Biochem. J. (1991) 274, 491–496 (Printed in Great Britain)

Characterization of the enzyme activity of human plasma lipoprotein (a) using synthetic peptide substrates

Matti JAUHIAINEN*, Jari METSO,* Pekka KOSKINEN,† and Christian EHNHOLM*‡
*National Public Health Institute, Mannerheimintie 166, SF-00300 Helsinki, and †First Department of Medicine, Helsinki University Central Hospital, SF-00290 Helsinki, Finland

The plasma concentration of lipoprotein (a) [Lp(a)] is correlated with the risk of atherosclerosis. It is a lipoprotein particle consisting of apoprotein (a) [apo(a)], a protein showing considerable amino acid sequence identity with plasminogen, bound to low-density lipoprotein. The apo(a) portion of Lp(a) was recently shown to have serine-proteinase-type amidolytic activity and to be able to degrade the adhesive glycoprotein fibronectin. To characterize this enzyme activity further, we used chromogenic peptide substrates and inhibitors. Of the substrates tested, those with arginine at the scissile bond [*N*-α-benzoyl-L-Arg *p*-nitroanilide (pNA), *N*-α-benzoyl-Ile-Glu-Gly-Arg-pNA, *N*-α-benzyloxycarbonyl-Arg-Gly-Arg-pNA] gave the highest hydrolysis rates. Synthetic substrates with plasmin specificity (Val-Leu-L-Lys-pNA and Val-Phe-L-Lys-pNA) were not hydrolysed by Lp(a). Neither tissue plasminogen activator nor urokinase had any effect on the enzyme activity. The addition of antibodies to these plasminogen activators did not inhibit the enzyme activity of Lp(a). Inhibition experiments with phenylmethanesulphonyl fluoride, carbodi-imide, dichloroisocoumarin and competitive peptide inhibitors demonstrated that Lp(a) has enzyme activity that closely resembles that of serine proteinases. Whether this serine-proteinase activity of Lp(a) plays any role in the genesis of atherosclerosis remains to be established.

INTRODUCTION

The serum concentration of lipoprotein (a) [Lp(a)] is an important independent risk factor for coronary heart disease [1–3]. Lp(a), first described by Berg [4], represents a quantitative genetic trait [5] which is associated with a size polymorphism of Lp(a) [6]. It is a lipoprotein with many physical and chemical properties in common with low-density lipoproteins (LDL). It has a similar lipid composition to, but a higher density and carbohydrate content than, LDL [7,8]. The protein moiety consists of apolipoprotein B (apoB) and the (a) protein [apo(a)] [8,9]. From the Lp(a) particle, apo(a) can be dissociated under reducing conditions [10]. Apo(a) displays genetically determined size heterogeneity, and different forms with molecular masses ranging from 280 to 710 kDa have been reported [11]. Utermann *et al.* [12] demonstrated that this size heterogeneity is controlled by a series of autosomal alleles at a single locus [13,14] closely linked to the plasminogen locus on chromosome 6.

Recently, the complete DNA sequence and the deduced amino acid sequence of apo(a) have been elucidated [15,16]. A comparison of apo(a) and plasminogen has revealed a striking sequence similarity between these two proteins. Structures showing sequence identity with the plasminogen elements termed kringle 4, kringle 5 and a *C*-terminal proteinase region are all present in apo(a). The proteinase domain of apo(a) has 88% amino acid sequence identity with plasminogen and, furthermore, the catalytic triad (Asp-His-Ser) essential for the proteolytic activity of serine proteinases is retained in apo(a) [15,17]. Plasminogen is an inactive zymogen and belongs to a family of proteins which include regulatory proteinases of the fibrinolytic and blood-coagulation systems. After being cleaved by tissue-type (t-PA) or urokinase (u-PA) plasminogen activators to form active plasmin, it is a protein essential for the lysis of blood clots [18]. In apo(a) the amino acid corresponding to the arginine at which the activating cleavage of plasminogen occurs is substituted by a serine residue, a mutation that has been considered to result in an inactive-proteinase region in apo(a) [15,19]. To our knowledge there has been only one early report describing enzyme activity associated with Lp(a) [20]. Recently Lp(a) has been shown to bind to, and degrade, human plasma fibronectin [21]. The proteolytic activity of Lp(a) was localized to apo(a) and was of the serine-proteinase type. On the basis of experiments with chromogenic peptide substrates and serine-proteinase inhibitors, we now describe some characteristics of this enzymic activity.

EXPERIMENTAL

Materials

Chemicals. Sepharose 4B and Heparin–Sepharose CL 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Dithioerythritol (DTE), dithiotreitol (DTT) and β-mercaptoethanol were purchased from E. Merck, Darmstadt, Germany. Trypsin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of the highest available grade.

Inhibitors. Phenylmethanesulphonyl fluoride (PMSF), trypsin inhibitor (from soybean, type I-S), leupeptin, *N*-ethylmaleimide (NEM) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDAC) were purchased from Sigma; aprotinin (Trasylol) was a product of Bayer, Leverkusen, Germany; Tos-Lys-CH_2Cl, tosyl-

Abbreviations used: Lp(a), lipoprotein (a); apo(a), apoprotein (a); apoB, apolipoprotein B; LDL, low-density lipoproteins; HDL, high-density lipoproteins; IRMA, immunoradiometric assay; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; -pNA, *p*-nitroanilide; Ph, *N*-α-benzoyl; Cbz, benzyloxycarbonyl; DTE, dithioerythritol; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane ('TLCK'); DCIC, 3,4-dichloroisocoumarin; APMSF, (4-amidinophenyl)methanesulphonyl fluoride; iPr_2P-F, di-isopropyl fluorophosphate; substrates S-2222 etc. are defined in the text; L-BAPA, *N*-α-benzoyl-L-Arg *p*-nitroanilide; L-TAME, tosyl-L-Arg methyl ester; chromozym PL, tosyl-Gly-Pro-Lys *p*-nitroanilide; PBS, phosphate-buffered saline (0.01 M-phosphate/0.14 M-NaCl, pH 7.4); DMSO, dimethyl sulphoxide.

‡ To whom correspondence should be sent.

lysylchloromethane ('TLCK'); 3,4-dichloroisocoumarin (DICC) and (4-amidinophenyl)methanesulphonyl fluoride (APMSF) were obtained from Boehringer, Mannheim, Germany; [1,3-^{3}H]di-isopropyl fluorophosphate (iPr_2P-F) was purchased from New England Nuclear Co., Boston, MA, U.S.A.

Substrates. Ph-Ile-Glu-Gly-Arg-pNA (where Ph is *N*-α-benzoyl and pNA is *p*-nitroanilide) (S-2222), Val-Leu-Lys-pNA (S-2251), Val-Phe-Lys-pNA (S-2390), Glu-Pro-Val-pNA (S-2484), *N*-α-Cbz-Arg-Gly-Arg-pNA (where Cbz is benzyloxycarbonyl) (S-2765) and Pro-Phe-Arg-pNA (S-2302) were obtained from Kabi Diagnostica, Stockholm, Sweden; *N*-α-benzoyl-L-Arg *p*-nitroanilide (L-BAPA), tosyl-L-Arg methyl ester (L-TAME) and tosyl-Gly-Pro-Lys-pNA (Chromozym PL) were purchased from Boehringer.

Antisera. Goat anti-[human tissue plasminogen activator (t-PA)] (IgG fraction) and goat anti-[human urokinase (U-PA)] (IgG fraction) were obtained from Biopool, Umeå, Sweden.

Methods

Isolation of lipoprotein (a). Plasma from healthy donors with Lp(a) concentrations of 100–150 mg/dl was obtained by plasmapheresis. Lp(a) was isolated as described [22] by sequential ultracentrifugation [23] followed by gel filtration on a Sepharose 4B column (2.5 cm × 100 cm) eluted with phosphate-buffered saline (PBS). To obtain apo(a), Lp(a) was reduced with 10 mM-DTE at 25 °C for 1 h and apo(a) separated by centrifugation in a 1.02–1.18 g/ml KBr density gradient [24] or by heparin–Sepharose chromatography after reduction with 10 mM-DTT at 37 °C for 2 h [25].

Determination of amidolytic activity. The amidolytic activities of Lp(a) and apo(a) were determined using chromogenic peptide substrates. The substrates S-2222, 2251, 2390, 2765 and L-BAPA were dissolved in distilled water, S-2484 in 8% dimethyl sulphoxide (DMSO) and L-TAME and Chromozym PL in 100 mM-Tris/HCl buffer, pH 8.0. In a typical assay, 650 μl of 100 mM-Tris/HCl buffer, pH 8.0, and 100 μl of purified Lp(a) solution were added to a cuvette, mixed and preincubated for 5 min at 25 °C. To initiate the reaction, 200 μl of substrate was added and the increase in absorbance at 405 nm due to liberated 4-nitroaniline was measured in a Shimadzu UV-260 spectrophotometer. The results are presented either as relative absorbance units or as catalytic units (μmol/min) using the linear millimolar absorption coefficient of 1.02 litre·mmol^{-1}·mm^{-1} for 4-nitroaniline, which is in accordance with the values for *p*-nitroaniline at 405 nm. The coefficient given by the peptide substrate manufacturer (Kabi), 1.27 litre·mmol^{-1}·mm^{-1}, was determined at a wavelength of 316 nm [26]. The L-TAME reaction was monitored at 247 nm.

Inhibition of amidolytic activity. The inhibitor stock solutions were prepared in distilled water, ethanol or DMSO. The final concentration of organic solvent in the assays did not exceed 1.5% (v/v). In a typical inhibition experiment purified Lp(a) in 100 mM-Tris/HCl buffer, pH 8.0, was preincubated with different concentrations of inhibitor for 15 min at room temperature. Thereafter the reaction was initiated by adding substrate and monitored for 10 min.

Other methods. Lp(a) assay is a solid-phase two-site immunoradiometric assay (IRMA) using two monoclonal antibodies directed toward different epitopes on apo(a) [27]. The coefficients of variation were 4.5% (intra-assay) and 4.9% (inter-assay). The standard curve range was 15–840 μg of Lp(a)/ml. ApoB was measured with an immunoturbidometric assay. The standard-curve range was 0.2–2.8 mg of apoB/ml (Orion, Helsinki, Finland). Cholesterol was measured by an enzymic method [28]. Protein was assayed according to Lowry *et al.* [29], with BSA as standard. PAGE was performed in 2–16% gradient slab gels using a Tris/HCl/borate electrophoresis buffer system as described by the manufacturer (Pharmacia, Uppsala, Sweden).

RESULTS

Lp(a) was isolated from plasma by a combination of ultracentrifugation and gel filtration on Sepharose 4B. When the fraction of density 1.05–1.12 g/ml was subjected to gel filtration, the elution pattern shown in Fig. 1 was obtained. The Lp(a) lipoprotein eluted as a symmetrical peak well resolved from high-density lipoprotein (HDL). The amidolytic activity determined from the fractions with the synthetic substrate S-2765 paralleled the Lp(a) concentration in these fractions. Thus the specific amidolytic activity eluted between 225 and 285 ml was 3.4 ± 0.46 μmol/min per mg of Lp(a). To study whether the amidolytic activity of Lp(a) resided in the apo(a) portion, this was isolated. When Lp(a) was subjected to density-gradient ultracentrifugation without prior reduction, the amidolytic activity could be demonstrated at a density of about 1.07 g/ml (Fig. 2*a*). The same density fractions also contained the Lp(a) protein. When Lp(a) that had been incubated under reducing conditions was centrifuged, cholesterol and apoB (not shown) could be demonstrated at a density of 1.04–1.05 g/ml, whereas the major portion of apo(a) and the amidolytic activity was measurable in the bottom fractions (Fig. 2*b*). Isolation of apo(a) using heparin–Sepharose chromatography (Fig. 3) also demonstrated that the amidolytic activity resides in the apo(a) portion of Lp(a).

To characterize the amidolytic activity of Lp(a), chromogenic peptide substrates were used. The rate of hydrolysis, using S-2765 or S-2222 as substrate at a concentration of 1.15 mM, was

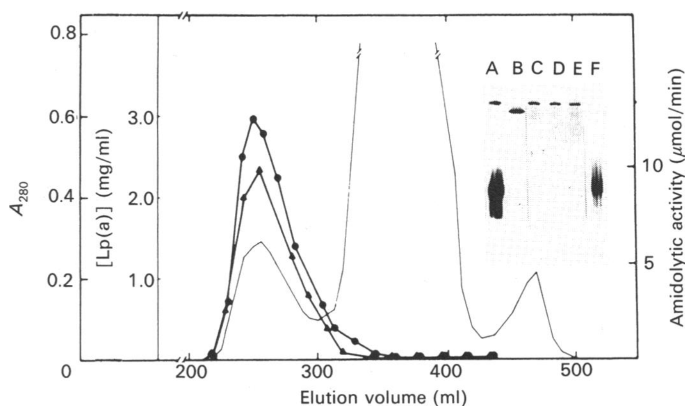

Fig. 1. Gel-filtration chromatography of human Lp(a)

The fraction of density 1.05–1.12 g/ml was chromatographed on a Sepharose 4B column (2.5 cm × 100 cm) equilibrated with PBS, pH 7.4, at a flow rate of 25 ml/h. The eluted fractions were analysed for A_{280} (——), Lp(a) concentration (●) and Lp(a) amidolytic activity (▲). For measurements of amidolytic activity, 50 μl of fraction was preincubated for 5 min in 100 mM-Tris/HCl buffer, pH 8.0, and thereafter 200 μl of 4 mM-S-2765 substrate was added and the release of *p*-nitroaniline monitored at 405 nm. The amidolytic activity was calculated as described in the Experimental section. The inset shows PAGE of different fractions eluted from the column; A, fraction of density 1.05–1.12 g/ml applied on to the column (40 μg of protein applied/lane); B, LDL fraction (density 1.02–1.05 g/ml; 20 μg of protein applied); C–F, aliquots from fractions eluted at the following volumes: C, 240 ml; D, 252 ml; E, 265 ml; F, 368 ml. The amount of protein loaded in each of the lanes (C–F) was 10 μg.

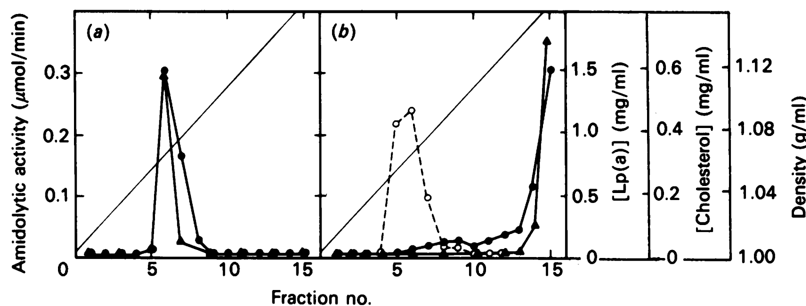

Fig. 2. Isolation of apo(a) by density-gradient ultracentrifugation

Isolated Lp(a) was ultracentrifuged either without prior reduction (*a*), or after reduction with DTE (*b*). Fractions (0.8 ml) were collected and assayed for Lp(a) (●), cholesterol (○), and amidolytic activity (▲). The density of the fractions (——) was determined by refractometry.

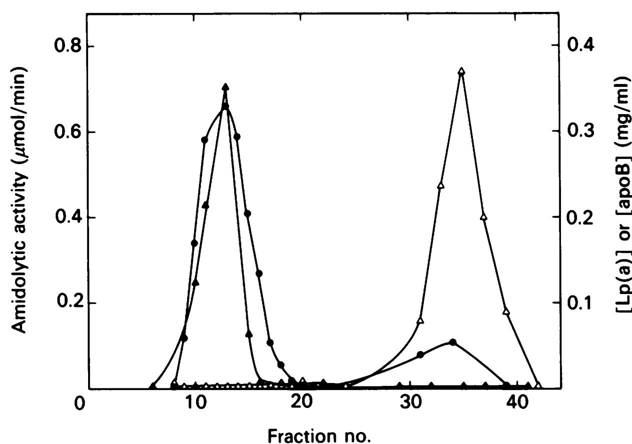

Fig. 3. Isolation of apo(a) by heparin–Sepharose affinity chromatography

Apo(a) was purified from isolated Lp(a). After reductive cleavage with 10 mM-DTT in PBS, pH 7.4, for 2 h at 37 °C, the reduced Lp(a) was passed over a Heparin–Sepharose column (1.5 cm × 4 cm) equilibrated with 20 mM-Tris buffer, pH 7.4, containing 1 mM-EDTA and 0.05 M-NaCl. The fraction bound to the column was eluted with equilibration buffer containing 0.5 M-NaCl, elution starting at fraction 22. The fractions were analysed for Lp(a) concentration (●), Lp(a) amidolytic activity (▲) and apoB (△) as described in the Experimental section.

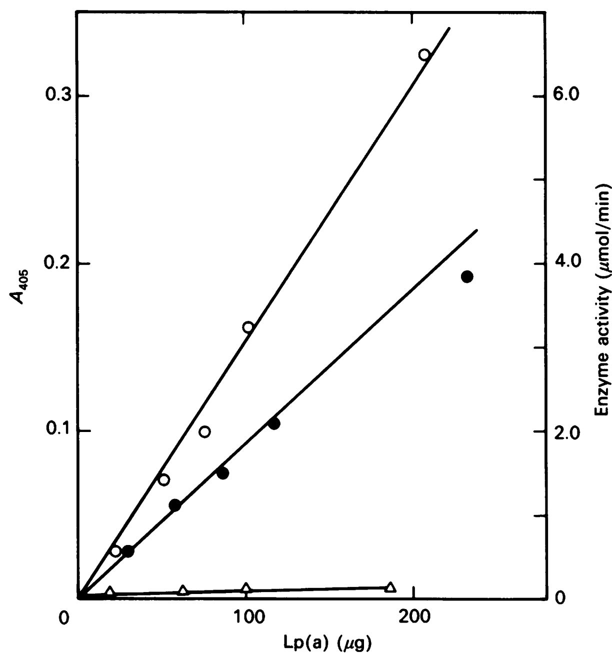

Fig. 4. Hydrolysis of chromogenic peptide substrates as a function of Lp(a) concentration

Increasing amounts of Lp(a) was preincubated in 100 mM-Tris/HCl buffer, pH 8.0, for 5 min at 25 °C. After equilibration, 150 μl of 4 mM-substrate was added and the reaction was monitored at A_{405}. Lp(a) amidolytic activity is expressed as μmol of *p*-nitroaniline liberated/min. The substrates used are: ○, S-2765; ●, S-2222; △, S-2390. The amidolytic activity has been calculated by using a molar absorption coefficient of 1.02 litre·mmol^{-1}·mm^{-1}. For measurement of the catalytic activity (*z*) of Lp(a), the rate of the catalysed substrate conversion per time unit was calculated according to the equation:

$$z = \frac{A \times V \times 1000}{\epsilon \times d \times t}$$

where *A* is the absorbance at 405 nm, *V* is the assay volume in litres, ϵ is the absorption coefficient in litres·mmol^{-1}·mm^{-1}, *d* is the pathlength in mm, and *t* is the time in minutes.

proportional to the amount of Lp(a) added to the assays (Fig. 4), and linear for at least 5 min (results not shown). A linear relationship ($r = 0.99$) was also found between the amount of apo(a) added to the assay and ΔA_{405} during the hydrolysis of substrate S-2765. The enzyme displayed a rather broad pH optimum, with maximal activity at about pH 8.0 (results not shown). The substrate specificity of the enzyme activity was assessed using synthetic substrates. Of the substrates tested, the one in which Arg-Gly is attached to a scissile L-arginine residue, namely S-2765, displayed the highest amidolytic activity. In Table 1 the relative enzyme activities towards synthetic substrates as compared with that observed using S-2765 are given. Those containing a *C*-terminal L-arginine residue are preferred. A similar substrate specificity was obtained with Lp(a) isolated from five different subjects. Only in the relative rate of hydrolysis could variation be observed among the five Lp(a) preparations (results not shown). The substrate specificity of the purified apo(a) was assessed in a manner similar to that used for Lp(a). The substrates having L-arginine at the scissile bond were the best substrates (S-2765 > L-BAPA > S-2222). Substrates with L-lysine or L-valine attached to *p*-nitroaniline were not hydrolysed by purified apo(a). Lp(a) amidolytic activity compared with that of trypsin (expressed as activity/mol of trypsin or Lp(a) and using S-2765 as a substrate) was approx. one-tenth that of trypsin. The plasmin substrates, S-2251 (results not shown) and S-2390 were not hydrolysed by Lp(a) (Fig. 4). The addition of t-PA (2 μg/assay) or u-PA (20 units/assay) had no significant effect on the Lp(a) proteinase activity (results not shown). Neither did the addition of anti-t-PA or anti-u-PA inhibit the Lp(a) proteinase activity (Table 2). Polyclonal antibodies to apo(a) did not inhibit the Lp(a) amidolytic activity. The inhibition of apo(a)

Table 1. Amidolytic activity of purified Lp(a) with chromogenic peptide substrates

Purified human Lp(a) (200 μg/assay) was preincubated in 100 mM-Tris/HCl buffer, pH 8.0, for 5 min at +25 °C. After addition of substrate to a final concentration of 1.14 mM (total volume of the reaction mixture was 1050 μl), the amidolytic activity of Lp(a) was determined and calculated as described in the Experimental section and in the legend to Fig. 4.

Substrate	Catalytic activity (μmol/min)	Relative activity (%)
S-2765	9.51	100
BAPA	7.04	74
S-2222	3.01	32
TAME	0.76	8
Chromozym PL	0.11	1
S-2390	0.02	0.2
S-2251	0	0
S-2484	0	0

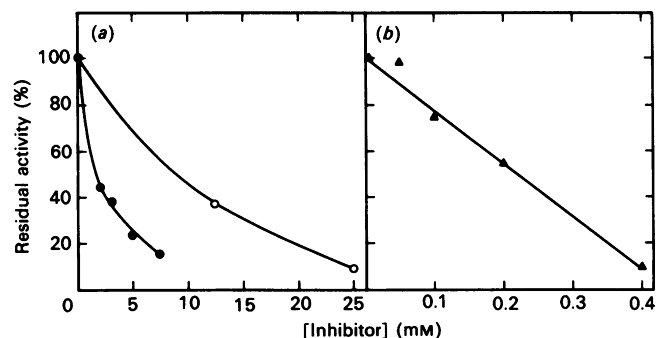

Fig. 5. Effects of serine, carboxy-group- and histidine-specific reagents on amidolytic activity of Lp(a)

Purified Lp(a) (120 μg/assay) was preincubated in 100 mM-Tris/HCl buffer, pH 8.0, with increasing amounts of PMSF (●), EDAC (○) (*a*) or DCIC (▲) (*b*) for 5 min at +25 °C. After the addition of substrate (S-2765, final concn. 1.14 mM), the mixture was incubated for 5 min at +25 °C, and the amidolytic activity was calculated as described in the Experimental section. The 100 % value for the Lp(a) amidolytic activity was 2.5 μmol of *p*-nitroaniline liberated/min.

Table 2. Effect of anti-t-PA and anti-u-PA on Lp(a) amidolytic activity

Freeze-dried antibodies against t-PA and u-PA were first dissolved in 20 mM-phosphate buffer, pH 7.3, containing 100 mM-NaCl. Known amounts of antibodies (shown as mg of IgG) were preincubated with purified Lp(a) (124 μg/assay) in assay buffer for 90 min at +25 °C before the addition of the substrate, S-2765. The blank values (the amidolytic activity caused by the antibodies, approx. 10–15 %) have been subtracted from the results.

	Lp(a) amidolytic activity (%)
Lp(a)	100
Lp(a)+anti-t-PA (0.28 mg)	113
Lp(a)+anti-t-PA (0.57 mg)	116
Lp(a)+anti-u-PA (0.28 mg)	102
Lp(a)+anti-u-PA (0.57 mg)	124

Table 3. Influence of inhibitors on kinetic parameters of Lp(a) amidolytic activity

Various substrate (S-2765) concentrations were used (0.2–1.4 mM). The Lp(a) concentration was 125 μg/assay. The concentrations of PMSF, EDAC and DCIC per assay were 2–5 mM, 2.5–5 mM and 0.05–0.3 mM respectively. The data shown are the means±S.D. of two determinations.

	$10^4 \times K_m$ (apparent) (M)	$V_{max.}$ (μmol/min)
Lp(a)	8.8 (±0.3)	6.9 (±0.5)
Lp(a)+PMSF	9.2 (±0.2)	4.3 (±0.4)
Lp(a)+EDAC	9.1 (±0.1)	1.3 (±0.2)
Lp(a)+DCIC	9.2 (±0.1)	0.7 (±0.1)

amidolytic activity was 45 % under the conditions used [incubation of purified apo(a) at 37 °C for 30 min with polyclonal anti-Lp(a) antibodies]. It is probable that the lack of antibody effect on Lp(a) amidolytic activity is due to the shielding effect of the LDL part of the particle, and the weak inhibitory effect on apo(a) may be either due to the rather low affinity of anti-Lp(a) antisera toward the apo(a) or due to antisera binding to catalytically non-essential regions on apo(a). In addition, anti-(Factor Xa) antibodies at increasing concentration/assay did not inhibit Lp(a) enzyme activity. Further, by immunoblotting the Lp(a) samples with anti-(Factor Xa) antibodies we could not detect any Factor Xa antigen in our Lp(a) preparations.

To study the mechanism underlying the amidolytic activity of Lp(a), reagents known to interfere with the catalytic triad (Asp-His-Ser) of serine proteinases were used. When the competitive peptide inhibitors leupeptin and aprotinin (known to block the active site of several serine proteinases) were used, leupeptin (10 μg/ml) and aprotinin (0.5 mg/ml) gave 40 % and 26 % inhibition respectively. The soybean trypsin inhibitor, which at the concentration used (0.1 mg/ml) effectively inhibits trypsin, had no effect on the amidolytic activity of Lp(a).

The carboxy-group reagent EDAC, a hydrophilic carbodiimide, inhibited the amidolytic activity in a concentration-dependent manner (Fig. 5*a*). The inhibition followed first-order reaction kinetics. Of reagents known to react with histidine, DCIC was the most effective inhibitor. Half of the activity was already abolished at 0.22 mM-DCIC (Fig. 5*b*). The inhibition did not follow first-order kinetics, but gave a relatively linear response to increasing DCIC concentrations. Another histidine-specific reagent, Tos-Lys-CH_2Cl, inhibited the enzyme only slightly (88 % residual activity at 10 mM-Tos-Lys-CH_2Cl; result not shown). Inhibition experiments with [^{3}H]iPr_2*P*-F indicated that the inhibitor did not bind to Lp(a), neither did it cause inhibition of enzyme activity, as measured with the synthetic substrate S-2765.

The serine-specific reagent PMSF inhibited the amidolytic activity of Lp(a) in a concentration-dependent manner (Fig. 5*a*). At 1.7 mM a 50 % inhibition occurred, and at 7 mM-PMSF, only 16 % residual activity remained. The addition of an amidino group to PMSF abolished the inhibitory effect. In addition, the inhibition of the amidolytic activity of purified apo(a) was tested with DCIC, PMSF and EDAC under the same experimental conditions as with Lp(a). As compared with the control activity [the 100 % value for the apo(a) amidolytic activity was 3.7 μmol of *p*-nitroaniline liberated/min; S-2765 used as a substrate] the remaining activities with these inhibitors were as follows: DCIC (0.3 mM), 16 %; DCIC (0.6 mM), 3 %; PMSF (5 mM), 2 %, and

EDAC (25 mM), 3 %. These data demonstrate that the amidolytic activity of Lp(a) closely resembles that of serine-proteinase enzymes.

To obtain data on the kinetics of Lp(a) amidolytic activity, the K_m (apparent) and $V_{max.}$ were determined with S-2765 as a substrate (at various concentrations) and a fixed amount of Lp(a) (Table 3). The effect of different inhibitors on these kinetic parameters was also evaluated. K_m (apparent) did not significantly change in the presence of inhibitors, but there was a clear reduction in $V_{max.}$. This suggests a non-competitive type of inhibition of Lp(a) amidolytic activity.

DISCUSSION

As Lp(a) has been reported to be an independent risk factor for coronary heart disease [1–3,30], our observation that apo(a) has serine proteinase-type amidolytic activity may be of importance in revealing the mechanism(s) whereby Lp(a) is atherogenic. The striking sequence similarity between plasminogen and apo(a) [15], especially in the proteinase region, suggests that apo(a) might be proteolytically active. By contrast, however, at the site where plasminogen is activated to plasmin, the arginine residue is replaced by a serine residue in apo(a), suggesting that apo(a) does not have proteolytic activity [15,19]. Only two reports describe amidolytic activity, whereas others do not [15,19]. The reason for this discrepancy may be explained by the difference in substrate specificity between plasmin and apo(a). Plasmin preferably cleaves substrates at the carboxy end of lysine [31,32]. We demonstrate that Lp(a) does not hydrolyse synthetic 'plasmin substrates' which have lysine at the scissile bond (S-2251 and S-2390) and that the activators of plasminogen, t-PA and u-PA, do not significantly affect the amidolytic activity of apo(a). These observations agree with previous studies reporting no plasmin-like activity [15,19]. However, using substrates containing arginine at the scissile bond, Lp(a) displays amidolytic activity. This specificity is similar to that reported by Jürgens *et al.* [20], who used L-TAME and L-BAPA substrates in their assays. Serine proteinases share a common catalytic mechanism, and their different specificities have been shown to arise from amino acid differences in the substrate-binding regions [17,33]. The residues which determine the substrate specificity, are located at positions -6 (at the bottom of the substrate-binding pocket), $+15$ to $+17$, and $+25$, relative to the serine at the active site [17]. A serine proteinase which also displays specificity for arginine is granzyme A, a serine proteinase found in granules of cytolytic T-lymphocytes [34]. In apo(a), aspartic acid at -6 is the same as that found in granzyme A.

To demonstrate that apo(a), besides having sequence identity with, also functions as, a serine-proteinase-type enzyme, we evaluated different proteinase inhibitors. Reagents known to react with catalytically essential amino acids and peptides which competitively bind to the active site have previously been employed to elucidate the structure and function of the catalytic triad Ser-His-Asp characteristic for serine proteinases [17,35–37]. It has been demonstrated that the amidolytic action of serine proteinases depends on the activation of the catalytic serine residue. In this process a histidine residue and the carboxy group of aspartic acid participate [17,38,39]. Our inhibition experiments revealed that serine-, histidine- and carboxylic-group-specific reagents, PMSF, DCIC and EDAC respectively, inhibited the amidolytic activity of purified Lp(a) and apo(a), observations which indicate that these residues are involved in catalysis. The inhibition with PMSF and EDAC followed first-order reaction kinetics, but the kinetics with DCIC were different. The reason for this is probably due to the two-step reaction pathway characteristic for DCIC. Harper *et al.* [40] reported that DCIC first acylates the serine at the active site, forming a reactive acyl chloride, which then forms an ester bond with a histidine at or near the active site.

Substrate-specificity and inhibition experiments on human plasma Lp(a) showed that Lp(a) and purified apo(a) 'prefer' substrates which have arginine at the scissile bond and that serine, histidine and carboxy groups are essential for the hydrolysis of these chromogenic peptide substrates. These findings demonstrate that human Lp(a) is also functionally closely related to serine-proteinase enzymes. The proteinase activity is localized to the apo(a) portion of Lp(a) particles. This conclusion is based on the following experimental observations. The proteinase activity co-migrated with the Lp(a) and apo(a) during their purification from plasma samples from different individuals. The pattern of proteolysis [21] and amidolysis caused either by Lp(a) or apo(a) was clearly different from that caused by either plasmin or kallikrein. Known plasminogen activators had no effect on the activity. In all Lp(a) preparations no kallikrein or Factor Xa could be detected by immunoblotting. Finally, polyclonal antibodies to apo(a) partially inhibited the amidolytic activity of apo(a). These results strongly suggest that Lp(a)/apo(a) has inherent proteinase activity. As apo(a) occurs in several phenotypes, it will be of interest to study whether all of them have the same degree of serine-proteinase activity. Several studies [41,42] have demonstrated that Lp(a) is a major constituent of atherosclerotic lesions. Whether the serine-proteinase activity of Lp(a) plays a role in the genesis of atherosclerosis remains to be established.

We thank Mrs. Liisa Ikävalko, Mrs. Ritva Keva, Mrs. Sirkka Metiäinen, Ms. Kaarina Emeleus and Ms. Mari Pursiainen for their expert technical assistance and Mrs. Liisa Palonen for help in preparing the manuscript. This work was supported by the Sigrid Juselius Foundation, Helsinki, Finland.

REFERENCES

1. Kostner, G. M., Avogaro, P., Cazzolato, G., Marth, E., Bittolo Bon, G. & Quinci, G. B. (1981) Atherosclerosis **38**, 51–61
2. Rhoads, G. G., Dahlen, G., Berg, K., Morton, N. E. & Dannenberg, A. L. (1986) J. Am. Med. Assoc. **256**, 2540–2544
3. Armstrong, V. W., Cremer, P., Eberle, E., Manke, A., Schulze, F., Wieland, H., Kreuzer, H. & Seidel, D. (1986) Atherosclerosis **62**, 249–257
4. Berg, K. (1963) Acta Pathol. Microbiol. Scand. **59**, 369–382
5. Harvie, N. R. & Schultz, J. S. (1970) Proc. Natl. Acad. Sci. U.S.A. **66**, 99–103
6. Utermann, G., Menzel, H. J., Kraft, H. G., Duba, H. C., Kemmler, H. G. & Seitz, C. (1987) J. Clin. Invest. **80**, 458–465
7. Simons, K., Ehnholm, C., Renkonen, O. & Bloth, B. (1970) Acta Pathol. Microbiol. Scand. **78**, 459–466
8. Ehnholm, C., Garoff, H., Renkonen, O. & Simons, K. (1972) Biochemistry **11**, 3229–3232
9. Utermann, G., Weber, W. (1983) FEBS Lett. **154**, 357–361
10. Gaubatz, J. W., Heideman, C., Gotto, A. M., Jr., Morrisett, J. D. & Dahlen, G. H. (1983) J. Biol. Chem. **258**, 4582–4589
11. Kratzin, H., Armstrong, V. W., Niehaus, M., Hilschmann, N. & Seidel, D. (1987) Biol. Chem. Hoppe-Seyler **368**, 1533–1544
12. Utermann, G., Kraft, H. G., Menzel, H. J., Hopferwieser, T. & Seitz, C. (1988) Hum. Genet. **78**, 41–46
13. Frank, S. L., Klisak, I., Sparkes, R. S., Mokandas, T., Tomlinson, J. E., McLean, J. W., Lawn, R. M. & Lusis, A. J. (1988) Hum. Genet. **79**, 352–356
14. Lindahl, G., Gersdorf, E., Menzel, H. J., Duba, C., Cleve, H., Humphries, S. & Utermann, G. (1989) Hum. Genet. **81**, 149–152
15. McLean, J. W., Tomlinson, J. E., Kuang, W.-J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M. & Lawn, R. M. (1987) Nature (London) **330**, 132–137
16. Tomlinson, J. E., McLean, J. W. & Lawn, R. M. (1988) J. Biol. Chem. **264**, 5957–5965

17. Kraut, J. (1977) Annu. Rev. Biochem. **46**, 331–358
18. Collen, D. (1980) Thromb. Haemostasis **43**, 77–89
19. Eaton, D. L., Fless, G. M., Kohr, W. J., McLean, J. W., Xu, Q.-T., Miller, C. G., Lawn, R. M. & Scanu, A. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 3224–3228
20. Jürgens, G., Marth, E., Kostner, G. M. & Holasek, A. (1977) Artery **3**, 13–26
21. Salonen, E.-M., Jauhiainen, M., Zardi, L., Vaheri, A. & Ehnholm, C. (1989) EMBO J. **8**, 4035–4040
22. Ehnholm, C., Garoff, H., Simons, K. & Aro, H. (1971) Biochim. Biophys. Acta **236**, 431–439
23. Havel, R. J., Eder, H. A. & Bragdon, J. R. (1955) J. Clin. Invest. **34**, 1345–1353
24. Fless, G. M., ZumMallen, M. E. & Scanu, A. M. (1985) J. Lipid Res. **26**, 1224–1229
25. Hajjar, K. A., Gavish, D., Breslow, J. L. & Nachman, R. L. (1989) Nature (London) **339**, 303–305
26. Geiger, R. & Fritz, H. (1984) in Methods of Enzymatic Analysis, vol. 5 (Bergmeyer, H. U., ed.), pp. 74–143, Verlag Chemie, Weinheim
27. Jansson, G. & Holmlund, E. (1988) Int. Symp. Atherosclerosis 8th, Rome, 418a
28. Röschlau, P., Bernt, E. & Gruber, W. (1974) Z. Klin. Chem. **12**, 226–230
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
30. Hoefler, G., Harnoncourt, F., Paschke, E., Mirtl, W., Pfeiffer, K. H. & Kostner, G. M. (1988) Arteriosclerosis **8**, 398–401
31. Teger-Nilsson, A. C., Friberger, P. & Gyzander, E. (1977) Scand. J. Clin. Lab. Invest. **37**, 403–409
32. Robbins, K. C., Summaria, L. & Wohl, R. C. (1981) Methods Enzymol. **80**, 379–387
33. Fersht, A. (1985) Enzyme Structure and Mechanism, 2nd edn., pp. 1–46, W. H. Freeman and Co., New York
34. Jenne, D. E. & Tschopp, J. (1988) Curr. Top. Microbiol. Immunol. **140**, 33–47
35. Stroud, R. M., Kay, L. M. & Dickerson, R. E. (1974) J. Mol. Biol. **83**, 185–208
36. Wright, C. S., Alden, R. A. & Kraut, J. (1969) Nature (London) **221**, 235–242
37. Umezawa, H. (1976) Methods Enzymol. **45**, 678–695
38. Blow, D. M., Birktoft, J. J. & Hartley, B. S. (1969) Nature (London) **221**, 337–340
39. Warshel, A., Naray-Szabo, G., Sussman, F. & Hwang, J.-K. (1989) Biochemistry **28**, 3629–3637
40. Harper, J. W., Hemmi, K. & Powers, J. C. (1985) Biochemistry **24**, 1831–1841
41. Walton, K. W., Hitchens, J., Magnani, H. N. & Khan, M. (1974) Atherosclerosis **20**, 323–346
42. Rath, M., Niendorf, A., Reblin, T., Dietel, M., Krebber, H-J. & Beisiegel, U. (1989) Arteriosclerosis **9**, 579–592

Received 19 June 1990/26 October 1990; accepted 13 November 1990