles have been described in many other proteins including plasminogen, tPA, urokinase, factor XII, prothrombin, and, recently, hepatic growth factor (Henkin *et al.*, 1991). Tertiary structures of isolated kringles have also been determined (de Vos *et al.*, 1992; Mulichak *et al.*, 1991).

It is currently believed that apo(a) is linked to apo B-100 via one or more disulfide bonds (Gaubatz *et al.*, 1983) although the cysteines involved had not been clearly designated. Recently, however, Guevara *et al.* (1993) have provided more evidence in favor of the involvement of Cys-3734 of apo B-100 in disulfide linkage with apo(a). Apo B-100 is a polypeptide chain containing 4536 amino acids (Knott *et al.*, 1986). Apo B-100 of LDL is a physiological ligand for the LDL receptor, whereas apo B-100 present in VLDL is a poor ligand for this receptor (Bradley & Gianturco, 1986). Therefore, the conformation of apo B-100 in apo B-100-containing particles is crucial to its recognition by the LDL receptor. Extensive studies have been carried out to determine the conformation of apo B-100 in LDL, using monoclonal antibodies (Marcel *et al.*, 1987), peptide mapping (Chen *et al.*, 1989), immunoelectron microscopy (Chatterton *et al.*, 1991), or constructions with truncated apo B-100 (Xiong *et al.*, 1991).

In contrast, little is known about the conformation of apo B-100 in the Lp(a) particle. For instance, conflicting evidence has accumulated regarding the interaction of apo B-100 present in Lp(a) with the LDL receptor (Armstrong *et al.*, 1985; Floren *et al.*, 1981; Havekes *et al.*, 1981; Martmann-Moe & Berg, 1981). Using monoclonal antibodies against known

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); apo B-100, apolipoprotein B-100; LDL, low-density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; BHT, butylated hydroxytoluene; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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epitopes of apo B-100, Zawadzki *et al.* (1988) found differences in apo B-100 conformation between LDL and Lp(a) which were reflected by differences in binding to the LDL receptor.

Equally, the structure of apo(a) itself and its relationships either with apo B-100 or with lipids have not been extensively investigated. Gaubatz *et al.* (1983) as well as Fless *et al.* (1986), using circular dichroism, showed that Lp(a) was much more disordered than LDL. This finding was attributed to the apo(a) moiety of the particle. After selective removal of apo(a) from Lp(a) with DTT, and further purification, the residual Lp(a−) particle was found to be similar to LDL (Fless *et al.*, 1985; Gaubatz *et al.*, 1987), indicating that apo(a) may only interact weakly with the lipid core of the particle. More recently, Phillips *et al.* (1993) have shown that the bulk of apo(a) was extended out into the aqueous phase in an Lp(a) particle reconstituted from LDL and recombinant apo(a).

Folded structural domains of proteins are relatively resistant to proteolytic cleavage as compared to the exposed flexible peptide regions that connect them. In order to further understand the structural domains of apo(a) and its interaction with apo B-100 in the Lp(a) particle, we have compared partial proteolytic digestions of Lp(a) and LDL particles using SDS–PAGE followed by immunoblotting with either anti-apo B-100 or anti-apo(a) antibodies, or more specific antibodies directed against specific sequences of these proteins. We have also compared the digestion patterns under reducing and nonreducing conditions in order to determine which domains of apo B-100 and apo(a) are linked together.

EXPERIMENTAL PROCEDURES

Isolation of LDL and Lp(a). LDL and Lp(a) were isolated from different plasmas to avoid cross-contamination of the particles. To prevent degradation of lipoproteins, PMSF (1 mM), BHT (4.4 μg/mL), gentalline (0.005%), NaN_3 (0.01%), and EDTA (0.01%) were added to the plasmas.

Particles were isolated by sequential ultracentrifugation in a 60 Ti rotor at 10 °C for 24 or 48 h at 45 000 rpm. Solvent densities were adjusted with KBr. LDL were isolated in the 1.025–1.050 g/mL density interval (24-h centrifugation) (Chapman *et al.*, 1988) and Lp(a) at 1.050–1.100 g/mL (48-h centrifugation).

The Lp(a)-enriched fraction was further purified on a gel filtration column (A15M, Bio-Rad) equilibrated with PBS. Fractions containing pure Lp(a) were pooled, concentrated against Aquacid II (Calbiochem), and dialyzed overnight against PBS (pH 7.0). For homogeneity of treatment, LDL were passed through the same column and concentrated in the same way. LDL containing exclusively apo B-100 were used.

The concentration of total protein was determined by the method of Lowry *et al.* (1951). The concentration of Lp(a) was determined by ELISA (Faucher *et al.*, 1993).

The purity of Lp(a) was checked by double-rocket electroimmunodiffusion (Gaubatz *et al.*, 1983). All preparations of Lp(a) used were more than 95% pure. To test the integrity of the particles, their lipid compositions were determined, and were found similar to those previously described (Seman & Breckenridge, 1986). The mean values were 31.7 ± 2% protein, 36.4 ± 1.4% cholesteryl ester, 8.2 ± 0.4% free cholesterol, 5 ± 1.5% triglycerides, and 18.8 ± 0.3% phospholipids.

The isoforms of Lp(a) were determined on 3.7% SDS–PAGE as previously described (Faucher *et al.*, 1993), and immunoblotting was performed using a sheep polyclonal anti-apo(a) antibody (Guo *et al.*, 1989).

Purification of Apo(a) from Lp(a). Purification of apo(a) was essentially carried out according to Armstrong *et al.* (1985). Lp(a) was reduced in 100 mM Tris, 50 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 (pH 7.6) containing 10 mM DTT for 3 h at 37 °C. The solution was then passed over a heparin–Sepharose CL-6B (Pharmacia) column equilibrated with the same buffer but omitting DTT. Apo(a) was not retained on the column, and the first peak was immediately dialyzed against PBS to remove DTT. Bound material was eluted by raising the NaCl concentration in the buffer to 0.5 M.

The first peak revealed the presence of the apo(a) antigen, and trace amounts of apo B-100. The second peak showed only the presence of apo B-100.

A recombinant apo(a) produced from human embryonic kidney cells (293S) (Koschinsky *et al.*, 1991) was kindly provided by Dr. M. Koschinsky.

Limited Proteolysis of LDL and Lp(a). Limited proteolysis was performed on LDL and Lp(a) with two enzymes of different specificity: thermolysin (from *Bacillus thermoproteolyticus*, 40 units/mg of lyophilisate:Boehringer), which primarily cleaves at neutral amino acids, and cathepsin D (from bovine spleen, 3.9 units/mg of protein; Sigma), which is more specific for clusters of hydrophobic amino acids.

Limited proteolysis by thermolysin was carried out at 37 °C by incubating LDL or Lp(a) (0.5 mg/mL) in 0.125 M Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, 0.01% EDTA, and 0.01% NaN_3 (pH 8.0) at a mass ratio of enzyme to substrate of 1:2000. Digestions by cathepsin D were performed in 50 mM sodium acetate, 0.1 M NaCl, 0.04% EDTA, and 0.01% NaN_3 (pH 4.8) at a mass ratio of enzyme to substrate of 1:400 or 1:600. The reaction was stopped by boiling with SDS (2%) for 1 min (thermolysin) or by adding Tris-HCl (pH 8.4) to a final concentration of 0.1 M and boiling with SDS (2%) (cathepsin D).

Peptide Mapping by Analytical SDS–PAGE and Immunoblotting. Unless otherwise stated, analytical SDS–PAGE was performed on 4–7% linear gradient slab minigels (Miniprotean II, Bio-Rad) using a discontinuous buffer system (Laemmli, 1970). Prior to electrophoresis, the proteolytic digests were combined with glycerol, bromophenol blue, and EDTA to final concentrations of 2%, 0.01%, and 0.5 mM, respectively. When DTT was added, its final concentration was 10 mM. Proteins were then electroblotted onto nitrocellulose (Amersham) and revealed by immunoblotting. Proteolytic fragments of apo B-100 were revealed using either a sheep polyclonal anti-apo B-100 antibody or rabbit antisera against sequence-specific synthetic peptides. Three different antibodies were used: antibody 4275 (residues 890–908), antibody 4285 (residues 2068–2091), and antibody 4296 (residues 4004–4021). All three were used to ascertain the position of the fragments in the digestion profile of apo B-100 in LDL and in Lp(a). These antisera were the kind gift of Dr. T. L. Innerarity (Innerarity *et al.*, 1987). Fragments of apo(a) were revealed using either a sheep polyclonal anti-apo(a) antibody or a mouse monoclonal antibody specifically directed against kringle V of apo(a) (antibody anti-kringle V). This antibody was the kind gift of Dr. G. Utermann. Revelation was done using as a secondary antibody, either an anti-sheep horseradish peroxidase-conjugated IgG (Dako) or anti-sheep, anti-mouse (Bio-Rad), and anti-rabbit (Sigma) alkaline phosphatase-conjugated antibodies. In the latter case, AMPPD, a chemiluminescent substrate of this enzyme, was used for the revelation (Bronstein *et al.*, 1992).

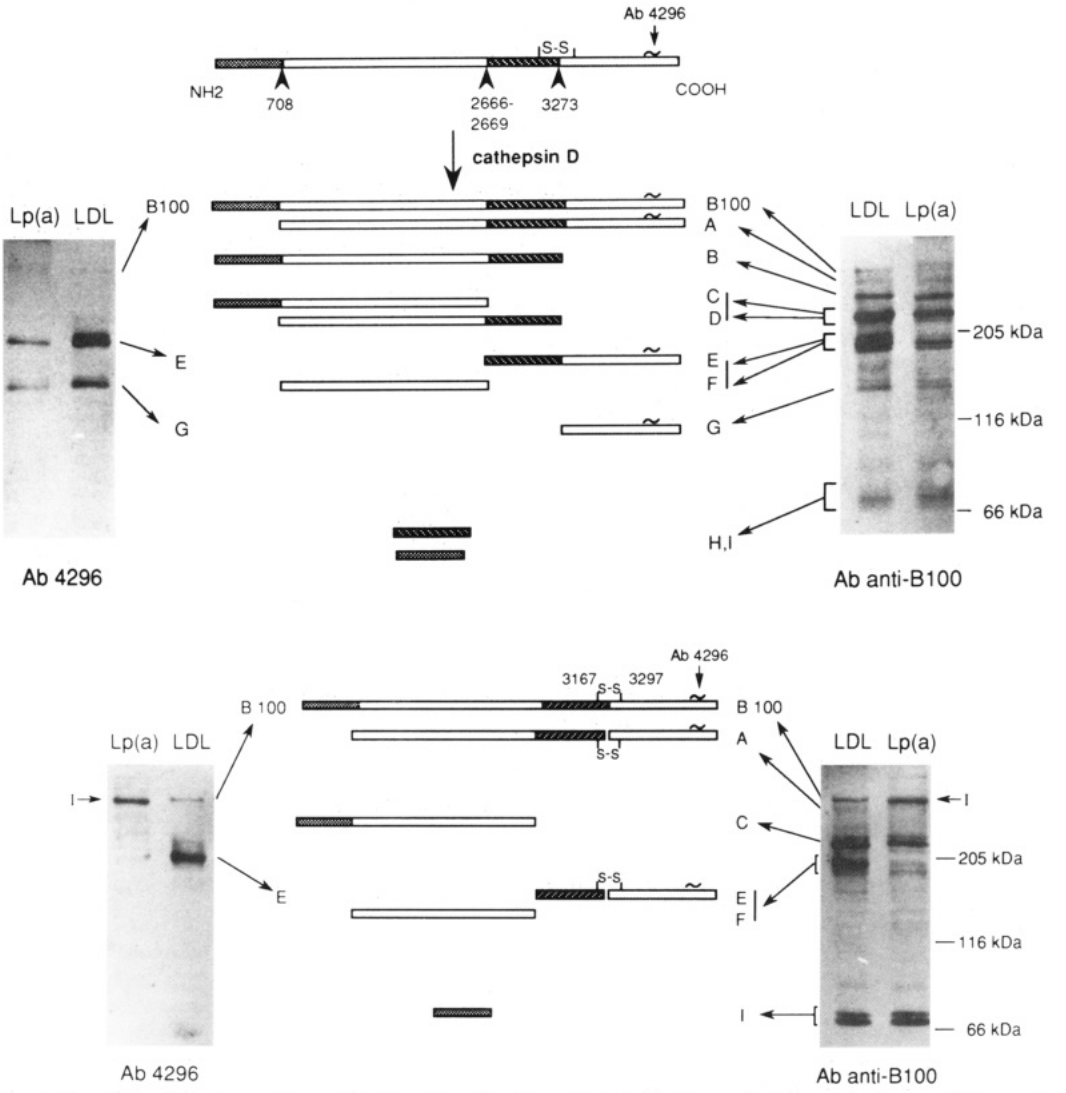

FIGURE 1: Comparison of the digestion profiles of LDL and Lp(a) with cathepsin D. (A, top) Under reducing conditions, samples were treated with 10 mM DTT. (B, bottom) Under nonreducing conditions, samples were loaded on the gel without DTT. Digestions were performed at 37 °C for 60 or 75 min for LDL and Lp(a), respectively (E:S = 1:600). LDL and Lp(a) were 0.5 mg/mL in protein, and 2 μg of protein was layered per lane. The mobilities of known apo B-100 fragments are indicated by arrows, and the fragments have been named from A to I from high to low molecular masses. The bands were detected using a chemiluminescent substrate of alkaline phosphatase, AMPPD. The panels on the right were revealed with the polyclonal anti-apo B-100 antibody, and the panels on the left with antibody 4296. Exposure time was 15 min for revelation with anti-apo B-100 antibody, and 2 h for antibody 4296.

RESULTS

Comparison of Digestion Patterns of Apo B-100 in LDL or Lp(a) under Reducing and Nonreducing Conditions. Certain exposed peptide regions of apo B-100 are highly susceptible to proteolytic cleavage in LDL, as demonstrated by Chen *et al.* (1989). Indeed, partial proteolysis of apo B-100 in LDL and in Lp(a) by cathepsin D resulted in a limited number of distinct bands, indicating that apo B-100 possesses a limited number of cleavage sites specific for this enzyme (Figure 1A). The digestion pattern of LDL by cathepsin D was comparable to the results of Chen *et al.* (1991) under reducing conditions. Apo B-100 of LDL is cleaved by cathepsin D at three different sites (residues 708, 2666 and/or 2669, and 3273), generating nine fragments under reducing conditions (Figure 1A, right). However, for clarity we have used a different nomenclature, and named the fragments A to I, the largest being A and the smallest being I.

Under reducing conditions, LDL and Lp(a) exhibited the same patterns of digestion of apo B-100 when revealed with the polyclonal anti-apo B-100 antibody (Figure 1A, right). The nine fragments were visible in both cases although not all with the same intensity. In particular, fragment A was not always visible, and several bands migrating at the level of fragments H and I could be due to atypic cleavages. To assess the validity of a direct comparison of the digestion patterns of apo B-100 in LDL and in Lp(a), we used different sequence-specific antibodies directed against the N-terminal, middle, and C-terminal parts of apo B-100 [see Experimental Procedures and Innerarity *et al.* (1987)]. The different patterns obtained for LDL and Lp(a) with these various antibodies were strictly superimposable. In particular, antibody 4296, raised against a synthetic peptide covering amino acids 4004–4021 of apo B-100, specifically recognized the COOH-terminal end of apo B-100 (Innerarity *et al.*, 1987). This antibody typically revealed only three fragments, A, E, and G, under reducing conditions (Figure 1A, left). In order to clearly detect the small fragments E and G, apo B-100 was almost entirely digested which explains its presence in trace amounts in Figure 1A. Fragment A was also only faintly visible as when revealed with anti-apo B-100 antibody (Figure 1A, right). Again, the same fragments were visible on digestion patterns of both LDL and Lp(a).

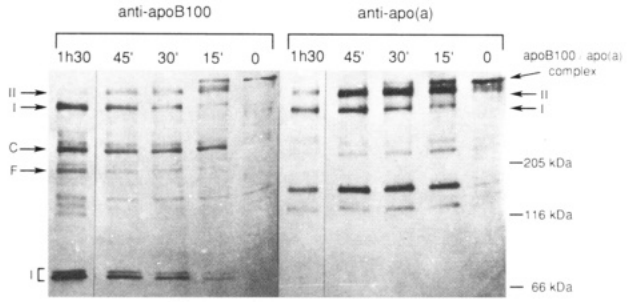

FIGURE 2: Digestion patterns of Lp(a) under nonreducing conditions. Digestion of Lp(a) (0.5 mg/mL) was carried out at 37 °C for 90 min with cathepsin D (E:S = 1:400). At the indicated times, two aliquots of 2 μg were taken from the digestion and analyzed by SDS–PAGE (4–7%). Both gels were electrophoresed at the same time; the proteins were transferred onto nitrocellulose and revealed with either anti-apo B-100 (left) or anti-apo(a) (right) antibodies. The position of the size markers is indicated on the right. The position of the known fragments of apo B-100 is indicated at the left. The same nomenclature was used as in Figure 1.

Under nonreducing conditions, the pattern was different (Figure 1B). Indeed, in the absence of DTT, the fragments linked by a disulfide bond remain associated and migrate at the same position as the corresponding noncleaved fragment. In particular, one disulfide bond in apo B-100 involves Cys-3167 and -3297 which flank one of the cleavage sites (at position 3273). Consequently, the individual fragments linked by this disulfide bond disappeared from the digestion profile. Only five major fragments were now visible on the apo B-100 digestion pattern of LDL when revealed with the polyclonal anti-apo B-100 antibody (Figure 1B, right).

Also, the apo B-100 digestion profiles of Lp(a) and LDL obtained under nonreducing conditions were not the same. In particular, one fragment migrating at the E/F level was absent. To determine which fragment was missing, antibody 4296 was used. Fragment E was still present in the LDL digestion profile, but was absent from the Lp(a) digestion profile (Figure 1B, left). Instead, a fragment of higher molecular mass, migrating just below apo B-100, and which we named band I (Figure 1B, left), was revealed in the Lp(a) profile, and absent in the LDL profile.

Fragment E of apo B-100 could be absent from the Lp(a) profile because it was linked to apo(a) or fragments of apo(a), via a disulfide bond. This hypothesis may also explain the origin of band I in the Lp(a) profile which could correspond to this association of apo B-100 and apo(a) fragments. Such complexes should be revealed by both anti-apo B-100 and anti-apo(a) antibodies.

Figure 2 shows the time course of digestion with cathepsin D of one Lp(a), revealed with either anti-apo B-100 or anti-apo(a) antibodies under nonreducing conditions. At time zero, only one band of very high molecular mass was revealed by both antibodies, which correspond to the apo B-100/apo(a) complex. At the end of the time course, the anti-apo B-100 antibody revealed the same pattern as that shown in Figure 1B, right. At intermediate times, one additional band (band II) was visible between the apo B-100/apo(a) complex and band I; these three bands were also revealed with the anti-apo(a) antibody, which indicated that they contained both apo B-100 and apo(a), or fragments of these proteins.

Digestion Profiles of Apo(a) under Reducing and Nonreducing Conditions. The same experimental procedure of partial digestion was used to examine the structure of the other protein component of Lp(a), apo(a). The marked polymorphism of this apolipoprotein necessitated the comparison of digestion profiles of several isoforms. Thus, several

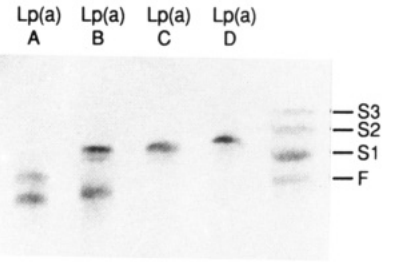

FIGURE 3: Determination of apo(a) isoforms of Lp(a)s. Proteins (100–400 ng) were loaded in the presence of 10 mM DTT prior to SDS–PAGE (3.7%). After transfer to a nitrocellulose membrane, apo(a) was probed with a polyclonal anti-apo(a) antibody and revealed with an alkaline phosphatase-conjugated rabbit anti-sheep Ig-G. The chemiluminescent substrate AMPPD was used for the revelation, and the exposure time was 20 min. An Lp(a) phenotyping standard (Immuno-France) containing isoforms F, S1, S2, and S3 [according to the nomenclature of Utermann *et al.* (1987)] was used as a reference.

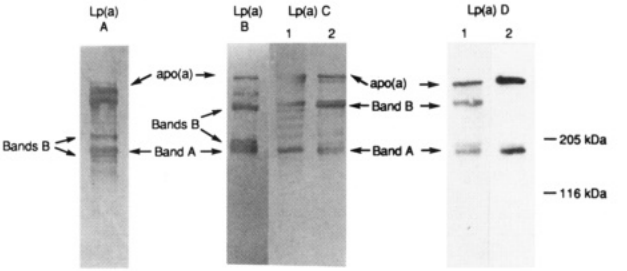

FIGURE 4: Digestion profiles of apo(a) in Lp(a) under reducing conditions. Lp(a)s A, C (lane 1), and D were digested with thermolysin (E:S = 1:2000) for 60 min at 37 °C. Lp(a)s B and C (lane 2) were digested with cathepsin D (E:S = 1:600) for 60 min at 37 °C. Protein concentration was 0.5 mg/mL. After enzymatic digestion, the reaction was stopped and 2-μg aliquots were treated with DTT (10 mM) prior to SDS–PAGE (4–7%) as described under Experimental Procedures. After transfer to nitrocellulose, fragments were revealed with an anti-apo(a) antibody. Lp(a) D, lane 2, was revealed with an anti-kringle V antibody. The positions of molecular mass standards are indicated on the right. The positions of the major digestion fragments A and B for each Lp(a) are indicated by arrows. The bands at the top of each gel correspond to the position of the intact apo(a) isoforms.

individual preparations of Lp(a) were used (Figure 3). Subjects A and B were heterozygous and displayed two apo(a) isoforms each. Subject C had only one isoform which migrated at the same level as the largest isoform of subject B. Subject D displayed also one isoform of high molecular mass. The smallest isoform of subject B had an intermediate migration between the two isoforms of subject A. These last three isoforms are of very low molecular mass since they migrate below or at the same level as the standard F isoform, which is one of the smallest isoforms described (Utermann *et al.*, 1987). However, it was difficult to attribute an exact molecular weight to each isoform, due to the high glycosylation content of apo(a) which modifies its migration on polyacrylamide gels. These four different Lp(a)s were cleaved with thermolysin and cathepsin D.

Under reducing conditions, the digestion profiles of Lp(a)s, revealed with anti-apo(a) antibody, showed one common feature: irrespective of the proteolytic enzyme used and the apo(a) isoform tested, one characteristic band of approximately 170 kDa (band A) was always present (Figure 4). In addition, other major bands denoted as bands B were present; their size and their number seemed to be related to the specific phenotype of the digested Lp(a). Lp(a)s C and D, which presented only one isoform, had only one band B, in contrast to Lp(a)s A and B, presenting two isoforms and two bands B. Moreover, the

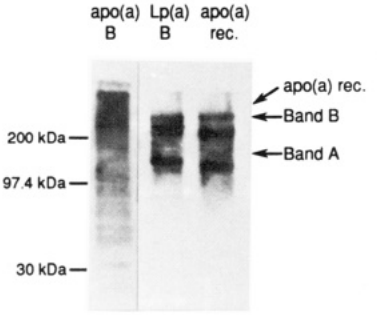

FIGURE 5: Digestion profiles of apo(a) under reducing conditions. Apo(a) was purified from Lp(a) as described under Experimental Procedures. Proteins were digested with thermolysin (E:S = 1:2000) for 30 min at 37 °C. After treatment with 10 mM DTT, 2 μg of each sample was analyzed by SDS–PAGE (4–20%). Proteins were transferred on nitrocellulose and detected with sheep polyclonal anti-apo(a) antibody and alkaline phosphatase-conjugated rabbit anti-sheep IgG. The bands were detected using AMPPD. The exposure time was 15 min. The smallest band of apo(a) B was detected at 28 kDa.

size of the B bands was a function of the size of the isoform: the smaller the isoform, then the smaller the band B.

As for apo(a) isoforms, their high glycosylation content made it difficult to measure precisely the molecular mass of these bands. However, under reducing conditions, the sum of bands A and B equalled approximately the mass of the corresponding isoform. Each band B could consequently be the fragment complementary to band A (Figure 4).

In addition to these two major bands, minor bands were also present, clearly visible above, but also present below, band A. These minor bands had equidistant migrations corresponding (when measurable) to a size difference between bands of approximately 12–14 kDa.

Major bands A and B appeared very early during the time course of digestion, under conditions in which apo B-100 was only slightly cleaved. However, these bands were very resistant to further proteolysis and were still visible even under conditions in which apo B-100 was almost completely digested (data not shown).

In order to determine the respective localization of bands A and B in the primary structure of apo(a), a specific anti-kringle V antibody was used. As shown in Figure 4, this antibody recognized apo(a) and band A but not the B band. Consequently, band A corresponds to the COOH-terminal end of apo(a).

The digestion of a recombinant apo(a) exhibited a comparable pattern (Figure 5). Two major bands were also visible; however, fragment A migrated slightly faster than in the case of Lp(a) digests (approximately at 150 kDa). On the contrary, apo(a) purified from Lp(a) by reduction with DTT followed by heparin–Sepharose chromatography exhibited a completely different digestion profile (Figure 5). No major bands were present, but the protein was cleaved into regular fragment multiples of 11–13 kDa. Using a 4–20% gradient gel, it was possible to detect the smallest band at 28 kDa.

Under nonreducing conditions, the apo(a) digestion profiles were completely different (Figure 6). There were no more bands migrating at 170 kDa, but high molecular mass complexes were revealed with either anti-apo(a) or anti-kringle V antibodies (Figure 6). As detailed in the previous section, these complexes (bands I and II) were constituted of apo B-100 and apo(a) fragments (Figure 2). Since anti-kringle V antibody could reveal these complexes, fragment A is part of them. In addition to these high molecular mass complexes, smaller bands approximately between 120 and 250 kDa were

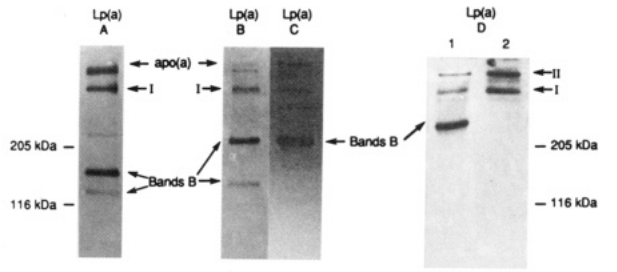

FIGURE 6: Digestion profiles of apo(a) under nonreducing conditions. Conditions were identical to those described in Figure 4 with the exception that samples were loaded without DTT and that all LP(a)s were digested with cathepsin D. Apo(a) fragments were revealed either by a polyclonal anti-apo(a) antibody [Lp(a) A, B, C, and D; lane 1) or by a monoclonal anti-kringle V antibody [Lp(a) D, lane 2]. Bands I and II, denoted by arrows, were revealed by both antibodies.

also revealed. Although they do not have the same migration as under reducing conditions, these bands have properties in common with bands B. First, these bands were not revealed with anti-kringle V antibody. Then, if the digested Lp(a) presented only one isoform [as Lp(a)s C and D], only one band of digestion could be seen; in contrast, when two isoforms were present [Lp(a)s A and B], two bands were visible (Figure 6). Moreover, the two Lp(a)s which have one isoform migrating at the same level [the largest isoform of Lp(a) B and the isoform of Lp(a) C] also presented one fragment of identical migration, which was the largest one for Lp(a) B. Finally, the size of these bands is related to the size of the apo(a) isoform of the digested Lp(a). The smallest isoform of Lp(a) B had an intermediate migration compared to both isoforms of Lp(a) A, and the fragments behaved similarly, the smallest fragment of Lp(a) B being intermediate between both bands of Lp(a) A. Consequently, we named these bands as bands B, as in the case of patterns obtained under reducing conditions.

When the same digestion was revealed with anti-apo B-100 antibodies, no fragments migrating like bands B were seen, indicating that these fragments of variable size were not linked to apo B-100 (Figure 2).

DISCUSSION

Among the major findings of our study was the observation that apo(a) can be cleaved into two structural domains: One was of constant size (170 kDa), which corresponded to the C-terminal part of apo(a), and was linked to apo B-100. The other domain, whose size varied according to the apo(a) isoform digested, was not linked to apo B-100. Finally, the structure of apo(a) could exist independently of interactions with apo B-100 or with lipids in the Lp(a) particle.

Apo B-100 and apo(a) are thought to be linked by one or several disulfide bonds. Gaubatz *et al.* (1983) first expressed this hypothesis after showing that apo(a) mobilities in SDS–PAGE in the reduced and nonreduced states were different. However, internal disulfide linkage within the kringles of apo(a) itself could be enough to interlock both proteins without any direct covalent bonding to apo B-100. Several studies, using sulfhydryl-selective fluorescence labeling (Sommer *et al.*, 1991; Coleman *et al.*, 1990; Guevara *et al.*, 1993), have been performed in order to determine the precise cysteine of apo B-100 involved in the disulfide bond with apo(a). The most recent one (Guevara *et al.*, 1993), involving a comparison of sulfhydryl-selective fluorescence-labeled LDL or Lp(a), provided additional evidence in favor of the involvement of cysteine-3734 of apo B-100 in the disulfide bond with apo(a).

Direct comparison of the digestion profiles of LDL and Lp(a), under reducing and nonreducing conditions, enabled us to confirm these results. Fragment E, which contains Cys-3734, was present in the digestion profile of LDL but was absent from that of Lp(a) (Figure 1B). Also, fragments of large molecular mass were present in the Lp(a) profile and not in that of LDL. These large fragments could be revealed either with anti-apo B-100 or with apo(a) antibodies, indicating that they contain fragments of both proteins.

We also studied the structure of apo(a) using the same approach of partial proteolytic digestion. In contrast to apo B-100, little is known about the structure of apo(a), although its primary structure has been deduced from cDNA sequencing (McLean *et al.*, 1987). To date, the only structural domain described in apo(a) has been the kringle. However, our results show that larger structural domains can be defined.

Two proteolytic enzymes of different specificity, thermolysin and cathepsin D, easily cleave apo(a) into two major fragments that we called A and B (Figure 4). These bands are very resistant to proteolysis, and could constitute structural domains of apo(a). Using different Lp(a)s representing different isoforms, we have shown that domain A is of constant size irrespective of the size of the apo(a) isoform, in contrast to domain B which is of variable size. This finding indicates that the cleavage site could be the same for all apo(a)s, but as the size of the protein differs from isoform to isoform, one of the fragments is of variable size. The fact that two isoforms of the same migration, from two different Lp(a)s, give rise to fragments B of identical size sustains this hypothesis. Using an anti-kringle V antibody, we have demonstrated that kringle V is part of fragment A which consequently represents the COOH-terminal part of apo(a) (Figure 4).

It is notable that partial digestion of a recombinant apo(a) gives rise to a pattern comparable to that of digested apo(a) in Lp(a) (Figure 5). Two major bands are visible, but fragment A has a slightly faster migration than that from Lp(a). This could result from a deficit in glycosylation of the recombinant apo(a). Indeed, it was produced from embryonic kidney cells which do not necessarily have the same glycosylation system as hepatocytes, the natural production site of apo(a). Under these conditions, recombinant apo(a) is produced as a protein. Consequently, the fact that recombinant apo(a) and authentic apo(a) in Lp(a) have similar cleavage patterns is an indication that the protection of the domains in Lp(a) is not due to interactions of apo(a) with lipids or with apo B-100. Also, the fact that the digestion profiles of apo B-100 were identical in LDL and Lp(a) suggests that apo(a) in Lp(a) does not protect apo B-100 against proteolysis. These results are in good agreement with the model described by Phillips *et al.* (1993) indicating that apo(a) in Lp(a) is extended out into the aqueous phase. However, it appears essential that the structure of the kringles remains intact in order to maintain the structural domains of apo(a). Indeed, apo(a) prepared from Lp(a) reduced with DTT has a completely different digestion pattern (Figure 5). No major bands are apparent, but apo(a) is cleaved at numerous sites. Measurement of the size interval between two adjacent bands gives an apparent molecular mass of 11–13 kDa. This interval could correspond to the size of a kringle domain, but our experiments cannot distinguish between intra- or interkringle cleavage sites.

In addition to the numerous cysteines involved in disulfide bonds to maintain the kringles' structure, one extra cysteine is present in kringle 36 of the sequenced apo(a) compared to plasminogen (McLean *et al.*, 1987). This extra cysteine could be the potential, and only, candidate for a disulfide bond

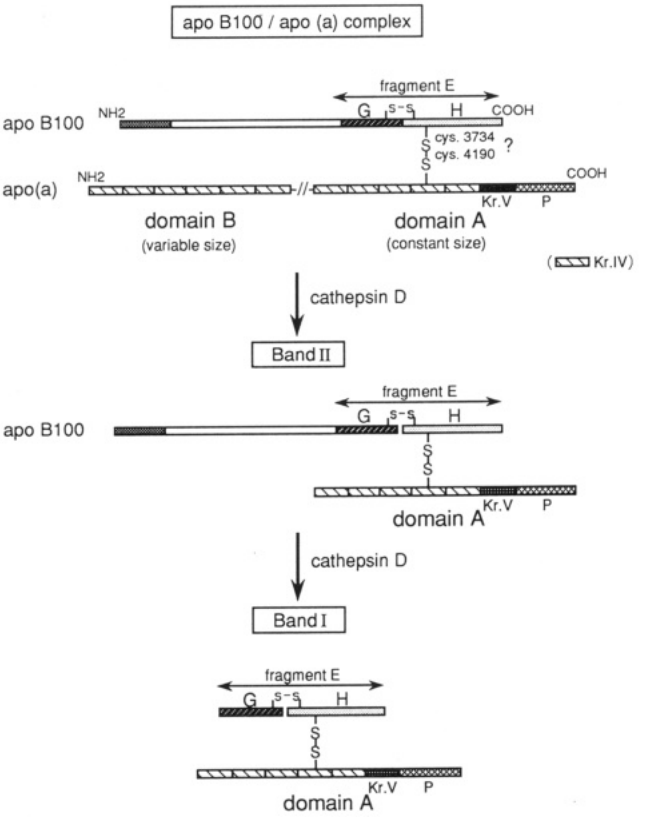

FIGURE 7: Schematic representation of the process of digestion of the apo B-100/apo(a) complex in the Lp(a) particle by cathepsin D.

between apo(a) and apo B-100. Indeed, if the structural organization of apo(a) can be strictly compared to that of plasminogen, then no other free cysteines are present in apo(a) since all the cysteines of the protease domain are also involved in disulfide bridges in plasminogen (Henkin *et al.*, 1991). However, it should be noted that the structural comparison is based on the sequence of only one apo(a) cDNA and the marked polymorphism of this apolipoprotein could also include punctual mutations. Consequently, it cannot be excluded that apo(a) and apo B-100 were linked by more than one disulfide bond.

The comparison of digestion patterns of apo(a) under reducing and nonreducing conditions has given indications on the interaction between apo(a) and apo B-100 in the particle. Domain A is present under reducing conditions, and absent under nonreducing conditions (Figures 4 and 6). Moreover, the anti-kringle V antibody revealed high molecular mass complexes under nonreducing conditions. Thus, domain A seems linked to apo B-100 via a disulfide bond. Consequently, the complex present in band I (Figures 2 and 6) could be constituted of domain A of apo(a) and fragment E of apo B-100.

In contrast, the variable domains B do not seem to be linked to apo B-100. Under nonreducing conditions, these fragments are not associated with apo B-100 fragments (Figure 2). The migration of these domains B is different under reducing and nonreducing conditions. However, they migrate faster under nonreducing conditions than under reducing conditions, indicating that they are not linked to apo B-100 fragments. This difference can be explained by the destruction of the kringle' structures under reducing conditions, leading to a less globular structure of the polypeptide, which migrates therefore more slowly on SDS–PAGE.

Taken together, our results can be summarized as shown in Figure 7. During proteolysis, the apo B-100/apo(a) complex

in the Lp(a) particle is cleaved to band II which appears very early during the time course of digestion. Among the two proteins, apo(a) and apo B-100, apo(a) is the most rapidly cleaved. Consequently, band II could be constituted by the entire apo B-100 and by one of the major fragments generated by the cleavage of apo(a). This fragment is fragment A, the C-terminal end of apo(a), since band II is revealed with the specific anti-kringle V antibody. During the time course of digestion, the cleavage of apo B-100 occurs more slowly than that of apo(a), and band I could result from the partial cleavage of apo B-100 in the band II complex.

It is known from the primary sequence of apo(a) (McLean *et al.*, 1987) that amino acids constituting the catalytic site of the serine protease domain are maintained in apo(a) as compared to plasminogen. However, apo(a) cannot be activated, a mutation at the cleavage site impairing the cleavage. Our results indicate that apo(a) can be easily cleaved into two domains. Furthermore, the cleavage site of apo(a) gives rise to a C-terminal fragment which is larger than the protease domain alone. However, we cannot exclude the possibility that elimination of a large domain containing the majority of the kringles induced a structural change, which could render the protease domain active. Work is currently in progress to test if other physiological proteolytic enzymes give rise to the same cleavage. The purification of domain A will also allow us to determine if it presents a serine protease activity.

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REFERENCES

Armstrong, V. W., Walli, A. K., & Seidel, D. (1985) *J. Lipid Res. 26*, 1314–1323.
Berg, K. (1963) *Acta Pathol. Microbiol. Scand. 59*, 369–382.
Boerwinkle, E., Leffert, C. C., & Lin, J. (1992) *J. Clin. Invest. 90*, 52–60.
Bradley, W. A., & Gianturco, S. H. (1986) *J. Lipid. Res. 27*, 40–48.
Bronstein, I., Voyta, J. C., & Murphy, O. J. (1992) *BioTechniques 12*, 748–753.
Chapman, M. J., Laplaud, P. M., & Luc, G. (1988) *J. Lipid Res. 29*, 442–458.
Chatterton, J. E., Phillips, M. L., & Curtiss, L. K. (1991) *J. Biol. Chem. 266*, 5955–5962.
Chen, G. C., Zhu, S., & Hardman, D. A. (1989) *J. Biol. Chem. 264*, 14369–14375.
Chen, G. C., Lau, K., & Hamilton, R. L. (1991) *J. Biol. Chem. 266*, 12581–12587.
Coleman, R. D., Kim, T. W., & Gotto, A. M., Jr. (1990) *Biochim. Biophys. Acta 1037*, 129–132.
de Vos, A. M., Ultsch, M. H., & Kelley, R. F. (1992) *Biochemistry 31*, 270–279.
Faucher, C., Doucet, C., & Baumelou, A. (1993) *Am. J. Kidney Dis. 22*, 808–813.
Fless, G. M., ZumMallen, M. E., & Scanu, A. M. (1985) *J. Lipid Res. 26*, 1224–1229.
Fless, G. M., ZumMallen, M. E., & Scanu, A. M. (1986) *J. Biol. Chem. 261*, 8712–8718.
Floren, C. H., Albers, J., & Bierman, E. L. (1981) *Biochem. Biophys. Res. Commun. 102*, 636–639.
Gaubatz, J. W., Heideman, C., & Gotto, A. M. (1983) *J. Biol. Chem. 258*, 4582–4589.
Gaubatz, J. W., Chari, M. V., & Nava, M. L. (1987) *J. Lipid Res. 28*, 69–79.
Guevara, J., Spurlino, J., & Jan, A. Y. (1993) *Biophys. J. 64*, 686–700.
Guo, H. C., Armstrong, V. W., & Luc, G. (1989) *J. Lipid Res. 30*, 23–37.
Havekes, L., Vermeer, B. J., & Brugman, T. (1981) *FEBS Lett. 132*, 169–173.
Henkin, J., Marcotte, P., & Yang, H. (1991) *Prog. Cardovasc. Dis. 34*, 135–164.
Innerarity, T. L., Young, S. G., & Poksay, K. (1987) *J. Clin. Invest. 80*, 1794–1798.
Knott, T. J., Pease, R. J., & Powell, L. M. (1986) *Nature 323*, 734–738.
Koschinsky, M. L., Tomlinson, J. E., & Zioncheck, T. F. (1991) *Biochemistry 30*, 5044–5051.
Laemmli, F. K. (1970) *Nature 227*, 680–685.
Lowry, V. H., Rosebrough, N. J., & Farr, A. L. (1951) *J. Biol. Chem. 193*, 265–275.
Marcel, Y. L., Innerarity, T. L., & Spilman, C. (1987) *Arteriosclerosis 7*, 166–175.
Martmann-Moe, K., & Berg, K. (1981) *Clin. Genet. 20*, 352–362.
McLean, J. W., Tomlinson, J. E., & Kuang, W. J. (1987) *Nature 330*, 132–137.
Mulichak, A. M., Tulinsky, A., & Ravichandran, K. G. (1991) *Biochemistry 30*, 10576–10588.
Phillips, M. L., Lembertas, A. V., & Schumaker, V. N. (1993) *Biochemistry 32*, 3722–3728.
Seman, L. J., & Breckenridge, W. C. (1986) *Biochem. Cell Biol. 64*, 999–1009.
Sommer, A., Gorges, R., & Kostner, G. M. (1991) *Biochemistry 30*, 11245–11249.
Utermann, G. (1990) *Curr. Opin. Lipidology 1*, 404–410.
Utermann, G., Menzel, H. J., & Kraft, H. G. (1987) *J. Clin. Invest. 80*, 458–465.
Xiong, W., Zsigmond, E., & Gotto, A. M. (1991) *J. Biol. Chem. 266*, 20893–20898.
Zawadzki, Z., Tercé, F., & Seman, L. J. (1988) *Biochemistry 27*, 8474–8481.