Comparison of the Lysine Binding Functions of Lipoprotein(a) and Plasminogen†

Jane L. Hoover-Plow,*,‡,§ Lindsey A. Miles,‡ Gunther M. Fless,∥ Angelo M. Scanu,∥ and Edward F. Plow‡,§

Committee on Vascular Biology (CVB-1), The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Medicine, University of Chicago, 5841 South Maryland Avenue, Chicago, Illinois 60637

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ABSTRACT: Regions of apoprotein(a) of lipoprotein(a) [Lp(a)] exhibit striking primary sequence homology to the kringles of plasminogen. The kringles of plasminogen are lysine binding structures and mediate interactions of plasmin(ogen) with substrates and inhibitors. In the current study, the lysine binding properties of Lp(a) have been compared to those of plasminogen and isolated kringle 4 of plasminogen (K4). An analytical assay was implemented to quantitate the interaction of kringle-containing molecules with lysine–Sepharose beads. Radioiodinated ligands, Lp(a), plasminogen, and K4, bound to the beads, and their interactions were inhibited by lysine analogues in a dose-dependent fashion. A series of ω-aminocarboxylic acids inhibited Lp(a), plasminogen, and K4 binding to the lysine–Sepharose beads, but marked differences in the effectiveness of these compounds were observed with each ligand. In this series of compounds, 6-aminohexanoic acid was the most potent inhibitor of binding to lysine-Sepharose for all three ligands. The pH had little effect on the inhibition of plasminogen binding by these compounds. For Lp(a), a low pH caused a marked decrease in inhibition by the 5-carbon and 4-carbon ω-amino acids. In addition, tranexamic acid was 750-fold more potent than lysine in inhibiting plasminogen and 55-fold more potent for K4 binding to the beads. In contrast, the differential potency of these compounds on Lp(a) binding was only 3-fold. These results suggest that the kringles of Lp(a) possess lysine binding functions which are similar, but not identical, to those of plasminogen and its K4. To test this conclusion in a biological context, ^{125}I-Lp(a) and ^{125}I-plasminogen binding to monocytoid cells and fibrin clots was compared. The potency of tranexamic acid was much higher than that of lysine for inhibiting plasminogen binding to cells and to fibrin clots. In contrast, the ratio of tranexamic acid to lysine efficacy was less pronounced for Lp(a) binding to cells and to fibrin. Thus, Lp(a) possesses lysine binding sites which can mediate its interaction with cells and with fibrin, but the fine specificity of these sites in Lp(a) and plasminogen is distinguishable.

Elevated plasma lipoprotein(a) [Lp(a)] is a highly significant and independent risk factor for the development of atherosclerosis, coronary artery disease, and thrombosis (Dahlen *et al.*, 1975; Koltringer & Jurgens, 1985; Armstrong *et al.*, 1986; Rhoads *et al.*, 1986; Utermann, 1989; Scanu, 1992). Lp(a) is similar to low-density lipoprotein (LDL) with respect to lipid composition and content of apoprotein B. Distinguishing Lp(a) from the LDL particle is the presence of apoprotein(a) [apo(a)] within Lp(a) (Fless *et al.*, 1984). Apo(a) has a primary structure which is markedly similar to that of human plasminogen (Eaton *et al.*, 1987). The plasminogen molecule contains five kringles, disulfide looped motifs with approximately 80 amino acids, and a protease domain (Sottrup-Jensen *et al.*, 1978). Human apo(a) is composed of a variable number of plasminogen kringle 4 (K4) repeats and a single kringle 5 homologue followed by a protease domain. The cDNA of the apo(a), which was sequenced by McLean *et al.* (McLean *et al.*, 1987), encoded 37 K4 repeats; these were 75–85% identical in primary amino acid sequence to K4 of plasminogen.

The kringles of plasmin(ogen), including K4, can bind to lysine and lysine analogues (Markus *et al.*, 1982; Thewes *et al.*, 1990; Rejante *et al.*, 1991). This functional activity is important in mediating the recognition by plasmin and plasminogen of specific lysine residues in substrates (Knudsen *et al.*, 1986; Lucas *et al.*, 1983), inhibitors (Wiman & Collen, 1978), and cellular receptors (Miles *et al.*, 1988). Lp(a) also has lysine binding properties, as evidence by its retention on lysine-Sepharose and its elution from this matrix by lysine analogues (Eaton *et al.*, 1987). In addition, Lp(a) interferes with certain functions of plasminogen which are mediated by its lysine binding site(s) (LBS) (Miles *et al.*, 1989; Edelberg *et al.*, 1989; Kluft *et al.*, 1989; Loscalzo *et al.*, 1990; Hajjar *et al.*, 1989). Such observations have given rise to the hypothesis that some of the pathogenic consequences of elevated Lp(a) may depend upon its LBS, which results in its interference with normal functions of plasminogen (Miles & Plow, 1990).

The individual LBS of plasminogen exhibit differences in fine specificity in their recognition of specific lysine analogues. Plasminogen itself binds preferentially to specific lysyl residues within a limited number of proteins (Lucas *et al.*, 1983; Knudsen *et al.*, 1986; Wiman & Collen, 1978), and the intact molecule, as well as its isolated kringles, can discriminate among lysine analogues with affinities that range over several orders of magnitude. For example, the affinities of plasminogen for tranexamic acid (*trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid) versus lysine differ by approximately 100-fold (Markus *et al.*, 1979); certain K5-containing fragments of plasminogen are not retained on lysine–Sepharose

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* To whom correspondence should be addressed.

§ Present address: Center for Thrombosis & Vascular Biology (FF20), The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195.

‡ The Scripps Research Institute.

∥ University of Chicago.

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and isolated K4 does not bind benzamidine, whereas benzamidine does bind to K5 (Thewes *et al.*, 1990; Rejante *et al.*, 1991). In characterizing the fine specificity of the LBS of plasminogen, it has been established that the carboxyl group, the ϵ-amino group, and the length of the intervening carbon backbone are of critical importance (Petros *et al.* 1989). While $N\alpha$-acetyl-L-lysine and lysine react with the LBS of plasminogen, $N\epsilon$-acetyl-L-lysine interacts poorly (Petros *et al.*, 1989). In a series of ω-aminocarboxylic acids of varying carbon length, the six-carbon chain, 6-aminohexanoic acid (6-AHA), is optimal for recognition by the LBS of plasminogen or isolated K4. The five-carbon, 5-aminopentanoic acid (5-APA), and seven-carbon 7-aminoheptanoic acid (7-AHA), compounds still interact but are less effective than 6-AHA (Violand *et al.*, 1978; Thorsen, 1975).

Based upon X-ray crystallography (Wu *et al.*, 1991; Mulichak *et al.*, 1991), the key amino acid residues in plasminogen K4 which are involved in forming the LBS include TRP72, ARG71, PHE64, TRP62, ASP57, ASP55, and LYS35. Other kringle-containing proteins conserve a number of these amino acids at these positions. For example, K2 of tissue plasminogen activator (t-PA) has the same amino acids at six of these seven positions. K2 of t-PA does have lysine binding function, but its fine specificity differs from that of plasminogen and its K4 (e.g., 7-AHA has a higher affinity than 6-AHA for K2 of t-PA) (Byeon *et al.*, 1991; DeVos *et al.*, 1992; Byeon & Llinás, 1991). In other proteins, the kringles have no lysine binding functions despite considerable conservation of structure at the key positions. For example, K2 of prothrombin has five of the seven key amino acids but has no measurable lysine binding function (Tulinsky *et al.*, 1988).

Of the kringles of apo(a), $K4_{37}$ is predicted to have lysine binding function, as it has six of the seven key amino acids and a conservative LYS-to-ARG substitution as the seventh position (McLean *et al.*, 1987). This kringle has, in fact, been directly implicated in the lysine binding functions of Lp(a) (Scanu *et al.*, 1993). Other kringles of apo(a) have only one or two amino acid differences at the seven key positions. Although the lysine properties of Lp(a) have been documented in multiple studies, the fine specificity of the lysine binding properties of Lp(a) has not been examined in detail. Such specificity could be extremely important in determining the functional activities of the Lp(a) particle, particularly with respect to its capacity to inhibit functions of plasminogen and other kringle-containing proteins. Accordingly, in the present study, we have sought to compare the lysine binding properties of Lp(a) with those of plasminogen and isolated K4 of plasminogen.

EXPERIMENTAL PROCEDURES

Materials. Sepharose 4B and Ultrogel AcA44 were purchased from Pharmacia (Piscataway, NJ); ^{125}iodine from Amersham (Pittsburgh, PA); iodine monochloride from Eastman Kodak (Rochester, NY); sodium dodecyl sulfate (SDS) and polyacrylamide gel from Bio-Rad (Hercules, CA); trasylol from FBA Pharmaceuticals (New York, NY); thrombin from Armour Pharmaceuticals (Collegeville, PA); Tween 80 from Fisher (Pittsburgh, PA); phenylmethanesulfonyl fluoride (PMSF), crystalline bovine serum albumin, Fraction V (BSA), and lysine (LYS) from Calbiochem (La Jolla, CA); tranexamic acid (TA) from Aldrich (Milwaukee, WI); gelatin (Type B from bovine skin endotoxin), 6-aminohexanoic acid (6-AHA), 4-aminobutanoic acid (4-ABA), 5-aminopentanoic acid (5-APA), 7-aminoheptanoic acid (7-AHA), 8-aminooctanoic acid (8-AOA), and HEPES from Sigma (St. Louis, MO); and Hank's balanced salt solution from ICN (Costa Mesa, CA). Peptides were synthesized as previously described (Miles *et al.*, 1991). All chemicals were of reagent grade.

Lp(a) Preparation and Radioiodination. Lp(a) was isolated as previously described (Snyder *et al.*, 1992). Briefly, this isolation entailed the collection of plasma from the selected donors (from plasmapheresis) into 0.15% EDTA and addition of 0.2 mM PMSF. Next, 0.3 g/mL of NaBr was added, and the plasma was centrifuged in a 50.2 Ti rotor at 49 000 rpm for 20 h at 20 °C to obtain the lipoproteins. The lipoprotein fraction was dialyzed into phosphate buffer and applied to a lysine–Sepharose column, and the bound Lp(a) was eluted with 0.2 M 6-AHA. Thus, the Lp(a) utilized was preselected for lysine binding function, and it is the fine specificity of this fraction (>80% in most individuals) that has been assessed. The eluate was made 7.5% by weight with CsCl and then recentrifuged in a 50.2 Ti rotor at 49 000 rpm for 20 h to separate the plasminogen (bottom), Lp(a) (middle), and VLDL (top). The Lp(a) fractions were dialyzed into 0.15 M NaCl, 0.01% Na_2 EDTA/0.01% NaN_3, pH 7.4 (EDTA–saline), filtered, and stored at 4 °C in the dark. Regardless of plasma apo(a) phenotype, all purified Lp(a) preparations were isolated to obtain a single apo(a) isoform. Plasminogen contamination of the Lp(a) preparations was assessed by Western blotting of SDS–PAGE, utilizing the antiserum isolated to obtain a single K1–3 region of plasminogen which does not crossreact with Lp(a) (Miles & Plow, 1986). The Lp(a) was radioiodinated by a modified ICl procedure (Bilheimer *et al.*, 1972); the labeling buffer, 0.2 M glycine, pH 10.3, included 0.2 M 6-AHA to protect LBS from iodination. Free iodine was removed by gel filtration on a PD 10 column followed by dialysis of the Lp(a) into EDTA–saline. Lp(a) protein was quantitated by the Lowry method; a molecular weight of 910 000 for Lp(a) protein was used to determine the Lp(a) concentration.

Plasminogen and Its Derivatives. Glu-plasminogen was purified from fresh human plasma by affinity chromatography on lysine–Sepharose followed by gel filtration on ACA-44 (Miles *et al.*, 1988; Deutsch & Mertz, 1970). The latter step was included routinely to eliminate contamination with Lp(a). Freedom of the plasminogen preparations from Lp(a) was assessed by Western blotting of SDS–PAGE using an antibody to K4 which crossreacts with Lp(a) (Plow & Collen, 1981). Plasminogen and K4 were radioiodinated by a modified chloramine T procedure (Miles & Plow, 1985), which does not alter the lysine binding function, the capacity to form plasmin, or the cellular binding function of the molecule. In addition, plasminogen also has been radioiodinated by the same ICl procedure used for Lp(a) labeling, and both preparations of radioiodinated plasminogen exhibited similar lysine binding properties. Derivatives of plasminogen, EDPII, which contains K4, and EDPI, which contains K1–K3, were prepared as described (Miles & Plow, 1986; Sottrup-Jensen *et al.*, 1978). The protein concentrations of plasminogen and K4 were determined spectrophotometrically at 280 mm using an extinction coefficient of 16.8 (Miles *et al.*, 1988).

Lysine–Sepharose Binding Assay. Sepharose 2B was activated with cyanogen bromide according to the method of Parikh *et al.* (Parikh *et al.*, 1974) and was coupled to lysine (Deutsch & Mertz, 1970) or to BSA. The beads were stored in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4 with 0.1% sodium azide. In a final volume of 250 μL, ^{125}I-plasminogen, ^{125}I-Lp(a), or isolated ^{125}I-K4 of plasminogen, at 10 nM in 0.01 M phosphate, pH 7.4, with 0.15 M NaCl and 0.1% BSA, was added to 20 nM to 200 mM lysine analogue and lysine–

Sepharose beads (60–90 μL/250 μL), mixed, and incubated at room temperature for 10 min. The reaction was terminated by centrifugation in a Beckman microfuge E for 3 min, and the supernatant was counted for radioactivity (Iso-Data Gamma Counter, Palatine, IL). The percent specific binding was calculated by the following equation:

$$(\text{cpm}_{200\text{ mM 6-AHA}} - \text{cpm}_{\text{exptl}}/\text{cpm}_{200\text{ mM 6-AHA}}) \times 100$$

Values in the tables and figures are the mean ± SEM of n number of replicates.

Cell Binding Assay. U937 monocytoid cell suspensions, at 4×10^6 cells/mL in Hanks's balanced salt solution, 0.05 M HEPES, pH 7.4 (HBSS), were incubated with 10 nM radiolabeled ligand at 37 °C for 150 min and inhibitors in the presence of a final concentration of 0.1% BSA. Bound and free ligands were separated by layering 50 μL of triplicate samples onto 300 μL of 20% sucrose in HBSS. The cells were recovered by centrifugation for 2.5 min in a Beckman microfuge. The tips of the tubes were cut off and counted for radioactivity. The number of molecules bound per cell was determined from the specific activity of the radiolabeled ligand.

Fibrin Binding Assay. Fibrinogen, prepared as previously described (Keckwick *et al.*, 1955), at 100 μg in 100 μL of PBS containing 50 KIU trasylol, was dried in individual wells of microtiter plates at 37 °C for 18 h. Thrombin, 0.1 units in 0.05 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.01% Tween 80, 50 KIU/mL trasylol, 1 mg/mL BSA, and 1 mg/mL gelatin (Buffer I), was incubated with the fibrinogen for 1 h at 22 °C, and the wells were washed 3 times with 150-μL aliquots of 0.05 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.01% Tween 80, and 50 units/mL trasylol. For the last wash, the buffer contained 0.1 unit/mL hirudin and was incubated with the fibrin for 30 min. To assess ^{125}I-ligand binding to the fibrin, triplicate 100-μL samples containing 10 nM radiolabeled plasminogen or Lp(a) and 0–200 mM tranexamic acid or lysine in Buffer I were placed in the fibrin-coated microtiter wells for 2 h at 22 °C on a rotary shaker. The test solutions were aspirated, and the wells were washed 3 times, individually removed, and counted for radioactivity (Iso-Data Gamma Counter). Percent specific inhibition of fibrin binding by the ligands was calculated by assuming that residual binding in the presence of EACA was nonspecific:

$$1 - \frac{\text{cpm exptl} - \text{cpm in presence of 6-AHA}}{\text{cpm buffer} - \text{cpm in presence of 6-AHA}} \times 100$$

RESULTS

Characterization of Lysine–Sepharose Binding Assay. An analytical lysine–Sepharose binding assay, similar to that described by Winn *et al.* (Winn *et al.*, 1980), was developed to compare the lysine binding functions of Lp(a), plasminogen, and K4 of plasminogen. The Lp(a) was radioiodinated by a protocol which protected its lysine binding properties and maintained the structural integrity of the lipoprotein particle (Snyder *et al.*, 1992). In preliminary experiments, conditions were standardized such that the same input concentration of ^{125}I-Lp(a), ^{125}I-plasminogen, and ^{125}I-K4 (10 nM final concentration) could be added to the beads to obtain significant binding of each ligand. During the course of these studies and utilizing several preparations of each ligand, 20–60% of the ligands added at the 10 nM concentration bound to the lysine–Sepharose. Removal of the unbound ligands, followed by their addition to a second aqliquot of lysine–Sepharose beads, resulted in a similar percent of binding. Furthermore, the measured interactions of each ligand with the lysine–Sepharose was specific, as the binding of each was inhibited by >99% by 0.2 M 6-aminohexanoic acid. Under these conditions, no specific binding of Lp(a) or other ligands to nonactivated or to cyanogen bromide-activated BSA–Sepharose was observed. Utilizing PBS as a buffer, time course studies indicated that the binding of all three ligands reached apparent equilibrium within 5 min at 22 °C. The order of addition of radiolabeled ligands and competitors to the beads also did not alter the extent of binding. In all reported experiments, the selected soluble lysine analogues and the radiolabeled ligands were added to the lysine–Sepharose beads, and binding was measured after a 10-min incubation at 22 °C.

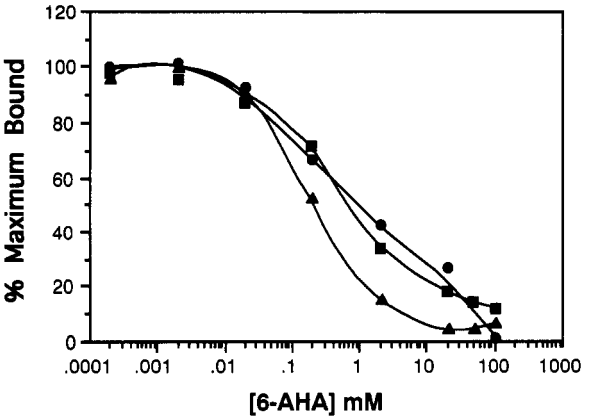

FIGURE 1: Inhibition of ^{125}I-Lp(a) (●), ^{125}I-plasminogen (■), and ^{125}I-K4 of plasminogen (▲) binding to lysine–Sepharose by AHA, 6-aminohexanoic acid. Radioiodinated Lp(a), plasminogen, and K4, at 10 nM, were bound to lysine–Sepharose beads in the presence of AHA (20 nM to 20 mM). IC_{50} values (mM, mean ± SE): Lp(a), 1.3 ± 0.2 (7); plasminogen, 1.0 ± 0.2 (10); K4, 0.2 ± 0.03 (5).

Lp(a) Binding to Lysine–Sepharose Is Mediated by One or More of Its Apo(a) Kringles. The interaction of radioiodinated Lp(a), plasminogen, and K4 with the lysine–Sepharose beads was inhibited by 6-AHA in a dose-dependent fashion (Figure 1). 6-AHA, a mimic of carboxyl-terminal lysyl residues in proteins, was an effective inhibitor of plasminogen and K4 binding to lysine–Sepharose. The IC_{50} values for inhibition of plasminogen and K4 binding to lysine–Sepharose by 6-AHA (1 and 0.2 mM, respectively) are similar to those reported by Winn *et al.* (Winn *et al.*, 1980). 6-AHA also produced a dose-dependent inhibition of Lp(a) binding to the lysine–Sepharose beads. Greater than 99% of the total binding of Lp(a) was inhibited by 0.2 M 6-AHA. Moreover, the IC_{50} value for Lp(a) was similar to that for plasminogen. This value was 1.3 ± 0.2 mM based on seven experiments. The IC_{50} of 6-AHA for K4 was 5-fold less than those for Lp(a) and plasminogen, indicating that 6-AHA is a more potent inhibitor of K4 binding to lysine–Sepharose beads.

Since Lp(a) preparations from different individuals exhibit heterogeneity within the apo(a) moiety (Soutar *et al.*, 1991), the lysine binding characteristics of homogeneous, single isoform Lp(a) preparations from three donors were examined (Figure 2). Lp(a) proteins, i.e., apo(a) plus apoB, from these three donors had molecular weights of 920, 1010, and 1200 kD as calculated from the molecular weight of whole Lp(a) and the percentage chemical composition (data not shown). By SDS–PAGE, the respective apo(a) isoforms had mobilities greater than, equal to, and smaller than that of $apoB_{100}$. The IC_{50} values for the three donors ranged from 0.9 to 8.0 mM (from two separate experiments with each donor). The curve for donor 3 was significantly different from that of donors 1 and 2 ($p < 0.01$, determined by an analysis of variance and a Tukey–Kramer multiple comparison test).

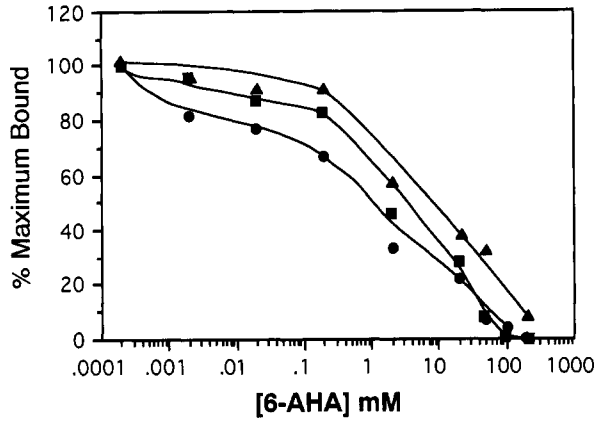

FIGURE 2: Inhibition of binding of ^{125}I-Lp(a) from three different donors to lysine–Sepharose by 6-AHA. Radioiodinated Lp(a) (10 nM) was bound to lysine–Sepharose beads in the presence of 6-aminohexanoic acid (20 nM to 200 mM). The molecular weights of the protein of apoB and apo(a) Lp(a) from donors 1 (●), 2 (■), and 3 (▲) were 920, 1200, and 1010 kD, respectively. Values are the means of two experiments.

Table I: Inhibition of Lp(a), Plasminogen, and K4 Binding to Lysine–Sepharose by Lysine Analogues

lysine analogue	IC_{50} (mM)		
	^{125}I-plasminogen[a]	^{125}I-Lp(a)	^{125}I-K4
lysine	11.0 ± 2.3 (7)[b]	3.8 ± 0.5 (6)	2.2 ± 0.2 (5)
6-aminohexanoic acid	1.0 ± 0.2 (10)	1.3 ± 0.2 (7)	0.2 ± 0.03 (5)
tranexamic acid	0.2 ± 0.1 (5)	1.3 ± 0.7 (5)	0.04 ± 0.01 (2)
*N*α-acetyllysine	1.6 ± 0.1 (4)	3.1 ± 0.9 (7)	0.9 ± 0.1 (2)
*N*ε-acetyllysine	>200 (4)	108 ± 32 (4)	180 ± 20 (3)

[a] Radiolabeled ligands at 10 nM were incubated with lysine–Sepharose beads in the presence of lysine analogues (20 nM–200 mM). Radiolabeled ligand binding to beads: plasminogen, 50–60%; Lp(a), 20–30%; and K, 40–45%. [b] Values are the mean ± SEM; (*n*) = number of experiments.

LDL, the lipoprotein which most resembles Lp(a) but does not contain apo(a), does not bind specifically to the lysine–Sepharose beads (Eaton *et al.*, 1987). That Lp(a) does bind to lysine–Sepharose in an interaction inhibited by 6-AHA implicates one or more of the apo(a) kringles of Lp(a) in the interaction. Still additional evidence for kringle involvement in Lp(a) binding to lysine–Sepharose was obtained with a polyclonal antibody to K4 of plasminogen. This antibody, which was previously shown (Plow & Collen, 1981) to inhibit the binding of K4 to lysine, also inhibited Lp(a) binding to lysine–Sepharose. At the maximum concentration producing no precipitation with either radiolabeled ligand, the percent inhibition of Lp(a) binding was 39 ± 8 (mean ± SE, n = 5) and of K4 binding was 44 ± 2 (n = 6). Lp(a) inhibition by an anti-EDPI, which reacts with plasminogen but not apo(a) by immunoblotting, was only 8% at the same dilution. The similarity in the inhibition of Lp(a) and K4 binding to lysine–Sepharose by an antibody specific for K4 further suggests that Lp(a) binding is mediated by its K4-like kringle(s).

Inhibition of Plasminogen, Lp(a), and K4 Binding to Lysine–Sepharose by Lysine Analogues. Radioiodinated Lp(a), plasminogen, and K4 binding to the lysine–Sepharose beads was inhibited by 6-AHA, lysine, and tranexamic acid in a dose-dependent fashion. IC_{50} values were derived from the inhibition curves and are summarized in Table I. IC_{50} values for 6-AHA and tranexamic acid were the same for Lp(a) and were 3-fold lower than that of lysine. The IC_{50} values for lysine and 6-AHA differed by 10-fold for plasminogen and K4. A comparison of the ratio of IC_{50} values of lysine to tranexamic acid indicated a marked difference between plasminogen and K4 (lysine/tranexamic acid = 55) vs Lp(a) (ratio = 3). The difference in these ratios indicates that the fine specificities of the LBS of plasminogen and Lp(a) are different.

Table II: Inhibition of ^{125}I-Ligand Binding to Lysine–Sepharose by Peptides with Lysine Residues

peptide[a]	percent inhibition	
	^{125}I-plasminogen[b]	^{125}I-Lp(a)[b]
C-Terminal		
DLKLVPPMEEDYPQFGSPK	68[c]	42
GSRGSTEDQMAK	67	35
C-Terminal and Internal		
HHLGGAKQAGDVGGYK	54	58
N-Terminal		
KYGGHHLGGAKQRGDV	5	0

[a] Final concentration of peptides was 2.5 mM. [b] Final concentration of ^{125}I-ligands was 10 nM. [c] Values are from a typical experiment. Means of triplicates from an experiment in which binding of both ligands was measured simultaneously. The results are similar to those obtained in two other experiments.

Table III: Differential Inhibition of Plasminogen, Lp(a), and K4 Binding to Lysine–Sepharose by ω-Aminocarboxylic Acids with Different Carbon Chain Lengths

lysine analogue	IC_{50} (mM)		
	^{125}I-plasminogen[a]	^{125}I-Lp(a)	^{125}I-K4
4-aminobutanoic acid	6.2 ± 1.0 (5)[b]	14.5 ± 4.9 (5)	0.7 ± 0.1 (5)
5-aminopentanoic acid	2.0 ± 0 (3)	3.0 ± 0.6 (3)	N/A[c]
6-aminohexanoic acid	1.0 ± 0.2 (10)	1.3 ● 0.2 (7)	0.2 ± 0.03 (5)
7-aminoheptanoic acid	5.5 ± 2.5 (2)	6.0 ± 2.0 (2)	2.5 ± 1.5 (5)
8-aminooctanoic acid	>200 (2)	>200 (2)	10.0 ● 0 (2)

[a] Radiolabeled ligands at 10 nM were incubated with lysine–Sepharose beads in the presence of lysine analogues (20 nM–200 mM). [b] Values are the mean ± SEM, (*n*) = number of experiments. [c] Not analyzed.

Role of the Amino and Carboxyl Groups in LBS Recognition of Lp(a) and Plasminogen. Previous studies have reported the importance of a carboxyl-terminal lysine for plasminogen binding to peptides and proteins (Skoza *et al.*, 1968; Miles *et al.*, 1988, 1991). For example, peptides with C-terminal lysines inhibit plasminogen binding to U937 cells by 80%, whereas peptides with internal and/or N-terminal lysines inhibit plasminogen binding to cells only minimally. A similar pattern exists for Lp(a) binding to lysine–Sepharose (Table II). Only peptides with carboxyl-terminal lysines were effective inhibitors of Lp(a) binding, whereas peptides with amino or internal lysines were ineffective. Acetylation (Table I) of the ε-amino group but not the α-amino group of lysine markedly reduced its ability to inhibit binding of all three ligands to lysine–Sepharose. While acetylation of the α-amino group of lysine increased affinity for plasminogen (6-fold) relative to lysine, this increase was not observed with Lp(a). Thus, the change and/or the bulkiness of the 2-carbonyl substitution group affects lysine analogue recognition by plasminogen but not by Lp(a).

Carbon Chain Length Requirement for Ligand Binding to Lysine–Sepharose. As previously reported in the literature (Violand *et al.*, 1978; Winn *et al.*, 1980; Rejante *et al.*, 1991), in the series of ω-aminocarboxylic acids with varying carbon chain lengths, maximal potency was observed with 6-AHA for both plasminogen and K4 (Table III). The 6-carbon ω-aminocarboxylic acid (6-AHA) was also the most potent inhibitor for Lp(a) (1.3 mM). For Lp(a), the IC_{50} for the 4–7-carbon ω-aminocarboxylic acids ranged from 1.3 to 14.5 mM. The IC_{50} for the 8-aminooctanoic acid on Lp(a) binding was similar to that of plasminogen (>200 mM). For Lp(a),

Table IV: Dependence of Inhibition of ^{125}I-Ligand Binding to Lysine–Sepharose on pH

pH	IC_{50} (mM)[a]	
	^{125}I-Lp(a)	^{125}I-plasminogen
	6-Aminohexanoic Acid	
4.0	4.2 ± 1.8 (6)	2.4 ± 1.4 (5)
7.4	1.2 ± 0.5 (7)	1.0 ± 0.6 (10)
	5-Aminopentanoic Acid	
4.0	133 ± 27 (5)[b]	1.9 ± 0.6 (3)
7.4	3.0 ± 0.6 (3)[b]	2.0 ± 0 (3)
	4-Aminobutanoic Acid	
4.0	20.5 ± 13.3 (4)	5.2 ± 1.1 (4)
7.4	14.5 ± 4.9 (5)	6.2 ± 2.2 (3)

[a] Values are the mean ± SEM, (n) is the number of experiments. [b] Values are significantly different at $p < 0.01$.

Table V: Effects of Tranexamic Acid and Lysine on Lp(a) and Plasminogen Binding by Fibrin and U937 Cells

	IC_{50} (mM)[a]	
	^{125}I-Lp(a)	^{125}I-plasminogen
	fibrin	
lysine	6.3 ± 1.5	4.5 ± 0.7
tranexamic acid	3.2 ± 1.2	0.3 ± 0
ratio[b]	2[c]	15
	U937 Cells	
lysine	3.6 ± 2.8	3.7 ± 0.3
tranexamic acid	0.11 ± 0.09	0.02 ± 0.002
ratio[b]	36[c]	214

[a] Values are the mean ± SE of 2–4 experiments. [b] Mean ratio of lysine IC_{50} tranexamic acid IC_{50} from 2–4 experiments. [c] Significant difference ($p < 0.05$) between Lp(a) ratio and plasminogen ratio.

the 4-carbon ω-aminocarboxylic acid (4-ABA) was a less potent inhibitor (14.5 mM) that for K4 (0.7 mM) and plasminogen (6.2 mM) (de Serrano *et al.*, 1992).

de Serrano et al. (de Serrano *et al.*, 1992) reported differences in recognition of ω-aminocarboxylic acids by a recombinant K2 variant of tissue plasminogen activator at acidic pH compared to pH 7.4. We observed that the percent of Lp(a) bound to the lysine–Sepharose decreased slightly ($p < 0.01$) at pH 4.0 (14 ± 2%, $n = 6$) compared to pH 7.4 (24 ± 2%, $n = 8$). Plasminogen binding was similar at pH 4.0 (52 ± 3%, $n = 7$) and pH 7.4 (55 ± 2%, $n = 8$). 5-APA and 4-ABA compounds were less potent inhibitors of Lp(a) binding to the lysine–Sepharose beads at acidic pH than at pH 7.4 (Table IV). This decrease in potency was more pronounced with 5-APA than with 4-ABA, with an increase in the IC_{50} for 5-APA from 3 mM at pH 7.4 to 133 mM at pH 4.0 ($p < 0.01$) (740-fold). For 4-ABA, the change was less pronounced, from 14.5 to 20.5 at pH 7.4 and pH 4.0 (1.4-fold), respectively ($p > 0.05$). With plasminogen, low pH did not affect the recognition of either 4-ABA or 5-APA. Thus, marked differences in the LBS of Lp(a) and plasminogen at acidic pH are apparent, and a striking difference in inhibition by the 5-carbon compound compared to the 6-carbon and 4-carbon ω-aminocarboxylic acids is observed for Lp(a).

LBS Recognition of Physiological Substrates. The relatively small difference in IC_{50} values of lysine and tranexamic acid on Lp(a) binding to lysine–Sepharose was also apparent in its binding to U937 cells and to fibrin (Table V). The ratio of lysine to tranexamic acid was smaller for fibrin binding for both Lp(a) and plasminogen. For Lp(a) the ratio was 18-fold higher for cells than fibrin, and for plasminogen the ratio was 14-fold higher. The difference in the IC_{50} values of lysine and tranexamic acid was 15-fold for plasminogen binding to fibrin but only 2-fold for Lp(a). Inhibition of plasminogen and Lp(a) binding to U937 cells by lysine and tranexamic acid showed a similar pattern; the ratio of lysine to tranexamic acid was 36 for Lp(a) but 214 for plasminogen. Thus, the reduced difference in potency of lysine and tranexamic acid for Lp(a) binding to lysine–Sepharose beads was also apparent in fibrin and cell binding.

DISCUSSION

Previous studies have demonstrated that Lp(a) can bind lysine and lysine analogues (Eaton *et al.*, 1987; Rouy *et al.*, 1992). Since lysine binding is a property of plasminogen but not LDL, and since antibodies to K4 of plasminogen can also inhibit the interaction of Lp(a) with lysine, it is the kringles of apo(a) that impart lysine binding functions to Lp(a). The fine specificity (i.e., the capacity to discriminate among lysine analogues) of the lysine binding functions of plasminogen and its kringles, as well as other kringle-containing molecules, has been analyzed in detail (Markus *et al.*, 1979; Thewes *et al.*, 1990; Rejante *et al.*, 1991; Petros *et al.*, 1989; Vali & Patthy, 1982; Thorsen, 1975). To our knowledge, the present study represents the first detailed analysis of the fine specificity of the lysine binding functions of Lp(a). Since it is the LBS of Lp(a) that allow it to interfere with numerous functions of plasminogen, information on their fine specificity is relevant to the thrombogenic property of this lipoprotein particle.

Overall, the fine specificity of the LBS of Lp(a) (defined as those site(s) which mediate its binding to lysine–Sepharose beads) is very similar to that of plasminogen. The following observations support this conclusion. (1) Lp(a) and plasminogen bound rapidly and specifically to lysine–Sepharose. (2) The LBS of each molecule exhibited a similar relative affinity for all lysine analogues (e.g., tranexamic acid > 6-AHA > lysine). (3) 6-AHA, a model ligand for the LBS, has a similar relative affinity for Lp(a) and plasminogen. (4) In a series of ω-aminocarboxylic acids, carbon chain length had a similar influence on recognition. (5) Peptides with carboxy-terminal lysines interacted with the LBS of Lp(a) and plasminogen, whereas amino-terminal and internal lysyl residues reacted poorly (or not at all). (6) For both Lp(a) and plasminogen, acetylation of the α-amino group of lysine enhanced recognition. (7) On the other hand, acetylation of the ε-amino group resulted in marked reduction of recognition. The striking similarities in recognition functions are consistent with the remarkable primary structural similarity between the kringles of apo(a) and plasminogen (McLean *et al.*, 1987).

The fine specificity of the LBS of Lp(a) also was similar to that of isolated kringle 4 of plasminogen. In most cases, however, the absolute IC_{50} values for Lp(a) were closer to those of plasminogen (e.g., 4-ABA, 6-AHA, and tranexamic acid) than to those of K4. Only for lysine, itself, were IC_{50} values for Lp(a) and K4 more similar than for plasminogen and Lp(a). The interaction of plasminogen with lysine–Sepharose is mediated primarily by the high-affinity LBS associated with its K1 (Lerch & Rickli, 1980). K4, in fact, may not be functional when integrated into the intact plasminogen molecule (Vali & Patthy, 1982). Thus, despite the absence of a K1 homologue within Lp(a), its LBS exhibit the functional characteristics of a K1. This conclusion is consistent with the capacity of Lp(a) to effectively inhibit the interactions of plasminogen which are mediated by its K1 moiety (Markus *et al.*, 1982; Miles *et al.*, 1989; Edelberg *et al.*, 1989; Kluft *et al.*, 1989; Loscalzo *et al.*, 1990). Recently, Rouy *et al.* (Rouy *et al.*, 1992) concluded that the recognition of fibrin by Lp(a) is mediated by a K1-like recognition.

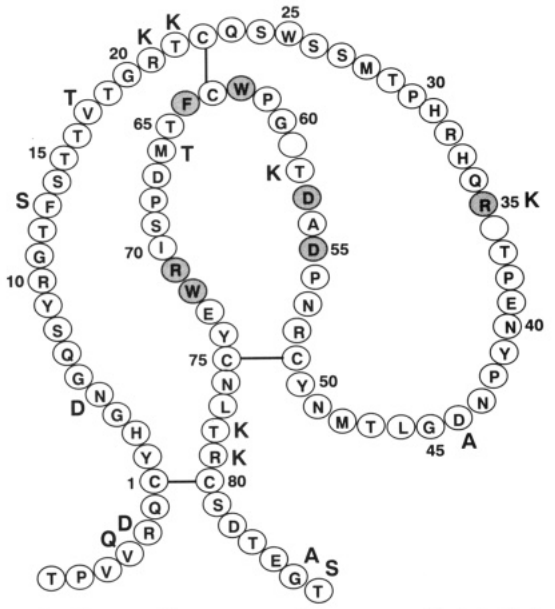

FIGURE 3: Comparative structure of human apo(a) kringle 4_{37} and human plasminogen K4. Amino acids of apo(a) are shown in the circles, and the differences in plasminogen are indicated outside the circles and include the positions 20, 21, 35, 58, 78, and 79. The numbering follows the convention for plasminogen K5. The amino acid sequences are from McLean *et al.* (1987) and Sottrup-Jensen *et al.* (1978). The shaded circles indicate residues which contact bound lysine analogues in the crystal structure (Wu *et al.*, 1991).

Isolated K4 and recombinant K1 differ primarily in their affinity for lysine and lysine analogues (Menhart *et al.*, 1991).

While the functional similarities between the LBS of Lp(a) and plasminogen are remarkable, certain differences in their fine specificity were noted. The IC_{50} values for 6-AHA, lysine, and tranexamic acid differed widely for both plasminogen and its isolated K4. However, for Lp(a), the IC_{50} values for these three compounds were very similar. In quantitative terms, the ratios of the IC_{50} values of lysine to those tranexamic for plasminogen and K4 were both 55, compared to 3 for Lp(a). This difference was observed not only in the recognition of the lysine–Sepharose but also in the recognition of physiological ligands of the LBS, fibrin and cell surface receptors. Another notable difference in the fine specificity of the LBS of plasminogen and Lp(a) was the potency of 5-APA at low pH vs pH 7.4. DeMarco *et al.* (DeMarco *et al.*, 1985) found that acidic pH reversibly altered the unfolding of plasminogen, and Sehl and Castellino (Sehl & Castellino, 1990) reported that the binding parameters of isolated K4 were not influenced by pH values between 5.5 and 8.2. Such pH sensitivity of Lp(a) is similar to that in the recognition of certain ligands by the LBS of variant t-PA (de Serrano *et al.*, 1992) and suggests that Lp(a) may also interfere with certain functions of t-PA which are mediated by its LBS. One such function is the activation of plasminogen by t-PA (Loscalzo *et al.*, 1990; Edelberg *et al.*, 1989). In fact, Lp(a) has been shown to bind directly to t-PA (Simon *et al.*, 1991) and inhibits t-PA binding to fibrinogen.

The crystallographic structure of K4 of plasminogen identifies seven amino acids in close contact with bound 6-AHA (Rouy *et al.*, 1992). As indicated in Figure 3, these residues are LYS35, ASP55, ASP57, TRP62, PHE64, ARG71, and TRP72. Of the kringles of human apo(a) that were sequenced by McLean *et al.* (McLean *et al.*, 1987), $K4_{37}$ was the most similar to K4 of plasminogen with respect to the conservation of the key amino acid residues contributing to the LBS of K4. The $K4_{37}$ kringle has a conservative ARG for LYS substitution at position 35 but is identical at the other key six positions. Other kringles of apo(a) have one or more nonconservative substitutions at the key positions, making it less likely that they would exhibit lysine binding functions, thereby suggesting that $K4_{37}$ plays a dominant role in the lysine recognition of apo(a). Two recent observations support this conclusion. First, Lp(a) from Rhesus monkeys exhibits a markedly diminished lysine binding capacity compared to human Lp(a). Each combination of amino acids of the seven key positions of the lysine binding site that are found in human apo(a) kringles also are present in Rhesus apo(a) with a single exception, $K4_{37}$ (Tomlinson *et al.*, 1989). At position 72, an ARG is present in the Rhesus apo(a) kringle instead of the TRP in the human apo(a) kringle. Molecular modeling suggests that this substitution of ARG for TRP would ablate the lysine binding function of the kringle (Scanu *et al.*, 1993). Second, it has recently been shown that it is variable repetition of $K4_2$ that gives rise to the size heterogeneity of Lp(a) (Gavish *et al.*, 1989; Koschinsky *et al.*, 1990). This kringle has been expressed in *Escherichia coli* and does not have lysine binding function (Li *et al.*, 1992). In comparing $K4_{37}$ to K4 of plasminogen, there are several features which might contribute to the observed differences in fine specificity of the LBS. First, the ARG substitution for LYS at position 35 might be contributory. In plasminogen K1, position 35 is also an ARG, which might account for the Lp(a) K1-like characteristics. Second, differences in the amino acids, other than those directly involved in contacting lysine, may be contributory. In this regard, K4 of plasminogen contains six lysyl residues, and all of these have been replaced in $K4_{37}$ of apo(a). Three of these replacements are with ARG residues, but at the other three positions, the replacements are with nonbasic amino acids. Thus, the overall surface charge on $K4_{37}$ is different from that of K4 of plasminogen, and $K4_{37}$ is also glycosylated, which would influence charge. As shown in Figure 3, LYS20, LYS21, and LYS35 in the outer loop of plasminogen K4 correspond to ARG20, THR21, and ARG35 in Lp(a); and in the inner loop, LYS58, LYS78, and LYS79 of plasminogen K4 corresponding to THR58, THR78, and ARG79 in Lp(a).

The inhibition curves for 6-AHA were constructed for three isoforms of Lp(a), and the curve for one donor was significantly different from the other two. At this juncture, we cannot determine whether those differences are solely isoform dependent. However, based upon data in the literature (Leerink *et al.*, 1992), it is reasonable to predict that Lp(a) will exhibit heterogeneity with respect to lysine binding properties. At the extreme, this heterogeneity results in Lp(a) which does not bind to lysine–Sepharose. Such heterogeneity could have a significant impact on the pathogenetic potential of Lp(a). Ultimately, systematic studies to compare the lysine binding properties of Lp(a) preparations from multiple individuals and of isolated kringles of apo(a) must be performed to clearly establish structure–function relationships and to assess functional heterogeneity. The present study provides a reference point and a facile assay system for such future comparisons.

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