Polymorphonuclear Cells Isolated from Human Peripheral Blood Cleave Lipoprotein(a) and Apolipoprotein(a) at Multiple Interkringle Sites via the Enzyme Elastase

GENERATION OF mini-Lp(a) PARTICLES AND apo(a) FRAGMENTS

(Received for publication, December 24, 1996)

Celina Edelstein‡§, James A. Italia‡, and Angelo M. Scanu¶

From the ‡Department of Medicine and the ¶Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Incubation of polymorphonuclear cells (PMN), isolated from human peripheral blood, with either lipoprotein(a) (Lp(a)) or free apolipoprotein(a) (apo(a)), derived from the parent Lp(a), caused in both cases a traceless fragmentation of apo(a) inhibited by methoxysuccinyl-Ala-Ala-Pro-Val-CH₂Cl, a specific elastase inhibitor. The major cut sites was at the interkringle region between apo(a) kringles IV-4 and IV-5 (Ile³⁵²⁰-Leu³⁵²¹). The other cleavages were between kringles IV-7 and IV-8 (Thr³⁸⁴⁶-Leu³⁸⁴⁷) and between kringles IV-10 and V (Ile⁴¹⁹⁶-Gln¹⁴⁹⁷). The elastase-induced fragmentation of apo(a) was the same whether free or as a member of Lp(a), indicating that the disulfide bond between apo(a) and the apoB100 component of Lp(a) did not hinder the elastase action. Lp(a) fragments containing kringles IV-9 retained the linkage to apoB100 via the disulfide bond, forming mini-Lp(a) particles in which the size of apo(a) varied according to the size of the fragments produced by the elastase digestion. The proteolytic fragmentation was unaffected by apo(a) size polymorphism within the range examined. PMN elastase also caused a partial proteolysis of apoB100 whether as a component of Lp(a), Lp(a) freed of apo(a), or authentic low density lipoprotein without an apparent destabilization of these lipoprotein particles. Proteolysis of Lp(a) by PMN was due to an elastase activity that was 3.5% of that observed when PMN were activated by N-formyl-Met-Leu-Phe. A portion of the released elastase was found to be associated in an active form with both Lp(a) and low density lipoprotein even in an ultracentrifugal field at high salt concentrations. Taken together, our results indicate that apo(a) undergoes important proteolytic modifications by PMN elastase, which exhibits specificity for peptide bonds located in the interkringle domains of apo(a). In the case of Lp(a), elastase cleavage causes the formation of mini-Lp(a) particles with a protein moiety containing a truncated apo(a). Elastase-mediated proteolytic events may occur in vivo under conditions associated with either an excessive leakage of elastase from PMN and/or deficiencies of natural inhibitors of this enzyme.

Lipoprotein(a) (Lp(a))¹ represents a class of lipoprotein particles containing apoB100 linked by a single disulfide bridge to apolipoprotein(a) (apo(a)), a multikringle structure with a high degree of homology with plasminogen (for reviews, see Refs. 1 and 2). Based on this homology, the apo(a) kringles have been classified into two major classes, IV and V. Ten subclasses of kringle IV exhibiting some differences in amino acid composition have been recognized and numbered from 1 to 10 (3). Of note, except for the domains linking the identical kringle IV-2 repeats, all of the other linkers differ significantly in amino acid sequence and length (4). Only one copy of kringle V has been reported thus far.

Human leukocyte elastase is a neutral serine protease that is stored in the cytoplasmic azurophilic granules of blood neutrophils (5–7). Upon release from these cells, the enzyme causes the cleavage not only of elastin but also of a broad range of substrates including kringle-containing proteins like plasminogen, where it generates miniplasminogen by cleaving in the interkringle region (8). In previous in vitro studies (9), we observed that the purified proteolysis of Lp(a) or apo(a) by limited preparations of either human leukocyte or porcine pancreatic elastase caused the cleavage of apo(a) at the Ile³⁵²⁰-Leu³⁵²¹ bond located in the linker region between kringles IV-4 and IV-5. This cleavage resulted in the generation of two main fragments, F1 and F2, with distinct structural, functional, and metabolic properties. Moreover, pancreatic and leukocyte elastase cleaved the apoB100 component of Lp(a) without apparently compromising the overall structural organization of this lipoprotein particle. Prompted by these findings and to gain a further insight into their potential biological relevance, we explored the effects attending the in vitro incubation of Lp(a) and free apo(a) with polymorphonuclear cells (PMN), freshly isolated from human peripheral blood. The results of these studies, which are the subject of this report, demonstrate that apo(a), whether free or a member of Lp(a), undergoes a time-dependent proteolytic fragmentation that is caused by the action of a PMN-released elastase, which has a specificity for peptide bonds located in the interkringle domains of apo(a), resulting in the formation of fragments of various length. The results also show that following incubation with PMN, Lp(a) particles become smaller as a consequence of the reduction in apo(a) size.

¹ The abbreviations used are: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); Lp(a), polymorphonuclear cell(s); LDL, low density lipoprotein; MeO-Suc, methoxysuccinyl; AMC, 7-amino-4-methylcoumarin; DFP, diisopropylfluorophosphate; KI, kallikrein inhibitor; β-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; EACA, ε-aminocaproic acid; KV, class V kringle; KIV, class IV kringle.

This paper was supported by National Institutes of Health Grant HL-18577. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed: Dept. of Medicine, MC5041, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637. Tel.: 312-702-1379; Fax: 312-702-4534; E-mail: celina@medicine.bsd.uchicago.edu.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
**EXPERIMENTAL PROCEDURES**

**Materials**—CNBr-Sepharose 4B, e-aminoacrid acid (EACA), phenylmethylsulfonyl fluoride, diisopropylfluorophosphate (DFP), EDTA, L-lysine, dithioerythritol, b-mercaptoethanol (b-ME), phosphate-buffered saline (PBS) packets, kallikrein inhibitor (KI), Tween 20, dextran (clinical grade, approximate molecular weight 60,000–90,000), N-formyl-Met-Leu-Phe, cytochalasin B, 7-amino-4-methylcoumarin (AMC), methotrexate (Met-Suc-Ala-Pro-Val-AMC), MeO-Suc-Ala-Pro-Val-CH2Cl, Suc-Ala-Ala-Phe-AMC, and human leukocyte elastase (EC 3.4.21.37) were from Sigma; benzoylxyycarboxyl-Gly-Leu-Phe-CH2Cl was a generous gift from Dr. James C. Powers at the Georgia Institute of Technology; RPMI 1640 media with HEPEPS buffer and penicillin—streptomycin was from Life Technologies, Inc.; Percoll was from Pharmacia Biotech Inc., and an enhanced chemiluminescent kit (ECL Western blotting detection kit) was from Amersham Corp. All other chemicals were reagent grade.

Antisera to purified preparations of apo(a), Lp(a), and LDL were raised in the rabbit. Antibodies to apo(a), Lp(a), and apoB100 were affinity-purified as described previously (10). Anti-Lp(a) and anti-apo(a) were shown to be devoid of immunoreactivity to LDL and plasminogen; anti-apoB100 was unreactive to apo(a). Monoclonal antibodies to apo(a) were prepared in our laboratory.

**Human Subjects Used for the Preparation of Lp(a)—**Subjects were healthy donors with plasma Lp(a) protein levels in the range of 15–43 mg/dl with a known apo(a) phenotype and genotype. Their plasma was obtained by plasmapheresis performed at the blood bank of the University of Chicago. All of the subjects used in the study gave a written informed consent. The steps for Lp(a) and LDL isolation were carried out immediately after blood drawing using the procedure outlined below.

**Preparation of Human Lp(a) and LDL—**To prevent lipoprotein degradation, the plasma obtained by plasmapheresis was adjusted with 0.15% EDTA, 0.01% NaN3, 10,000 units/liter KI, and 1 mm phenylmethlysulfonfluoride. Lp(a) was isolated by sequential ultracentrifugation and lysine-Sepharose chromatography as described previously (11). The purity of the isolated Lp(a) was assessed by electrophoresis on precast 1% agarose gels (Ciba-Corning, Palo Alto, CA). Western blotting of SDS-PAGE utilizing anti-Lp(a) and anti-apoB100. The LDL preparations used in this study were isolated at d 1.030–1.050 g/ml by sequential flotation as described previously (12).

**Phenotyping and Genotyping of Apo(a)—**Apo(a) phenotyping was performed on isolated apo(a) samples by SDS-PAGE followed by immunoblotting using anti-Lp(a) (13). The mobility of the individual apo(a) bands was compared with isolated apo(a) isoforms of known molecular weights (11). For apo(a) genotyping, DNA plugs were prepared from blood mononuclear cells and subsequently fractionated by pulsed-field gel electrophoresis, and the blots were developed with an apo(a)-specific probe essentially as described earlier (14). The Lp(a) species used in this study had a single apo(a) isoform. The isoforms examined were between 5 and 88 kDa.

**Isolation of Apo(a) from Lp(a)—**Apo(a) was isolated from Lp(a) essentially as described previously (13). Briefly, Lp(a), 1 mg/ml protein was incubated with diethyryothithyl at a final concentration of 1.5–2 mM. EACA to a final concentration of 100 mM was then added, and the mixture was incubated at room temperature for 1 h under nitrogen gas. An equal volume of 60% sucrose was added, and the resulting mixture was placed into a TLA 100.3 titanium rotor and spun in a tabletop ultracentrifugation system. Columns were packed with lysine-Sepharose at a specific gravity of 1.5 g/ml. Chromatography was performed at 22 °C on a Bio-Rad Econo Chromatography system. Columns were packed with lysine-Sepharose at a ratio of 5 ml of packing material to 1 mg of Lp(a) protein and equilibrated with PBS containing 1 mM EDTA and 0.02% NaN3. After loading, the column was washed with at least 5 column volumes of equilibrating buffer followed by 3 column volumes of 500 mM NaCl to elute nonspecifically absorbed material and with 200 mM EACA for elution of specifically bound components.

**Electrophoretic Methods—**SDS-PAGE, and 8% polyacrylamide) was performed on a Novex system (Novex, San Diego, CA) for 1.5 h at constant voltage (120 V) at 22 °C. The samples were prepared by heating at 95 °C for 5 min in sample buffer, which consisted of 94 mM phosphate buffer, pH 7.0, 1% SDS, and 2% urea with without 5% b-ME. Immediately after electrophoresis, the gels were placed onto Immobilon-P sheets (Millipore Corp., Bedford, MA), which were previously wetted with a buffer containing 45 mM Tris, 39 mM glycine, pH 8.9. Blotting was performed on a horizontal semidy electroblot apparatus (Pharmacia) at 0.8–1 mA/cm2 for 45 min at 22 °C.

**Immunoblotting—**After electrophoresis, the Immobilon-P sheets were blocked for 1 h. The primary antibody (anti-apo(a) or anti-apoB100 antibody) was used. After washing the membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-labeled IgG. Subsequently, the membranes were developed with the ECL Western detection reagent according to the manufacturer's instructions.

**Immunoprecipitation—**Fractions obtained by elution with PBS from
lysine-Sepharose columns were concentrated with Amicon microconcentrators, and 45-μl aliquots were incubated with 7.5 μl of anti-KV antibodies (10:1 protein:antibody ratio, w/w) at 22 °C for 1 h. The mixture was then added to 25 μl of Protein A-Sepharose and rotated for 1 h at 22 °C. The immunoprecipitates were washed twice with 250 μl of a buffer of 0.5 × NaCl, 0.1 × NaHCO₃, 2 mM EDTA at pH 5.0 and once with a buffer containing 20 mM Tris-HCl, 140 mM NaCl at pH 8.0. To 25 μl of the immunoprecipitate-Protein A-Sepharose mixture was added an equal volume of 2 × sample buffer, 1.5 μl of β-ME (final 3%) followed by boiling for 5 min. The complete mixture was then applied onto a slab of 8% polyacrylamide SDS gel.

**Amino-terminal Sequence Analyses—Apo(a) fragments (10–30 μg)** were electrophoresed under reducing conditions as outlined above. After electrophoresis, the gels were electroblotted onto Immobilon PSQ sequence grade membranes (Millipore) as described above under “Immunoblotting.” The blots were rinsed in distilled water, stained with Coomassie Blue R250 (0.025% in 40% methanol), and destained with 50% methanol. The stained bands were cut from the membrane, further washed with 40% methanol, and allowed to air dry. Reduction with dithiothreitol and alkylation with iodoacetamide was performed directly on the PSQ membrane, which was then subjected to automated Edman degradation on an Applied Biosystems 477A unit using procedures recommended by the manufacturer. In the case of proteins with blocked amino termini, the PSQ membranes containing the protein bands were first treated with polyvinylpyrrolidone so that the enzyme, pyroglutamyl aminopeptidase, would not absorb to it. The PSQ membrane was then digested with the aminopeptidase to remove the first blocked amino terminus, and then the membrane was subjected to sequencing. All sequencing procedures were carried out in the core laboratory of the Macromolecular and Structural Analysis Facility at the University of Kentucky.

**Lipoprotein and Apolipoprotein Analyses—Lp(a) and LDL protein were quantitated by a sandwich enzyme-linked immunosorbent assay essentially as described previously (10) except that anti-Lp(a) IgG was used as the capture antibody and anti-apoB100 IgG conjugated to alkaline phosphatase was used as the detection antibody. For the enzyme-linked immunosorbent assay quantitation of apo(a), anti-apo(a) IgG conjugated to alkaline phosphatase was used as the detection antibody. Subsequently, an extinction coefficient (ε₄₀₅ nm = 1.31 ml mg⁻¹ cm⁻¹) was established for apo(a) in the 30% sucrose solution. Protein determinations were performed by the Bio-Rad DC protein assay.**

**RESULTS**

**Time Course of Proteolysis of Apo(a) and Lp(a) by PMN—** The ability of PMN to hydrolyze apo(a) and Lp(a) was assessed using anti-apo(a) immunoblots of SDS-polyacrylamide gels. Preliminary experiments were carried out to determine the conditions required to elicit proteolysis of apo(a) and Lp(a). Using various ratios of cell number to apo(a) and Lp(a) protein concentrations in the incubation mixture, we arrived at the concentrations in the incubation mixture, we arrived at the results, we concluded that PMN causes a time-dependent progressive proteolysis of apo(a) and that the proteolytic pattern is comparable for free apo(a) and apo(a) bound to LDL. In subsequent studies we chose 45 min as the incubation time.

**The Enzyme That Cleave Apo(a) and Lp(a) upon Incubation with PMN Is an Elastase—** Based on our previous observation that apo(a) and Lp(a) can be fragmented by incubation with a purified preparation of human leukocyte elastase (9), we wanted to determine whether an elastase may cause the cleavage of Lp(a) and apo(a) following incubation with PMN. To this end, PMN were incubated only in the presence of RPMI for 45 min, and this medium was then brought to d 1.21 g/ml with solid NaBr and centrifuged overnight in a tabletop TL100 ultracentrifuge 412,160 × g for 18 h. The sedimenting fraction was then incubated with apo(a) or Lp(a) for 45 min in the absence and presence of the specific elastase inhibitor, MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl, in a final concentration of 1% MeOH. As shown in Fig. 2A, in the absence of the elastase inhibitor, the proteolysis of apo(a) and Lp(a) (lanes 2 and 7, respectively) was similar to that elicited by a purified preparation of leukocyte elastase (lanes 4 and 9). On the other hand, in the presence of the elastase inhibitor, there was only a single band in the position of apo(a) whether the enzymatic source was PMN (lanes 3 and 8) or leukocyte elastase (lanes 5 and 10), indicating that no proteolysis had occurred. In turn, in the presence of 1% MeOH alone no inhibition of enzymatic activity was observed (data not shown). Since the primary granules of PMN also contain cathepsin G, we wanted to rule out the potential participation of this enzyme in the digestion of apo(a) or Lp(a). To this end, the d 1.21 g/ml sedimentation fraction of the conditioned PMN medium was incubated with apo(a) and Lp(a) in the presence of the specific cathepsin G inhibitor, benzoxycarbonyl-Gly-Leu-Phe-CH₂Cl (100 μM). No significant inhibition of the digestion of either apo(a) or Lp(a) took place. The SDS-PAGE exhibited a profile similar to that shown in Fig. 2A, lanes 2 and 7. We also examined whether either the sedimenting fraction of PMN conditioned medium or the purified leukocyte elastase could digest apoB100 in the Lp(a) particle using as a criterion anti-apoB100 immunostained 6% SDS-PAGE run under reduced conditions (Fig. 2B). In the absence of the elastase inhibitor, several bands with a faster mobility were ob-

![Fig. 1. Time course of proteolysis of apo(a) and Lp(a) by PMN.](image)
Elastase Fragments of Lipoprotein(a) and Apolipoprotein(a)

FIG. 2. Western blots of apo(a) and Lp(a) after incubation with the d > 1.21 g/ml conditioned medium fraction or with purified leukocyte elastase in the absence and presence of the specific elastase inhibitor. A, PMN conditioned medium was brought to d 1.21 g/ml with solid NaBr and centrifuged overnight in a tabletop TL100 ultra centrifuge at 412,160 × g. The sedimenting fraction was incubated for 45 min at 37 °C with apo(a) or Lp(a) in the absence and presence of the specific elastase inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH2Cl at a final concentration of 1% MeOH. Human leukocyte elastase was diluted 1000-fold in 50 mM Tris-HCl, 0.1 M NaCl, pH 8.0. One microliter of the diluted enzyme/7.5 μg of Lp(a) protein or 15 μg of apo(a) was incubated at 37 °C for 30 min in the absence and presence of the elastase inhibitor. The samples were reduced with 3% β-ME, separated by 6% SDS-PAGE, and Western blotted. Rabbit anti-apo(a) was used to probe the blot. Lane 1, controlapo(a) incubated for 45 min in the d > 1.21 g/ml fraction of RPMI medium without PMN; lanes 2 and 3, apo(a) incubated with the PMN conditioned sedimenting fraction in the absence and presence of inhibitor, respectively; lanes 4 and 5, apo(a) incubated with purified leukocyte elastase in the absence and presence of inhibitor, respectively; lane 6, control Lp(a) incubated for 45 min in the d > 1.21 g/ml fraction of RPMI medium without PMN; lanes 7–10, Lp(a) under the same conditions as for lanes 1–5. B, rabbit anti-apoB100 immunostained blots of reduced samples run on 6% SDS-PAGE. Lanes 1–5, the same samples described for A, lanes 6–10.

TABLE I

| Sample | Elastase activity* | Elastase activity relative to control* |
|--------|--------------------|---------------------------------------|
| PMN    | 36.1 ± 6.6         | 1.0                                  |
| PMN + Lp(a) | 115.6 ± 29.5     | 3.2                                  |
| PMN + LDL | 126.3 ± 52.4      | 3.5                                  |
| PMN + cytocholasin B’ + N-formyl-Met-Leu-Phe* | 3240.2 ± 661.0 | 89.8                                  |

* Average ± the range of single determinations from four experiments. Values are pmol of substrate cleaved/min/0.05 ml of sample.

** Calculated as the ratio of the activity when PMN were incubated with the indicated lipoproteins or agonist divided by the activity when PMN were measured alone.

The cytocholasin B concentration was 1 μg/ml and the N-formyl-Met-Leu-Phe concentration was 1 μM.

In the presence of the elastase inhibitor, there was no proteolytic degradation of apoB100 (lanes 3 and 5).

In subsequent studies, the proteolytic activity contained in the d 1.21 g/ml sedimenting fraction of the conditioned medium from the PMN/Lp(a) and PMN/LDL incubation systems was tested against MeO-Suc-Ala-Ala-Pro-Val-AMC, (53 μM), a substrate specific for elastase. Following incubation, the released AMC in the medium d > 1.21 g/ml fraction was measured by fluorescence spectroscopy at excitation and emission wavelengths of 370 and 460 nm, respectively. As shown in Table I, the incubation of PMN with Lp(a) resulted in a 3.2-fold increase in released elastase activity as compared with controls without Lp(a) and was similar to that observed for LDL. In each case, the released activity was negligible when Suc-Ala-Ala-Phe-AMC (74 μM), a synthetic substrate specific for cathepsin G, was incubated with the same d 1.21 g/ml sedimenting fraction. Moreover, we pretreated PMN with the microfilament-disrupting agent, cytochalasin B (5 μg/ml), followed by stimulation of the cells with the chemotactic peptide, N-formyl-Met-Leu-Phe, a system known to cause 90–100% release of the PMN elastase activity (18). The amount of cathepsin G activity released by these cells was 0.18% of the total elastolytic activity (data not shown). In this context it should be noted that the elastase activity released from PMN by Lp(a) represented about 3.5% of that released by N-formyl-Met-Leu-Phe-stimulated PMN (Table I). From these data we came to the conclusion that the PMN enzyme that specifically cleaves apo(a) and Lp(a) is an elastase and that the amount of enzyme released by either of these two products represents only a relatively small percentage of the total cellular elastase activity.

Lysine-Sepharose Chromatography of the Products Obtained by Digestion of Apo(a) with PMN and Purified Leukocyte Elastase—Free apo(a) digested with PMN was applied to a lysine-Sepharose affinity column, which was then washed with three column volumes of PBS, 500 mM NaCl, and 200 mM EACA (Fig. 3A). Two major peaks were observed, one eluting with PBS and one with EACA. Electrophoretic analysis on reduced gels (6%) probed with anti-apo(a) (Fig. 3B), showed that the unbound fraction eluting with PBS represented one major band migrating in the 220-kDa position, corresponding to what we called previously F1 (9), and a set of repeating bands of lesser intensity differing in size by 20 kDa, the apparent size of a single kringle (Fig. 3B, lane 3). The fraction eluting with EACA consisted of six bands, designated F2–F6, migrating at positions corresponding to 167, 133, 112, 78, and 68 kDa, respectively (Fig. 3B, lane 4). Similar results were obtained with apo(a) digested with purified leukocyte elastase both in terms of elution behavior on lysine-Sepharose (data not shown) and electrophoretic profiles (Fig. 3B, lanes 6 and 7). To determine which of the PBS and EACA eluting fragments contained the COOH-terminal region of apo(a), we used an anti-KV antibody to probe for the presence of KV on 8% SDS-PAGE run under...
nonreduced conditions. The PBS eluting fraction contained F1 and F7; the latter exhibited a band migrating at 38 kDa, faintly staining with anti-apo(a) (Fig. 3C, lane 1). However, when probed with anti-KV, F7 was intensely stained, while F1 was not (lane 3). The fraction eluting with EACA contained fragments F2–F6 (lane 2), of which only F2 and F4 reacted against anti-KV (lane 4).

**Amino-terminal Sequence Analyses of the Proteolytic Fragments Obtained from Digested Apo(a)**—Fig. 4 shows the partial NH2-terminal sequences of F1–F7, obtained as described above (see Fig. 3). In addition, fragment F7 in the PBS fraction was separated from F1 and the low intensity 20-kDa repeating bands by immunoprecipitation with anti-KV. The cleavage sites for each of the fragments were located by aligning the NH2-terminal sequences of F1 through F7 with those of apo(a). Fragment F1 corresponded to the NH2-terminal sequence of the first kringle in apo(a), KIV-1. Fragments F2, F3, and F6 exhibited identical NH2-terminal sequences to those produced after cleavage at the IleEn Leu bond in the linker region between kringles IV-4 and IV-5. Fragments F4 and F5 had the same NH2-terminal sequence as those resulting from the cleavage at the Thr-Leu bond in the linker region between kringles IV-7 and IV-8 and F7 resulted from the cleavage at the IleEn-Gln bond in the linker region between kringles IV-10 and KV. In the case of F7, Gln was the first amino acid in the sequence; however, yields were very poor, suggesting that the cleavage at the IleEn-Gln bond released a Gln, which readily cyclized to pyroglutamic acid. On this premise, we treated the blot containing F7 with the enzyme pyroglutaminate and then conducted sequence analyses. The fact that Val was the first amino acid sequenced, confirmed that the NH2 terminus of F7 was a cyclized Gln that had been removed by pyroglutaminase. Moreover, we detected no amino acids in some of the positions predicted from the cDNA sequence (4) as Ser and Thr when we examined the NH2-terminal sequences in the linker regions, particularly those in fragments F4 and F5. It is likely that the lack of detection of Ser and Thr was due to either phosphorylation or glycosylation of these residues. PMN have been reported to cause phosphorylation of proteins (19). Since Ser and Thr remained undetected even in fragments generated by a purified leukocyte elastase, the data suggest, but do not prove, that Ser and Thr in those positions were O-glycosylated.

Fig. 5 gives a summary of the properties of the fragments of apo(a) based on NH2-terminal sequence data, affinity for lysine-Sepharose, apparent molecular weight derived from electrophoretic data, and the presence of KV by immunoblot analysis. Elastase cleaved apo(a) at three sites in the linker regions between KIV-4 and IV-5, between KIV-7 and KIV-8, and between KIV-10 and KV, generating seven distinct fragments, F1–F7. The fragments that bound to lysine-Sepharose comprised the following regions: F2, KIV-5 through the protease region; F3, KIV-5 through KIV-4; F4, KIV-8 through the protease region; F5, KIV-8 through KIV-10; and F6, KIV-5 through KIV-7. The two fragments that lacked lysine-binding capability were F1 (the NH2-terminal domain of apo(a) containing KIV-1 through KIV-4) and F7 (comprising KV and the protease region).

**Properties of the Products Obtained by Digestion of Lp(a) with PMN—**Lp(a) was incubated with PMN, DFP was added to stop the enzymatic reaction, and the cells were sedimented by low speed centrifugation. The digest was then subjected to 6% SDS-PAGE immunoblot analysis using a rabbit anti-apo(a) polyclonal antibody. Fig. 6A, shows the immunostained gel run under nonreduced and reduced conditions. Control Lp(a) are in lanes 1 and 5. Digested Lp(a), in the nonreduced gel (lane 2), exhibited two major bands, one migrating just above the 203 kDa marker and a doublet, less intensely stained, migrating somewhat faster than Lp(a) and co-localizing with apoB100.
Fig. 4. Alignment of the partial amino acid sequences of F1–F7. The fragments were excised from gels and prepared for microsequencing as described under “Experimental Procedures.” Apo(a) is composed of repeats of KIV numbered 1–10, one KV, and a protease domain (P) according to the nomenclature of Scanu and Edelstein (1). The first 20 amino acids obtained by sequencing F1 were aligned to the amino acid sequence of the mature apo(a) without the signal peptide sequence. The dashed lines refer to undetected amino acids. The dotted lines represent continuation sequences upstream and downstream of the protein sequence. Alignments to the deduced amino acid sequence of apo(a) (4) were performed with the ClustalW sequence alignment program.

(Fig. 6B, lane 2). On the reduced gels, the banding pattern of digested Lp(a) (Fig. 6A, lane 6) resembled that of an apo(a) digest as shown in Fig. 3B, lane 2. Notably, the doublet that migrated underneath the Lp(a) in the nonreduced gel was no longer present.

Based on the data summarized in Fig. 5, we reasoned that digested Lp(a) fragments containing KIV-9, i.e. F2–F5, should remain covalently attached to apoB100 of LDL via the inter-chain disulfide bond and thus readily separable by ultracentrifugal flotation from the fragments not containing KIV-9, i.e. F1, F6, and F7. On that premise, we diluted PMN-digested Lp(a) 1:1 (v/v) with 60% sucrose in 10 mM phosphate buffer containing 200 mM EACA to a final density of 1.127 g/ml, and the mixture was centrifuged in a TL 100 tabletop ultracentrifuge at 15 °C, 412,160 × g for 18 h. Thereafter, we collected two fractions: a sedimenting fraction (1 ml) and a top one fraction (0.5 ml).

The sedimenting fraction on the anti-apo(a) immunostained reduced gels (Fig. 6A, lane 7) contained two major bands, F1 and F6, and some minor repeating bands. Immunostaining with anti-apoB100 showed that the sedimenting fraction was devoid of apoB100 (Fig. 6B, lanes 3 and 7). In addition to F1 and F6, immunodetection with the anti-KV antibody on 8% SDS-PAGE showed a band migrating at 38 kDa, corresponding to F7 (Fig. 6C, lane 3) which was only faintly visible in the anti-apo(a)-stained gel (lane 1).

Lysine-Sepharose affinity chromatography (data not shown) of the sedimenting fraction showed that the unbound component eluting with PBS contained F1 and the 38-kDa fragment, F7. Fragment F7 was immunoprecipitated from the PBS fraction and treated as described above for the apo(a)-derived fragment. The partial amino acid sequence of fragments F1, F6, and F7 corresponded to the sequences of the apo(a)-derived fragments shown in Fig. 4.

The top floating d 1.127 g/ml fraction, on nonreduced gels, showed a doublet that migrated faster than Lp(a) (Fig. 6A, lane 4) and co-localized with apoB100 (Fig. 6B, lane 4). In keeping
with our previous studies (9), we called this apoB100:apo(a) fraction mini-Lp(a). Upon reduction, the doublet disappeared and was replaced by bands representing fragments F2–F5 (Fig. 6A, lane 8), which were unreactive to the anti-apoB100 antibody, (data not shown).

Furthermore, the mini-Lp(a) was reduced with 1.5 mM di-thioerythritol in the presence of 100 mM EACA and centrifuged in 30% sucrose according to the method that we utilized for the isolation of free apo(a) (13). The sedimenting fraction bound specifically to lysine-Sepharose and was eluted with EACA (data not shown). By gel electrophoretic analysis, the EACA eluting fraction contained fragments F2–F5 (Fig. 6C, lane 2) exhibiting sequences similar to those of the apo(a) fragments in Fig. 4. Immunostaining with anti-KV antibody showed that only fragments F2 and F4 contained this kringle (Fig. 6C, lane 4).

Thus, limited proteolysis of Lp(a) by PMN elastase produced a lipid-rich fraction, which floated at $d = 1.127$ g/ml, and a sedimenting one. The lipid-rich fraction consisted of four species of mini-Lp(a) particles, each containing apoB100 disulfide linked to individual apo(a) fragments, namely F2, F3, F4, and F5. The sedimenting fraction consisted of apo(a) fragments, F1, F6, and F7. Whether starting from Lp(a) or free apo(a), incubation with PMN produced seven fragments as a consequence of the cleavage at three sites on apo(a) in the interkringle linker regions between KIV-4 and KIV-5, between KIV-7 and KIV-8, and between KIV-10 and KV.

**Effect of Apo(a) Size Polymorphism on Elastase Action**—Within the range of the molecular weights studied, there was no apparent effect of apo(a) size polymorphism on the extent of proteolytic fragmentation of Lp(a) and apo(a) by PMN elastase or purified leukocyte elastase. Except for F1, fragments F2–F6 were consistently released independently of apo(a) size. Since the repeat KIV-2 was a component of fragment F1, the size of the latter varied according to the number of those repeats.

**Association of PMN Elastase with Lp(a) and LDL**—Following preincubation for 45 min at 37 °C with Lp(a) or LDL, PMN were sedimented by centrifugation at $150 \times g$ for 10 min in the absence of DFP to allow unlimited elastase digestion. The supernatant was then recentrifuged at $400 \times g$ for 10 min and thereafter at $d = 1.21$ g/ml at $412,160 \times g$ for 18 h. The floating fraction containing either digested Lp(a) or LDL was then used as an enzyme source in incubation studies with fresh Lp(a). We examined the floating fraction before incubation with fresh Lp(a) on-anti-apo(a)-immunostained 6% SDS-PAGE run under reduced conditions. Fig. 7A, lane 1, shows that in the absence of DFP, apo(a) in Lp(a) underwent extensive digestion, since no bands were detected. In contrast, the apoB100 component of Lp(a) was only partially hydrolyzed (Fig. 7B, lane 1) and remained as a lipid-protein complex. When this floating fraction was incubated with fresh intact Lp(a) for 45 min at 37 °C and the reaction was stopped with DFP, a partial hydrolysis of Lp(a) occurred (Fig. 7A, lane 2). When incubated in the presence of the specific elastase inhibitor, MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl, no proteolysis of Lp(a) was observed (Fig. 7A, lane 3). We also found that LDL preincubated with PMN caused proteolysis of Lp(a) (Fig. 7A, lane 4); in turn, proteolysis was minimal in the presence of the elastase inhibitor (Fig. 7A, lane 5). We also examined the possibility that during the ultracentrifugation of the PMN-conditioned medium, an elastase gradient formed, causing a portion of the enzyme to float without a true association with either Lp(a) or LDL. To rule out this possibility, we centrifuged at $d = 1.21$ g/ml the RPMI medium following incubation with PMN. We then incubated the floating fraction with Lp(a). Lane 6 shows that Lp(a) remained undigested. In addition, Lp(a) under the same conditions did not undergo self-degradation (lane 7), nor did LDL degrade apo(a) (lane 8). Thus, the elastase that is released from PMN can affiliate with either Lp(a) or LDL, and once bound, the enzyme remains active even in the presence of high salt concentrations and a high gravitational field.

**DISCUSSION**

The results of the current studies have shown that apo(a), either free or a constitutive component of the parent Lp(a), undergoes cleavage at multiple interkringle sites upon incubation with PMN freshly isolated from human peripheral blood. The involvement of an elastase enzyme in this fragmentation is
supported by several lines of evidence: 1) similarity in results between PMN-dependent proteolysis and that caused by a purified human leukocyte elastase preparation; 2) capacity to hydrolyze the synthetic substrate MeO-Suc-Ala-Ala-Pro-Val-Arg, which is hydrolyzed by human leukocyte elastase; 3) capacity to cause peptide bond cleavage. The notion that PMN elastase can effect a limited proteolysis of Lp(a) and apo(a) by a purified leukocyte elastase preparation; 2) capacity to apply to lysine-Sepharose column chromatography, a complementary technique commonly used in Lp(a) purification. An inhibition of the enzyme. In the latter instance, apo(a) fragments and mini-Lp(a) particles may also be generated topically at inflammatory sites favored by a microenvironment, where activated PMNs would release the elastase enzyme in amounts unopposed by physiological inhibitors. Apo(a) fragments have been reported in the atherosclerotic plaque (26), and it is tempting to speculate that they might be derived from an elastase action. Should this be the case, two possibilities may be considered, although they are not mutually exclusive; the fragments are formed in the plasma and then transferred to the arterial wall and/or they are formed at a tissue site by an elastase action unopposed by physiological inhibitors of the enzyme. In the latter instance, apo(a) fragmentation may be contributed by the action of an elastase released from activated resident macrophages, an assumption based on the finding that macrophage elastase has structural and enzymatic properties similar to those of PMN elastase (27).

Another interesting aspect of our studies was the observation that an active PMN elastase can associate with Lp(a). The interaction appears to occur predominantly through the LDL component of Lp(a) and may be hydrophobic in nature, since the enzyme remains associated in the presence of high salt concentrations. In our studies, the Lp(a) isolated from the PMN conditioned medium had an elastase activity that was able to cleave the apo(a). Ultracentrifugation is an important procedure used to separate Lp(a) from the other lipoproteins and proteins of the plasma. It is now apparent that this procedure does not assure elastase-free Lp(a) products. This would also apply to lysine-Sepharose column chromatography, a complementary technique commonly used in Lp(a) purification. An elastase “contamination” must be considered in reports (28) associating proteolytic events in either Lp(a) or derivatives. In this vein, of significance are our past studies (29) showing that the in vitro incubation of LDL with PMN results in the release into the medium of a proteolytic activity that co-elutes with LDL by column chromatography at a physiological ionic strength. The similarity of the LDL results with those obtained with Lp(a) is further corroborated by the observation that for both lipoprotein particles, the proteolytic activity was due to an elastase based on its inhibition by MeO-Suc-Ala-Ala-Pro-Val-

Elastase Fragments of Lipoprotein(a) and Apolipoprotein(a)

**FIG. 7.** Association of PMN elastase with Lp(a) and LDL. Following incubation for 45 min at 37°C with Lp(a) or LDL, PMN were sedimented by centrifugation at the absence of DFP to allow unlimited elastase digestion.

The supernatant was then recentrifuged at 400 g/ml flotation co-

fused Lp(a) incubated with fresh Lp(a) in the absence and presence of PMN; 4) the nature of the cut sites, which were between PMN-dependent proteolysis and that caused by a purified human leukocyte elastase preparation; 2) capacity to apply to lysine-Sepharose column chromatography, a complementary technique commonly used in Lp(a) purification. An inhibition of the enzyme. In the latter instance, apo(a) fragments and mini-Lp(a) particles may also be generated topically at inflammatory sites favored by a microenvironment, where activated PMNs would release the elastase enzyme in amounts unopposed by physiological inhibitors. Apo(a) fragments have been reported in the atherosclerotic plaque (26), and it is tempting to speculate that they might be derived from an elastase action. Should this be the case, two possibilities may be considered, although they are not mutually exclusive; the fragments are formed in the plasma and then transferred to the arterial wall and/or they are formed at a tissue site by an elastase action unopposed by physiological inhibitors of the enzyme. In the latter instance, apo(a) fragmentation may be contributed by the action of an elastase released from activated resident macrophages, an assumption based on the finding that macrophage elastase has structural and enzymatic properties similar to those of PMN elastase (27).

Another interesting aspect of our studies was the observation that an active PMN elastase can associate with Lp(a). The interaction appears to occur predominantly through the LDL component of Lp(a) and may be hydrophobic in nature, since the enzyme remains associated in the presence of high salt concentrations. In our studies, the Lp(a) isolated from the PMN conditioned medium had an elastase activity that was able to cleave the apo(a). Ultracentrifugation is an important procedure used to separate Lp(a) from the other lipoproteins and proteins of the plasma. It is now apparent that this procedure does not assure elastase-free Lp(a) products. This would also apply to lysine-Sepharose column chromatography, a complementary technique commonly used in Lp(a) purification. An elastase “contamination” must be considered in reports (28) associating proteolytic events in either Lp(a) or derivatives. In this vein, of significance are our past studies (29) showing that the in vitro incubation of LDL with PMN results in the release into the medium of a proteolytic activity that co-elutes with LDL by column chromatography at a physiological ionic strength. The similarity of the LDL results with those obtained with Lp(a) is further corroborated by the observation that for both lipoprotein particles, the proteolytic activity was due to an elastase based on its inhibition by MeO-Suc-Ala-Ala-Pro-Val-

Elastase Fragments of Lipoprotein(a) and Apolipoprotein(a)

**FIG. 7.** Association of PMN elastase with Lp(a) and LDL. Following incubation for 45 min at 37°C with Lp(a) or LDL, PMN were sedimented by centrifugation at 150 × g for 10 min in the absence of DFP to allow unlimited elastase digestion. The supernatant was then recentrifuged at 400 × g for 10 min and immediately after at d 1.21 g/ml at 412,160 × g for 18 h. The floating fraction containing either digested Lp(a) or LDL was then used as an enzyme source in incubation studies with fresh Lp(a). A, anti-apo(a)-immunostained 6% SDS-PAGE run under reduced conditions. Lane 1, d < 1.21 g/ml floating fraction of digested Lp(a); lanes 2 and 3, floating fraction containing digested Lp(a) incubated with fresh Lp(a) in the absence and presence of the elastase inhibitor, respectively; lanes 4 and 5, floating fraction containing digested LDL incubated with fresh Lp(a) in the absence and presence of the elastase inhibitor, respectively; lane 6, control Lp(a) incubated with d < 1.21 g/ml floating fraction of RPMI medium without PMN; lane 7, control Lp(a) incubated with d < 1.21 g/ml floating fraction of PMN conditioned medium; lane 8, d < 1.21 g/ml floating fraction of control Lp(a) and LDL incubated together with RPMI medium without PMN. B, anti-apoB100 blot of a nonreduced gel. Lane 1, sample as in lane 1 of A.
CH$_2$Cl. Moreover, in the case of the LDL, there was no evidence for a participation of oxidative events as assessed by lipid peroxidation in the thiobarbituric assay (29). The potential for oxidative events contributing to Lp(a) proteolysis was not examined in the current studies. However, we believe that, if present, they were of a relatively minor nature, since the proteolysis was inhibited by a specific elastase inhibitor and was equally observed in Lp(a)/apo(a) incubated with either PMN or a purified preparation of leukocyte elastase. Of interest, LDL modified by PMN elastase was shown to enhance the uptake of the lipoprotein particle by human monocyte-derived macrophages (30) in the absence of reactive oxygen species. It would be of interest to conduct similar studies with elastase-derived fragments of apo(a) as well as mini-Lp(a) in an attempt to better define the site(s) on apo(a) responsible for the cellular uptake and degradation of Lp(a) and also their relative pathogenicity.

Finally, the availability of products derived from the proteolytic fragmentation of Lp(a)/apo(a) should pave the way for in depth structural and functional studies directed at establishing potential differences among the various apo(a) domains. In this context, it would be important to carry out systematic studies on the content and composition of carbohydrates of the interkringle linkers to assess a potential heterogeneity among apo(a) domains. These studies are in progress in this laboratory.

Acknowledgments—We are grateful to Dr. James C. Powers at the Georgia Institute of Technology for the gift of the specific inhibitor to cathepsin G. We also thank Carol Beach at the Macromolecular and Structural Analyses Facility, University of Kentucky, for helpful discussions and advice on sequencing methodology. We gratefully acknowledge Jose Santiago of Core B of the Program Project HL 18577 for the preparation of Lp(a) and LDL.

REFERENCES
1. Scarn, A. M., and Edelstein, C. (1995) Biochim. Biophys. Acta 1256, 1–12
2. Utermann, G. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriver, C.R., Beaudet, A., Sly, W.S, and Valle, D., eds) pp. 1887–1912, McGraw Hill Inc., New York
3. Guevara, J., Jan, A. Y., Knapp, R., Tulinsky, A., and Morrisett, J. D. (1993) Arterioscler. Thromb. 13, 758–770
4. McLean, J. W., Tomlinson, J. E., Kuang, W.-J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M., and Lown, R. M. (1987) Nature 330, 132–137
5. Schmidt, W., and Havemann, K. (1974) Hoppe-Seylers Z. Physiol. Chem. 355, 1077–1082
6. Trincic, R., Raugh, R., Giles, P. J., D. J., Bewen, J., and Reilley, C. F. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Jonoff, A., eds) pp. 118, Urban & Schwarzenberg, Baltimore
7. Ohlsson, K., and Olsson, I. (1974) Eur. J. Biochem. 42, 519–527
8. Moroz, L. A. (1981) Blood 59, 97–104
9. Edelstein, C., Italia, J. A., Kleczewitch, O., and Scanu, A. M. (1996) J. Lipid Res. 37, 1786–1801
10. Fless, G. M., Snyder, M. L., and Scanu, A. M. (1989) J. Lipid Res. 30, 651–662
11. Fless, G. M., Snyder, M. L., Furbee, J. W. J., Garcia-Hedo, M. T., and Mora, R. (1994) Biochemistry 33, 13492–13501
12. Schumaker, V. N., and Puppione, D. L. (1986) Methods Enzymol. 128, 155–170
13. Edelstein, C., Mandala, M., Pfaffinger, D., and Scanu, A. M. (1995) Biochemistry 34, 16483–16492
14. Lackner, C., Boerwinkle, E., Leffert, C. C., Rahmig, T., and Hobbs, H. H. (1991) J. Clin. Invest. 87, 2153–2161
15. Pelacek, D., Byrne, R. E., Barreus, M., and Scanu, A. M. (1984) J. Biol. Chem. 259, 14531–14536
16. Castillo, M. J., Nakajima, K., Zimmerman, M., and Powers, J. C. (1979) Anal. Biochem. 99, 53–64
17. Wilkie, S. D., and Landry, D. (1988) Biochromatography 5, 205–215
18. Sklar, L. A., McNeil, V. M., Jesaitis, A. J., Painter, R. G., and Cochrane, C. G. (1982) J. Biol. Chem. 257, 5471–5475
19. Babior, B. M. (1988) Arch. Biochem. Biophys. 264, 361–367
20. Oida, K., Takai, H., Maeda, H., Takahashi, S., Shimada, A., Sushi, J., Tamai, T., Nakai, T., and Miyabo, S. (1992) Clin. Chem. 38, 2244–2248
21. Mooser, V., Seabra, M. C., Abedin, M., Landschulz, R. T., Marevova, S., and Hobbs, H. H. (1996) J. Clin. Invest. 97, 858–864
22. Mooser, V., Marevova, S. M., White, A. L., and Hobbs, H. H. (1996) J. Clin. Invest. 98, 2414–2424
23. Egbring, R., Schmidt, W., Fuchs, G., and Havemann, K. (1977) Blood 49, 219–231
24. Maeda, S., Abe, A., Seishima, M., Makino, K., Noma, A., and Kawade, M. (1989) Atherosclerosis 78, 145–150
25. Kario, K., Matsuo, T., Kobayashi, H., Matsuo, M., Asada, R., and Koide, M. (1995) Thromb. Haemostasis 74, 1020–1024
26. Hoff, H. F., O’Neill, J., Smejkal, G. B., and Yashiro, A. (1994) Chem. Phys. Lipids 67, 271–280
27. Senior, R. M., Cambell, E. J., Landis, J. I., Cox, F. R., Kuhn, C., and Koren, H. S. (1982) J. Clin. Invest. 69, 384–393
28. Pursiainen, M., Jauhiainen, M., Kovanen, P. T., and Ehnholm, C. (1994) Chem. Phys. Lipids 67, 25–33
29. Polacek, D., Byrne, R. E., Fless, G. M., and Scanu, A. M. (1986) J. Biol. Chem. 261, 2057–2063
30. Polacek, D., Byrne, R. E., and Scanu, A. M. (1988) J. Lipid Res. 29, 797–808