Characterization of the N-Terminal and C-Terminal Domains of Human Apolipoprotein(a): Relevance to Fibrin Binding†

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ABSTRACT: The structural domains of human apolipoprotein(a) (apo(a)) in the lipoprotein(a) (Lp(a)) particle have been recently investigated by limited proteolysis [Huby, T., Doucet, C., Dieplinger, H., Chapman, J., & Thillet, J. (1994) *Biochemistry 33,* 3335−3341]. We have shown that apo(a) can be cleaved into two structural domains: one was of constant size (170 kDa) and corresponded to the C-terminal (C_{ter}) domain of apo(a). This domain was linked by a disulfide bond to apo B100. By contrast, the N-terminal (N_{ter}) domain, whose size varied according to the digested apo(a) isoform, was not linked to apo B100. We now describe the purification of these apo(a) domains and their interaction with fibrin surfaces in an *in vitro* binding assay. The N_{ter} domain of apo(a) was purified as a soluble protein in a two-step procedure which involved sequential use of a heparin−Sepharose column and a lysine−Sepharose column. The C_{ter} domain of apo(a), which remained in disulfide linkage with apo B100 of Lp(a), was isolated as a lipoprotein particle by a combination of chromatographic steps on heparin−Sepharose and Q-Sepharose columns. This particle, termed "mini-Lp(a)", appeared homogeneous in nondenaturing polyacrylamide gels and exhibited a particle size (285 Å) which was intermediate between that of Lp(a) (300 Å) and LDL (265 Å). The cleavage site between the respective apo(a) domains was determined by N-terminal sequencing of the purified C_{ter} domain. Such cleavage occurred between residues 3532 and 3533, which are located in the interkringle region between apo(a) kringles 4_4 and 4_5. Consequently, the C_{ter} domain of apo(a) was composed of kringles 4_5 to 4_{10}, kringle V, and the protease domain. The binding properties of the purified N_{ter} domains and mini-Lp(a) were investigated on intact and on plasmin-modified fibrin and compared to those of Lp(a). We demonstrated that the C_{ter} domain, in the form of mini-Lp(a), binds to fibrin in a lysine-specific manner that approached saturation, in contrast to the N_{ter} domains which did not bind either to fibrin or to plasmin-degraded fibrin. The apparent K_d for the mini-Lp(a) (380 ± 30 nM) was slightly different from that of Lp(a) (150 ± 15 nM) in binding to either plasmin-degraded or intact fibrin. This finding indicates that the C_{ter} domain of apo(a) mediates the interaction of Lp(a) with fibrin. On intact fibrin, the B_{max} values for Lp(a) and for mini-Lp(a) were 8.5 and 25 fmol/well, respectively; these values increased, upon plasmin digestion of fibrin, to 25 and 100 fmol/well, respectively. The increased number of accessible binding sites in the case of mini-Lp(a) may result from a decreased steric hindrance as compared to Lp(a).

Elevated plasma levels of lipoprotein(a) (Lp(a)) have been correlated with the development of coronary artery disease (Scanu, 1988; Utermann, 1990). This lipoprotein was first described by Berg in 1963 (Berg, 1963). Lp(a) consists of an LDL-like particle containing one additional highly glycosylated protein, called apolipoprotein(a) (apo(a)), which is linked to apo B100 by a single disulfide bond (Brunner *et al.*, 1993; Guevara *et al.*, 1993; Koschinsky *et al.*, 1993). The primary structure of this protein was deduced from the sequence of a cloned apo(a) cDNA (McLean *et al.*, 1987). The apo(a) cDNA contained 37 copies of a sequence which closely resembles the fourth kringle of plasminogen, followed by sequences highly similar to the kringle V and protease domains of plasminogen. The apo(a) kringles have been classified into ten kringle 4 subtypes which differ in both their respective amino acid substitutions and in their relation to the plasminogen kringle 4 primary sequence (Guevara *et al.*, 1992; McLean *et al.*, 1987). With the exception of the first kringle 4 ($K4_1$), the next 28 kringles exhibited the same primary sequence ($K4_2$), while the last 8 kringles ($K4_3$ to $K4_{10}$) differed by amino acid substitutions from $K4_2$. The apo(a) gene exhibits a high degree of size polymorphism, and indeed 34 different alleles have been described at this locus (Lackner *et al.*, 1993). Similarly, 34 different isoforms have been detected at the protein level (Marcovina *et al.*, 1993). Van der Hoek *et al.* (1993) have recently demonstrated that sequences corresponding to apo(a) $K4_1$ and $K4_3$ to $K4_{10}$ are present at the mRNA level in all individuals. Therefore, the size heterogeneity of apo(a) has been attributed to a difference in the number of the identically repeated kringle 4_2.

A kringle is a highly conserved protein motif represented as a triple loop polypeptide structure in which the amino acid chain is interconnected by three disulfide bonds. Kringle domains exhibit essentially specific ligand binding

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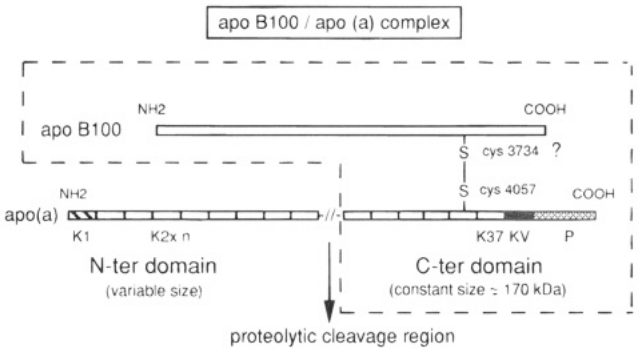

FIGURE 1: Schematic representation of the apo(a)/apo B100 complex showing the cleavage of apo(a) into two domains after proteolytic digestion of the Lp(a) particle. Under nonreducing conditions, the C_{ter} domain remains associated with apo B100.

function. It has been demonstrated that Lp(a) is able to compete with plasminogen for binding to both fibrin (Harpel *et al.*, 1989; Rouy *et al.*, 1991) and cell surfaces (Hajjar *et al.*, 1989; Miles *et al.*, 1989). Other studies have also suggested that Lp(a) can inhibit the direct activation of plasminogen by t-PA (Edelberg *et al.*, 1990) and the plasmin generating activity of streptokinase (Edelberg *et al.*, 1989). These complex and multiple interactions can be promoted by various parts of Lp(a) and/or by different kringles.

We have recently demonstrated that apo(a) in the Lp(a) particle is composed of two structural domains (Huby *et al.*, 1994). This finding was obtained following analysis of partial proteolytic digestion patterns of several Lp(a) particles. Lp(a) digestion profiles, revealed by immunoblotting with a polyclonal anti-apo(a) antibody, have shown that apo(a) was always cleaved into two fragments. Both apo(a) fragments exhibited resistance to further proteolysis and therefore were described as structural domains of apo(a) linked by a region sensitive to enzymatic cleavage. One apo(a) domain was disulfide-linked to apo B100 and was revealed by immunoblotting with a monoclonal antibody specific for the kringle V region of apo(a). This domain constituted the C_{ter} domain of apo(a) and always exhibited a constant size (170 kDa) irrespective of the isoform size of the digested apo(a). The other domain, which was of variable size according to the digested apo(a) isoform, was the N_{ter} domain and was not linked to apo B100. These results are summarized in Figure 1.

In the current study, we describe the purification of both domains of apo(a). The N_{ter} domain has been purified as a soluble protein in the absence of apo B100 or lipids. The C_{ter} domain, which is disulfide-linked to apo B100, has been purified as a lipoprotein particle, termed "mini-Lp(a)". Moreover, we have determined the precise site of cleavage between both domains by protein sequencing. In addition, we assessed the ability of each purified domain to bind either to fibrin or to plasmin-degraded fibrin in an *in vitro* system and compared such binding affinities with those of the corresponding purified Lp(a). Our results indicate that mini-Lp(a) binds both to fibrin and to degraded fibrin in a lysine-specific manner. On the contrary, the purified N_{ter} domains did not bind to either surface.

MATERIALS AND METHODS

Isolation of LDL and Lp(a). LDL and Lp(a) were isolated from the same regenerate fluid eluted from the dextran sulfate

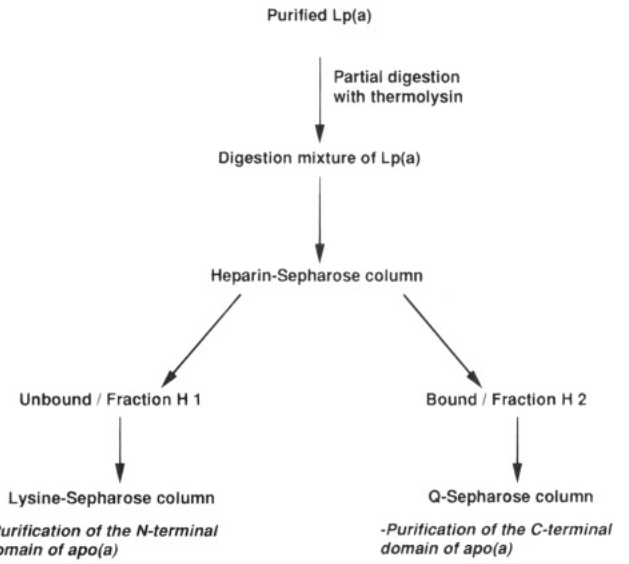

FIGURE 2: Flow diagram summarizing the preparative isolation of the N_{ter} and C_{ter} domains of apo(a).

cellulose columns of an automatic LDL apheresis system. This material was obtained from a unique subject heterozygous for apo(a) isoforms (see below). To prevent degradation of lipoproteins, PMSF (1 mM), AEBSF (Pefablock SC; Interchim; 0.1 mM), BHT (4.4 μg/mL), gentalline (0.005%), NaN_3 (0.01%), and EDTA (0.01%) were added to the regenerate fluid.

Lipoproteins were isolated by sequential ultracentrifugation in a 50.2 Ti rotor at 10 °C for 24 or 48 h at 45 000 rpm. Solvent densities were adjusted with solid KBr. LDL were isolated in the 1.025−1.050 g/mL density interval (24 h centrifugation) and Lp(a) at 1.050−1.100 g/mL (48 h centrifugation).

To eliminate any contamination by Lp(a), LDL were subjected to gel filtration on a column (A15M, Bio-Rad; 90 × 2.5 cm), equilibrated with 50 mM Tris-HCl, 150 mM NaCl, and 0.01% NaN_3 (pH 8). Fractions containing only LDL were pooled, concentrated against Aquacid II (Calbiochem), and dialyzed against PBS (0.01% EDTA, pH 7.4).

The lipoproteins isolated in the 1.050−1.100 g/mL interval were dialyzed against 20 mM Na_2HPO_4 and 0.01% NaN_3 (pH 7.3) and applied to a lysine−Sepharose column (Pharmacia) equilibrated in the same buffer. The column was 10 cm in length and 1.5 cm in diameter, and the flow rate was 25 mL/h. After washing with the same buffer containing 150 mM NaCl, the Lp(a) was eluted from the column with a lysine analogue, ϵ-aminocaproic acid (ϵ-ACA) (200 mM), in a buffer containing 100 mM Na_2HPO_4 and 150 mM NaCl at pH 7.3. The purified Lp(a) was then dialyzed against PBS containing EDTA 0.01%, pH 7.4, to remove ϵ-ACA.

The lipid composition of isolated lipoproteins was determined enzymatically with Biomerieux kits (Marcy l'Etoile, 69280, France). The concentration of total protein was determined by the method of Lowry *et al.* (1951).

Purification of Apo(a) Domains. The different steps of the procedure are shown in Figure 2.

(1) Limited Proteolysis of Lp(a). The limited proteolysis of Lp(a) (1 mg/mL) by thermolysin (Boehringer; 40 units/mg of lyophilizate) was carried out at 37 °C in 125 mM Tris-HCl, 150 mM NaCl, 4 mM $CaCl_2$, and 0.01% NaN_3 (pH 7.8) at a mass ratio of enzyme to substrate of 1:500. The reaction was stopped after 30 min by adding 10 mM

EDTA, and the digestion mixture was dialyzed overnight at 4 °C against the heparin−Sepharose buffer A (see below).

(2) Heparin−Sepharose Column. The digestion mixture dialyzed against 10 mM Tris-HCl, 50 mM NaCl, and 0.01% NaN_3 (pH 7.6) (buffer A) was passed through a heparin−Sepharose column (Pharmacia; 10 × 1 cm) equilibrated in the same buffer. The flow rate was 20 mL/h. The unabsorbed material constituted fraction H1. The bound material was eluted with the equilibrating buffer containing 500 mM NaCl and constituted fraction H2.

(3) Lysine−Sepharose Column. The heparin−Sepharose fraction H1 was concentrated against Aquacid II, dialyzed overnight against 20 mM Na_2HPO_4 and 0.01% NaN_3 (pH 7.3), and passed over a lysine−Sepharose column (1.5 × 10 cm) equilibrated in the same buffer. The flow rate was 20 mL/h. After washing, the NaCl concentration was raised to 150 mM. Then a third peak was eluted with 200 mM ϵ-ACA, 500 mM NaCl, and 100 mM Na_2HPO_4 (pH 7.3).

(4) Q-Sepharose Column. The heparin−Sepharose fraction H2 was dialyzed overnight against 10 mM Tris-HCl and 0.01% NaN_3 (pH 8.0) and passed through a Q-Sepharose column (Pharmacia; 1.5 × 10 cm) equilibrated in the same buffer. The flow rate was 25 mL/h. The elution of different fractions was effected by increasing NaCl concentration in the buffer.

Electrophoresis and Immunoblotting. SDS−PAGE was performed on 4.5% acrylamide slab minigels (Mini protean II, Bio-Rad) using a discontinuous buffer system (Laemmli, 1970). Prior to electrophoresis, samples were combined with glycerol, bromophenol blue, and EDTA to final concentrations of 2%, 0.01%, and 0.5 mM, respectively. Reduced samples were prepared by boiling at 100 °C for 4 min in the presence of 10 mM DTT. Proteins were then electroblotted onto nitrocellulose and revealed by immunoblotting. Fragments of apo B100 and apo(a) were revealed using respectively sheep polyclonal anti-apo B100 and anti-apo(a) antibodies. Both antibodies were peroxidase conjugated, and the revelation was performed by chemiluminescence (ECL-Amersham).

Particle Size Determination. Particle size determination was performed on nondenaturing polyacrylamide gradient gels, 2−16% (PAA 2/16; Pharmacia), according to Chapman *et al.* (1988). Latex beads (38 nm) and a set of standard proteins with known hydrated diameters (thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; lactate dehydrogenase, 81 Å; bovine serum albumin, 71 Å; HMW electrophoresis calibration kit, Pharmacia) were run as a reference marker. From the migration distances of the different lipoproteins and those of the standards, it was possible to calculate the Stokes diameters of lipoprotein particles.

Amino-Terminal Sequence Analysis. Fraction QS2 of the Q-Sepharose column (see Results) was used to determine the amino-terminal sequence of the C_{ter} domain of apo(a). Sample (20−30 μg) was prepared as described above in the Electrophoresis and Immunoblotting section under reducing conditions. Samples were applied to SDS−PAGE using a homogeneous 4.5% acrylamide slab gel (13 × 16 × 0.15 cm). After electrophoresis, proteins were electroblotted onto a poly(vinylidene difluoride) (PVDF) membrane at 90 V for 2 h. CAPS (10 mM; pH 11) containing 10% methanol was used as the transfer buffer to reduce the level of Tris and glycine contamination from the polyacrylamide gel. The PVDF membrane was rinsed several times with Milli-Q H_2O after the transfer. Proteins were then either visualized by staining with Coomassie Blue R250 (0.1% in 50% methanol) or revealed by immunoblotting with anti-apo B100 or anti-apo(a) antibodies. The immunoblots revealed no contamination of apo(a) fragments by apo B100 fragments which could interfere with its sequencing. The stained protein bands were cut out of the membrane and were washed three times with 0.1 mL of a methanol/water (50:50 v/v) solution to remove additional glycine. The membrane pieces were then placed in the blot cartridge of the instrument and sequenced with the standard blot cycle. The sequence was performed with the pulsed liquid phase protein sequencer 473A from Applied Biosystem (USA) with online PTH detection.

Fibrin Binding Assays. Human fibrinogen (American Diagnostic, type L) was depleted of plasminogen by affinity chromatography on lysine−Sepharose and of fibronectin by affinity chromatography on gelatin−Sepharose (Pharmacia) as previously described (Engvall & Ruoslahti, 1977). Fibrinogen was then extensively dialyzed against 100 mM Na_2HPO_4, 300 mM NaCl, and 0.01% NaN_3, pH 7.4, and stored in aliquots (0.46 g/L) at −70 °C.

Microtitration plates (Costar; EIA/RIA high binding) were coated with fibrinogen (100 μL containing 25 μg of protein/mL of 100 mM bicarbonate coating buffer, pH 9.5) and incubated overnight at 4 °C. The wells were washed four times in 50 mM Na_2HPO_4, 150 mM NaCl, 0.01% Tween 20, and 0.2% BSA, pH 7.4 (buffer I). Fibrinogen was then converted into fibrin by treatment with human thrombin (Sigma; 75 μL at 20 NIH units/mL) at 37 °C for 1 h in buffer I containing 1 mM $CaCl_2$. After three washes with buffer I, human plasmin (American Diagnostic; 50 μL at 25 nM for 30 min at 37 °C) was used to degrade fibrin surfaces. The wells were washed three times with buffer I and then incubated (2 × 15 min at room temperature) with 100 μL of buffer I containing the serine protease inhibitor AEBSF (0.5 mM) and ϵ-amino caproic acid (ϵ-ACA; 0.5 M) to respectively inhibit and remove plasmin from fibrin surfaces. The wells were washed with buffer I and then with 50 mM Na_2HPO_4, 150 mM NaCl, 0.01% Tween 20, 0.4% BSA, and 2 mM EDTA, pH 6.8 (buffer II). Various amounts of Lp(a), purified N_{ter} domains, and C_{ter} domain (mini-Lp(a); see Results), diluted in buffer II containing 4% BSA with or without 0.25 M ϵ-ACA, were added to the wells (50 μL) for 16 h at 4 °C. After washing with buffer II and then with buffer I, a polyclonal peroxidase-conjugated anti-apo(a) antibody was added (50 μL/well of a 1/5000 dilution in buffer I). After a 2-h incubation at 37 °C, the wells were washed and 100 μL of the peroxidase substrate, *O*-phenylenediamine dihydrochloride (Sigma; 1 mg/mL dissolved in 50 mM phosphate−citrate buffer containing 0.03% sodium perborate, pH 5.0), was added. The color development was followed at 405 nm with a microtiter plate reader (BIO TEK; kinetics reader EL 340). The results were obtained as the change in absorbance per minute. The molar concentrations were calculated by assuming a molecular mass of 3.4×10^6 Da, 4.6×10^5 Da, and 2.5×10^6 Da for Lp(a), N_{ter} domains, and mini-Lp(a), respectively. Reaction rates of binding assays were then transformed into moles by using standard curves established for each component. Standard curves were constructed by coating 50 μL of Lp(a) or the purified domains in serial dilutions in the wells of microtitration plates

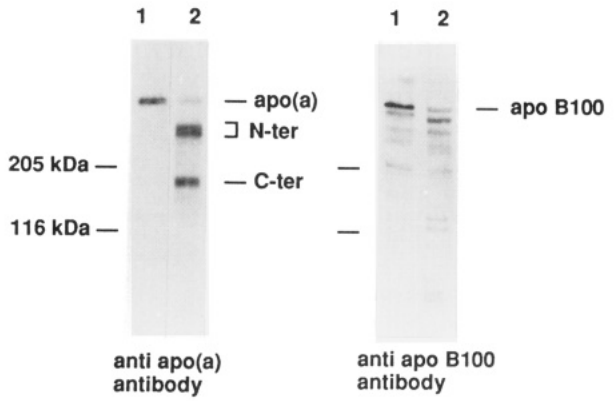

FIGURE 3: Immunoblots of Lp(a) under reducing conditions, before (lane 1) and after (lane 2) proteolytic digestion. Lp(a) (1 mg/mL) was digested with thermolysin (E:S = 1:500) for 30 min at 37 °C. Aliquots of the purified Lp(a) and of the digestion mixture (50 ng for apo(a) detection and 300 ng for apo B100 detection) were loaded on SDS−PAGE (4.5%) after treatment as described in the Materials and Methods section. After transfer to nitrocellulose, proteins were detected with either anti-apo(a) (left) or anti-apo B100 (right) antibodies. The positions of the molecular weight markers (high range; Bio-Rad) are indicated on the left of both immunoblots. The positions of the fragments of apo(a) are indicated on the right of the immunoblots revealed with the anti-apo(a) antibody.

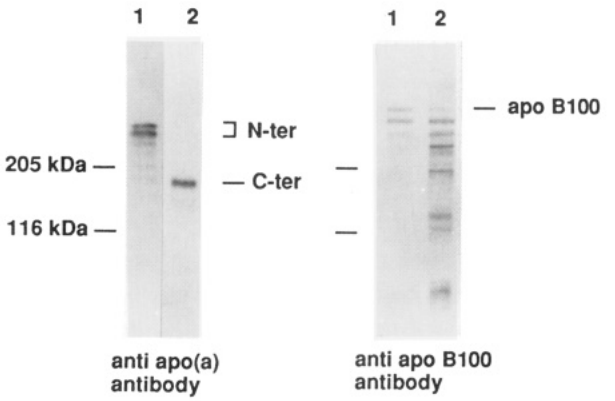

FIGURE 4: Fraction H1 (lane 1) and H2 (lane 2) of heparin−Sepharose column chromatography were analyzed on SDS−PAGE under reducing conditions, as described under Materials and Methods. After electroblotting onto nitrocellulose, they were revealed with either anti-apo(a) (left) or anti-apo B100 (right) antibodies, both peroxidase conjugated. The bands were revealed by using a chemiluminescent reagent. The exposure time was between 10 and 30 s.

(in 100 mM bicarbonate buffer, pH 9.5). After overnight incubation at 4 °C, followed by saturation with 3% BSA in buffer I, the detecting antibody was applied under the same conditions as for the binding assays. The binding isotherms obtained were fitted to the Langmuir equation on the assumption of single-site binding:

$$[\text{Fn-Lp(a)}] = [\text{Fn}_0]\frac{K[\text{Lp(a)}]}{1 + K[\text{Lp(a)}]} \quad (1)$$

where $[\text{Fn}_0]$ represents the total number of fibrin binding sites, [Fn-Lp(a)] the number of molecules of Lp(a) (or domains) adsorbed on fibrin, and K the association constant.

RESULTS

Proteolytic Digestion of Lp(a). Figure 3 shows the characteristic digestion patterns of a representative preparation of Lp(a), under reducing conditions; the blots were revealed with either an anti-apo(a) (left) or an anti-apo B100 antibody (right). The Lp(a) used in this study was isolated from a heterozygous subject and exhibited two apo(a) isoforms of similar size, which were not separated on a 4.5% acrylamide gel but were resolved on a 3.7% acrylamide gel (data not shown). These two isoforms generated two N_{ter} domains of different sizes (Figure 3, left). The apo B100 was cleaved into several fragments (Figure 3, right).

In order to obtain a maximal amount of both domains, the ratio of enzyme to substrate was chosen so as to almost totally digest the apo(a) protein (Figure 3, left). We have previously observed that, during the time course of Lp(a) digestion, the cleavage of apo B100 occurred more slowly than that of apo(a) (Huby *et al.*, 1994). As a matter of fact, entire apo B100 was still visible on the digestion pattern of Lp(a) revealed by the anti-apo B100 antibody (Figure 3, right).

Lp(a) (11.25 mg; protein content) was digested as described in the Materials and Methods section. The reaction was stopped with EDTA. After dialysis, the digestion mixture was loaded onto a heparin−Sepharose column as the first purification step. Two different peaks were eluted. One fraction (fraction H1) was not retained on the column, and the other one was eluted with 500 mM NaCl in the buffer (fraction H2). The protein content of H1 and H2 peaks was analyzed by electrophoresis and immunoblotting with both anti-apo(a) and anti-apo B100 antibodies (Figure 4). Fraction H1 was mainly constituted of the N_{ter} domains of apo(a) but was contaminated by uncleaved and large fragments of apo B100 (Figure 4, lane 1). Fraction H2 (Figure 4, lane 2) was constituted of the C_{ter} domain of apo(a) associated with the same apo B100 fragments as those visible on the digestion profile of Lp(a) (Figure 3).

Purification of N-Terminal Domains. To further purify the N_{ter} domains, fraction H1 from the heparin−Sepharose column was loaded onto a lysine−Sepharose column (Figure 5A). Three fractions were obtained and analyzed (Figure 5B). The first fraction was eluted with the equilibrating buffer (LS1) and contained a small amount of apo B100, faintly visible on the immunoblot (Figure 5B, lane 1). The second fraction (LS2) was eluted by raising the NaCl concentration of the buffer to 150 mM and contained the purified N_{ter} domains without any contaminants (Figure 5B, lane 2). No lipids could be detected in the LS2 fraction. The third fraction (LS3) was eluted from the column with 0.2 M ϵ-ACA. This fraction contained apo B100 fragments as well as residual C_{ter} and N_{ter} domains of apo(a) (Figure 5B, lane 3).

The LS2 fraction presented minor degradation products of the N_{ter} domains under reducing conditions (Figure 5B, left). These bands could be confused with the C_{ter} domain although they did not migrate exactly to the same position. In order to confirm that the N_{ter} domains were free of the C_{ter} domain, fraction LS2 was loaded onto SDS−PAGE without DTT (Figure 5B, lane 4). Under these nonreducing conditions, only two bands were visible; as previously observed (Huby *et al.*, 1994), they migrated faster than under reducing conditions. On the contrary, under nonreducing conditions, the C_{ter} domain migrated more slowly than under reducing conditions, since it remained in association with apo B100 (Huby *et al.*, 1994). Consequently, if the C_{ter} domain had been present as a contaminant in the LS2

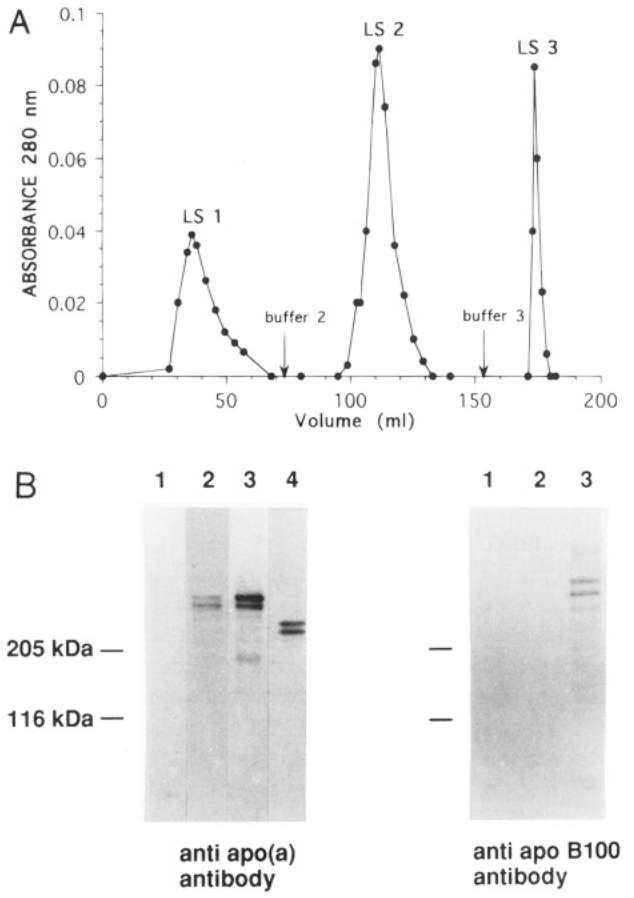

FIGURE 5: (A) Lysine−Sepharose chromatography profile of fraction H1. Fraction H1 (5.5 mL), concentrated with Aquacid II at 0.4 mg of protein/mL and dialyzed against 20 mM Na_2HPO_4 and 0.01% NaN_3, pH 7.3 (lysine−Sepharose buffer 1), was applied onto the column at a flow rate of 20 mL/h. Peak LS1 constituted the protein fraction eluted with buffer 1. The elution of the bound proteins was first achieved with buffer 1 containing 150 mM NaCl (buffer 2) and with 100 mM Na_2HPO_4, 500 mM NaCl, and 200 mM ϵ-ACA, pH 7.3 (buffer 3). These buffers released fractions LS2 and LS3, respectively. (B) Fractions LS1 (lane 1), LS2 (lane 2), and LS3 (lane 3) were loaded onto SDS−PAGE (4.5%), blotted onto nitrocellulose, and revealed with either anti-apo(a) (left) or anti-apo B100 (right) antibodies. Samples were analyzed under reducing conditions, except for fraction LS2 which was loaded under nonreducing conditions (lane 4). The positions of the molecular weight markers are indicated on the left of both blots.

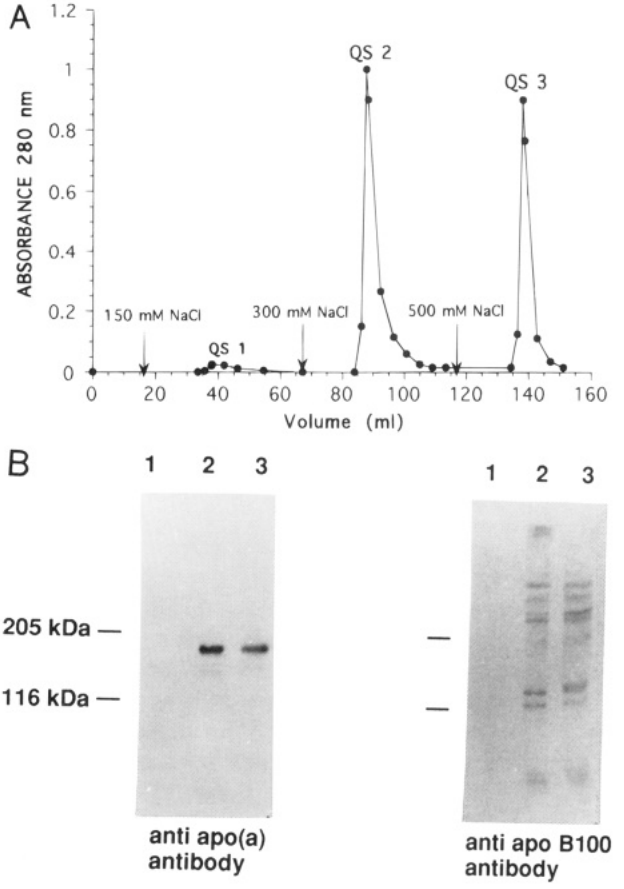

FIGURE 6: (A) Q-Sepharose chromatography profile of fraction H2. The column, equilibrated in 10 mM Tris-HCl and 0.01% NaN_3, pH 8.0, was loaded with 8.5 mL of fraction H2 (0.9 mg of protein/mL), dialyzed against the same buffer, at a flow rate of 25 mL/h. Proteins were eluted from the resin by increasing NaCl concentration in the buffer. Fractions QS1, QS2, and QS3 were eluted with 150, 300, and 500 mM NaCl in the buffer, respectively. (B) Immunoblots of the Q-Sepharose fractions. Each fraction was concentrated, dialyzed against PBS (pH 7.4), and analyzed on SDS−PAGE under reducing conditions. Lane 1, Fr QS1; lane 2, Fr QS2; lane 3, Fr QS3.

fraction, it would have been clearly visible in the upper part of the gel.

Purification of C-Terminal Domain. To further purify the C_{ter} domain of apo(a), fraction H2 from the heparin−Sepharose column was passed over a Q-Sepharose column (Figure 6A). A linear gradient step elution ranging from 0 to 1 M NaCl was first used to establish the optimal purification conditions. Then, three NaCl concentrations were successively applied, giving three fractions. The first fraction (QS1) was eluted with 150 mM NaCl and contained only small proteolytic fragments, which were almost undetectable when analyzed by immunoblotting (Figure 6B, lane 1). The second and the third fractions were eluted with 300 mM (QS2) and 500 mM NaCl (QS3), respectively, in the elution buffer. Electrophoresis and immunoblotting of the QS2 fraction (Figure 6B, lane 2) revealed that it contained the C_{ter} domain, associated with the same apo B100 fragments as those visible on the Lp(a) digestion profile (Figure 3). Fraction QS3 has the same protein composition as fraction QS2. However, when the proteolytic digestion of apo(a) was not complete, the intact apo(a) was recovered in this third fraction.

Fraction QS2 was further analyzed to determine if it was organized as a lipoprotein particle. The chemical composition of two different preparations of this fraction was determined and compared to those of Lp(a) and LDL particles of the same subject (Table 1). The percentage of each of the lipid components of fraction QS2 was within the normal range, and its protein content (29.6 ± 1.6%) was intermediate between that of Lp(a) and LDL (respectively 35 ± 2.1% and 26.2%).

Fraction QS2 was also analyzed on a nondenaturing polyacrylamide gradient gel (Figure 7). The respective sizes of the Lp(a) and the LDL particles of the same subject were estimated as 300 and 265 Å, respectively, while fraction QS2 was revealed as a unique particle exhibiting an intermediate size of 285 Å. We have termed this particle, containing the C_{ter} domain of apo(a) associated with the apo B100 fragments and lipids, "mini-Lp(a)".

The purification yields of both domains for one typical experiment are given in Table 2. Twenty percent and 70% of the total amount of protein loaded onto the heparin−

Table 1: Comparison of the Percent Weight Chemical Composition of Fraction QS2, LDL, and Lp(a) Isolated from the Same Subject[a]

sample	no. of samples	free cholesterol	cholesteryl ester	triglycerides	phospholipids	protein
Lp(a)	4	7.3 ± 0.3	36.1 ± 1.8	3.4 ± 0.7	18.1 ± 1.0	35 ± 2.1
fraction QS2	2	7.6 ± 1.6	40.8 ± 4.6	2.3 ± 1.3	19.7 ± 0.04	29.6 ± 1.6
LDL	1	8.4	39.4	5	21	26.2

[a] Values are the means ±SD of several determinations for Lp(a) and fraction QS2 of the Q-Sepharose chromatography. LDL composition was determined once on this subject and corresponded to the mean values determined in our laboratory (Chapman *et al.*, 1988).

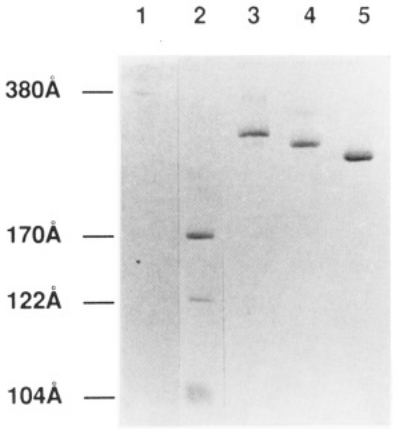

FIGURE 7: Nondenaturing 2−16% polyacrylamide gradient gel. Lane 3, Lp(a); lane 4, fraction QS2; lane 5, LDL. The amount of protein loaded per lane was 10 μg. Samples were applied in parallel with a series of marker proteins of known Stokes diameter (lanes 1 and 2). The gel was stained with Coomassie Brilliant Blue. The Stokes diameters of the particles were calculated as indicated in the Materials and Methods section.

Table 2: Purification Yields of N-Terminal Domains and Mini-Lp(a)[a]

step	protein (mg)	total recovery[b] (%)	yield[c] (%)
(1) digestion mixture of Lp(a)	11.25		
(2) heparin−Sepharose			
fraction H1	2.3	20.4	
fraction H2	7.8	70	
(3) lysine−Sepharose			
LS1	0.08	0.7	3.5
LS 2	0.92	**8.2**	40
LS 3	0.32	2.8	14
(4) Q-Sepharose			
QS 1	0.025	0.2	0.3
QS 2	3.15	**28**	40.3
QS 3	1.3	11.5	17

[a] Each fraction was concentrated and dialyzed against PBS before protein determination. [b] Total recovery was calculated from the quantity of the starting material (11.25 mg). [c] The yields of the lysine and Q-Sepharose columns were calculated from the quantity of fraction loaded on these columns, H1 and H2, respectively.

Sepharose column were recovered in fractions H1 and H2, respectively. Approximatively 1 mg (8.2%) of purified N_{ter} domains was recovered after the lysine−Sepharose column (fraction LS2), and 3.2 mg (28%) was recovered as mini-Lp(a) (fraction QS2). The total recovery was approximatively 50%.

Determination of the Cleavage Site. The N-terminal sequencing of the C_{ter} domain of apo(a) was performed to identify the location of the proteolytic cleavage site between the two structural domains of apo(a). The following sequence was obtained in two different experiments: FVP-PNVILAP(S)LEAFFEQ(A)(L). Consequently, the cleavage occurs between alanine 3532 and phenylalanine 3533 according to the numbering of the primary amino acid sequence deduced from the apo(a) cDNA sequence published by McLean *et al.* (1987).

Fibrin Binding Assays. The binding of the purified N_{ter} domains and of mini-Lp(a) to fibrin or to plasmin-degraded fibrin surfaces was studied in parallel with that of Lp(a). Figure 8 illustrates the results obtained. Nonspecific binding in the presence of 0.25 M ϵ-ACA was subtracted to yield values for lysine-specific binding. The various proteins bound to fibrin were quantified by ELISA. The binding of Lp(a) to fibrin approached saturation, with an apparent K_d of 150 ± 15 nM. The maximal amount of Lp(a) bound to the surface of fibrin (B_{max}) was 8.5 fmol/well (Figure 8). Plasmin treatment of fibrin increased the binding of Lp(a) (Figure 8, insert), as previously described (Harpel *et al.*, 1989), and B_{max} reached a value of 25 fmol/well without affecting the apparent K_d value (145 ± 15 nM). Under the same conditions, no binding was observed for the N_{ter} domains, on either intact or degraded fibrin, in the presence or absence of ϵ-ACA. On the contrary, mini-Lp(a) exhibited a specific binding that approached saturation, with an apparent K_d of 380 ± 30 nM for both surfaces and with B_{max} values of 25 fmol/well and 100 fmol/well for intact (Figure 8) and plasmin-degraded (Figure 8, insert) fibrin, respectively.

DISCUSSION

We have previously demonstrated (Huby *et al.*, 1994) that apolipoprotein(a) in the lipoprotein(a) particle is composed of two structural domains, a N_{ter} domain whose size varied according to apo(a) isoform and a C_{ter} domain of constant size (170 kDa), and linked to apo B100 by a disulfide bond (Figure 1). We presently describe the purification of these domains and the location of the cleavage site in apo(a). In addition, the binding of the purified domains to fibrin in an *in vitro* assay system was evaluated and revealed that the C_{ter} domain, in the form of "mini-Lp(a)", displayed a specific and saturable binding to fibrin and to plasmin-degraded fibrin. On the contrary, the N_{ter} domains did not bind to fibrin surfaces.

Due to the propensity of apo(a) to denature upon reduction, because of the intrakringle disulfide bonds, the purification of apo(a) domains was performed without any reducing agents. These conditions allowed us to maintain the structural integrity of both domains. Under these conditions, the C_{ter} domain of apo(a) was still linked to apo B100 and could be purified as a lipoprotein particle. On the contrary, the N_{ter} domain was purified as a soluble protein.

In each case, only two purification steps were necessary to obtain pure domains. The N_{ter} domains were purified by a combination of a heparin−Sepharose column and a lysine−Sepharose column (Figure 5). Phillips *et al.* (1993) have recently studied the association of a recombinant apo(a) with LDL. Their results suggested that r-apo(a) had probably no

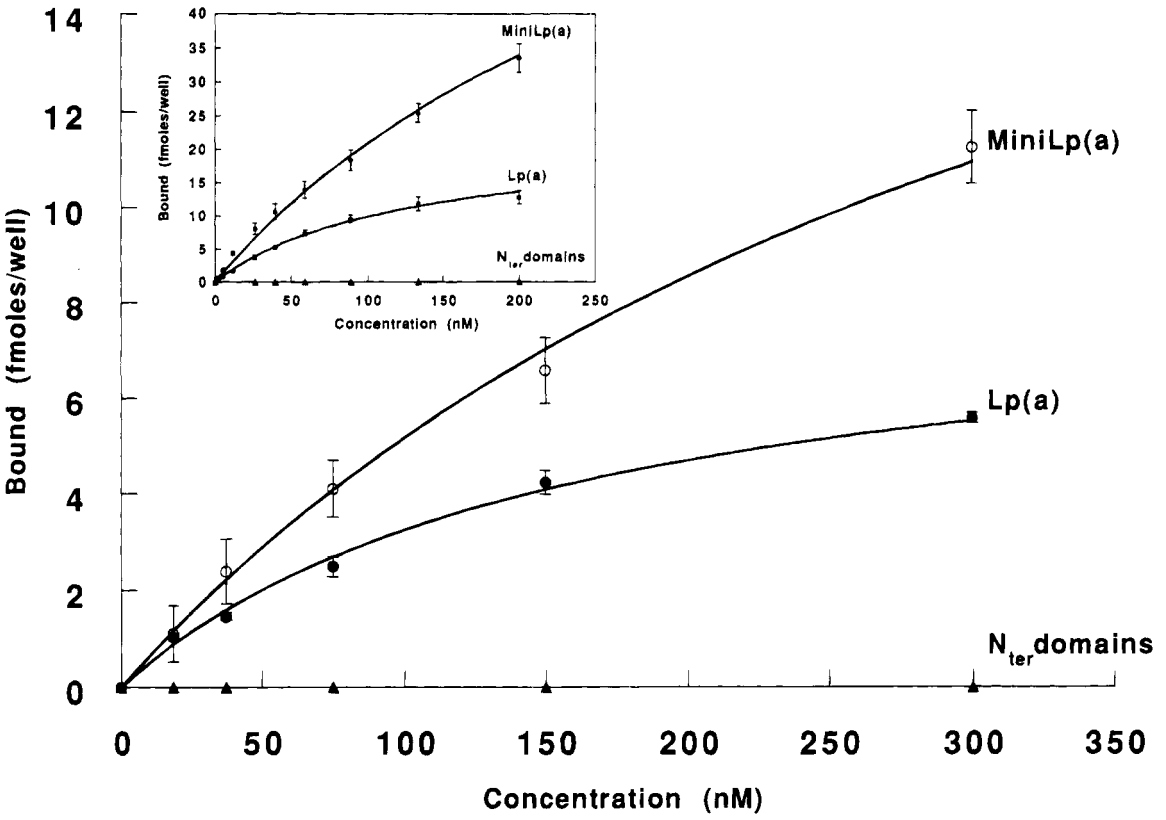

FIGURE 8: Binding of Lp(a) (●), mini-Lp(a) (○), and apo(a) N_{ter} domains (▲) to intact or plasmin-degraded (insert) fibrin surfaces. Each experimental point represents the mean ± SD of two to three individual experiments. The fibrin surfaces were prepared as indicated in the Materials and Methods section. Lysine-specific binding was obtained by subtracting the binding in the presence of ε-ACA from total binding. The binding was quantified by ELISA, expressed as ΔA_{405nm}/min, and transformed into moles by using standard curves established for each component.

more than one or two points of attachment to the LDL particle and that the rest of the protein was extended into the aqueous phase. During the purification procedure, the N_{ter} domains of apo(a) were eluted from a heparin–Sepharose column at low NaCl concentration (50 mM) and neutral pH (7.6), while the C_{ter} domain was retained. This result can be explained by the fact that, at low ionic strength, the N_{ter} part of apo(a) does not interact, or interacts weakly, with the rest of the particle. Such an interpretation is consistent with the conclusion of Phillips *et al.* (1993).

The C_{ter} domain of apo(a) was also isolated in two steps by a combination of heparin–Sepharose and Q-Sepharose chromatography (Figure 6). Like the N_{ter} domains, the apo(a) C_{ter} domain was purified without the use of reducing agents to avoid the disruption of the internal disulfide bridges of kringles. Consequently, the C_{ter} domain of apo(a) was purified while linked to the apo B100 moiety by the disulfide bond. Apo B100 is a highly hydrophobic protein which interacts intimately with the lipid core of lipoprotein particles. Therefore, we attempted to purify the C_{ter} domain of apo(a) as a lipoprotein particle. As a matter of fact, the purified fraction QS2 migrated as a lipoprotein particle on nondenaturing polyacrylamide gradient gel electrophoresis (Figure 7). Also the lipid content of this fraction was similar to those determined for Lp(a) and LDL particles in the same subject (Table 1). This finding indicated that the lipid core of the particle constituting fraction QS2 was conserved. We have termed this particle "mini-Lp(a)". Mini-Lp(a) exhibited a protein content intermediate between that of Lp(a) and LDL as expected by the loss of the apo(a) N_{ter} domain (Table 1). Therefore, the apo B100 content may have been modified to only a minor degree. In fact, the apo B100 digestion pattern of the mini-Lp(a) preparation (Figure 6B, lane 2) was similar to that of apo B100 in Lp(a) (Figure 3, right). These data suggest that the mini-Lp(a) preparation is composed of particles containing intact apo B100 or apo B100 fragments embedded in the lipid core. However, we cannot evaluate the question as to whether all the mini-Lp(a) particles possessed the same apo B100 content. Nevertheless, this mini-Lp(a) preparation appeared homogeneous in particle size on nondenaturing polyacrylamide gradient gel (Figure 7), its size being intermediate between those of the native Lp(a) and LDL particles.

The N-terminal sequencing of the C_{ter} domain of apo(a) was performed with the purified "mini-Lp(a)". We have shown that the cleavage, generated by thermolysin, occurred between alanine 3532 and phenylalanine 3533 according to the numbering of the primary sequence deduced from the apo(a) cDNA sequence published by McLean *et al.* (1987). These residues are located in the interkringle region between kringles 4_4 and 4_5 (Table 3). The interkringle regions of apo(a) are composed of 36 amino acid residues, with the exception of the regions $K4_6$–$K4_7$ and $K4_{10}$–KV which span 28 and 26 residues, respectively (Guevara *et al.*, 1992; McLean *et al.*, 1987). These interkringle regions are rich in serine and threonine residues which could be at the origin of the high O-glycosylation content of apo(a) (Kratzin *et al.*, 1987). These regions are also rich in proline residues. The interkringle region $K4_4$–$K4_5$ exhibited a marked decrease in its content of serine, threonine, and proline residues as compared to the other interkringle regions of apo(a). Notably, two proline residues (numbered 15 and 27 in Table

Table 3: Comparison of the Sequences of the Apo(a) Interkringle Regions[a]

	10	20	30
K1-K2	S D A E G T A V A P P T V T P V P S L E A P S E Q A P T E Q R P G V Q E		
K2-K3	S D A E G T A V A P P T V T P V P S L E A P S E Q A P T E Q R P G V Q E		
K3-K4	S D A E G T A V A P P T I T P I P S L E A P S E Q A P T E Q R P G V Q E		
K4-K5	S D A E W T A ↓ F V P P N V I L A P S L E A F F E Q A L T E E T P G V Q D		
K5-K6	L V T E S S V L A T L T V V P D P S T E A S S E E A P T E Q S P G V Q D		
K6-K7	P V T E S S V L A T S T A V - - - - - - - - S E Q A P T E Q S P T V Q D		
K7-K8	P V M E S T L L T T P T V V P V P S T E L P S E E A P T E N S T G V Q D		
K8-K9	P V T E S S V L T T P T V A P V P S T E A P S E Q A P P E K S P V V Q D		
K9-K10	S E T E S G V L E T P T V V P V P S M E A H S E A A P T E Q T P V V R Q		
K10-KV	S D T E G T V V A P P T V I Q V P S L G P P S E Q - - - - - - - - - - D		

[a] The arrow indicates the cleavage site.

3), which are conserved in the other interkringle regions, are replaced by leucine residues in the $K4_4-K4_5$ region. Another proline (22), which is conserved to a lesser extent, is substituted by a phenylalanine. These substitutions could create a different structure in the $K4_4-K4_5$ region, since proline is well-known to disrupt secondary structures by creating turns. Equally, one threonine (12) and one serine (23), present in all interkringle regions, are respectively replaced by an asparagine and a phenylalanine residue in this $K4_4-K4_5$ region. One threonine (14), which has also been described as a putative O-glycosylation site (Kratzin *et al.*, 1987), is replaced by an isoleucine in the $K4_4-K4_5$ region. These Ser/Thr substitutions could induce a diminished O-glycosylation pattern of this interkringle region compared to the others. Finally, this sequence exhibits the unusual presence of four aromatic amino acids (one tryptophan and three phenylalanine residues), not found in the other interkringle regions. All these changes characterize the $K4_4-K4_5$ region and could be at the origin of a greater accessibility to enzymatic cleavage.

Van der Hoek *et al.* (1993) have recently demonstrated that the sequences corresponding to apo(a) $K4_1$ and $K4_3$ to $K4_{10}$ were present in all individuals, at the mRNA level. This result suggested that the differences in size between the apo(a) isoforms were due to a variable number of identical K4 repeats ($K4_2$). Since the apo(a) C_{ter} domain was identical in size in all apo(a) isoforms, we hypothesized that it was composed of the $K4_5$ to $K4_{10}$, KV, and serine protease domains. Consequently, the N_{ter} domain is composed of the $K4_1$, the repeated identical $K4_2$, $K4_3$, and $K4_4$.

It has now been demonstrated that apo(a) of Lp(a), like plasminogen, binds to lysine residues of fibrinogen or fibrin (Harpel *et al.*, 1989; Rouy *et al.*, 1991). The lysine binding site present in the kringle 4 domain of plasminogen has been well described (Mulichak *et al.*, 1991; Wu *et al.*, 1991). Since each type of apo(a) kringle 4 contains amino acid substitutions relative to the kringle 4 of plasminogen, the question has been raised as to whether the apo(a) kringle(s) may be implicated in the lysine binding affinity of Lp(a). We have consequently tested the fibrin binding properties of both domains of Lp(a) to determine which part of the particle was responsible for the binding. The C_{ter} domain or mini-Lp(a) bound specifically to fibrin surfaces (Figure 8) with an apparent K_d (380 ± 30 nM) similar to that obtained for Lp(a) (145 ± 15 nM). On the other hand, the N_{ter} domain did not bind to fibrin or degraded fibrin at the tested concentrations (up to 400 nM) either in the presence or in the absence of ϵ-ACA. These results suggest that the fibrin binding capacity of Lp(a) resides in the C_{ter} domain. It is notable that LoGrasso *et al.* (1994), using an *in vitro* system similar to our own, have shown that a bacterial recombinant $K4_{10}$ was able to bind to plasmin-treated fibrinogen, but with an EC50 value much higher than the apparent K_d of Lp(a) (20 μM versus 1 nM). They suggested the presence of a second site in order to explain the overall Lp(a) binding. If this was the case, then we can restrict the presence of the second site to the region comprising $K4_5$ to $K4_9$, according to the structure of the C_{ter} domain. It can also be noticed that Sangrar *et al.* (1994) have shown that a recombinant $K4_{10}$ expressed in eucaryotic cells did not exhibit fibrinogen binding, but under very different experimental conditions. Our results also revealed that the number of binding sites is greater for mini-Lp(a) than for Lp(a) (100 fmol/well versus 25 fmol/well on plasmin-treated fibrin). The increased number of accessible sites for mini-Lp(a) can be explained by the fact that the molecular mass of this particle is smaller than that of Lp(a), and thus steric hindrance may be reduced. This hypothesis is in agreement with two recent studies (Hervio *et al.*, 1993; Leerink *et al.*, 1994) which have shown a negative correlation between apo(a) isoform size and the maximal number of Lp(a) particles bound to plasmin-treated fibrin. Such differences in maximal binding were also observed between plasminogen and a recombinant apo(a) (Rouy *et al.*, 1992).

In conclusion, mini-Lp(a) presents the unique advantage of allowing the study of a domain common to all apo(a)

isoforms ($K4_5$ to $K4_{10}$). Furthermore, each purified domain of apo(a) constitutes a biochemical tool which may facilitate further understanding of the interactions of Lp(a) with other proteins involved in the fibrinolytic process or with proteins of extracellular matrices such as fibronectin (Salonen *et al.*, 1989).

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