Fibrin-Bound Lipoprotein(a) Promotes Plasminogen Binding but Inhibits Fibrin Degradation by Plasmin†

Jian-ning Liu,‡ Peter C. Harpel,§ and Victor Gurewich*,‡

Vascular Research Laboratory, Institute for the Prevention of Cardiovascular Disease, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02215, and Division of Hematology, Mount Sinai Medical Center, New York, New York 10029

Received September 15, 1993; Revised Manuscript Received December 30, 1993⊗

ABSTRACT: Conflicting results have been obtained from studies of the effects of lipoprotein(a) [Lp(a)] on plasminogen binding to fibrin and fibrin-dependent activation by tissue plasminogen activation (t-PA). We performed binding studies of Glu-plasminogen (0–16 μM) to immobilized D-dimer ± Lp(a) (0.20 μM). In the absence of Lp(a), Scatchard analysis revealed a binding constant of K_D = 1.01 ± 0.18 μM, with two plasminogen binding sites per D-dimer. In the presence of Lp(a), a lower affinity (K_D 3.10 ± 0.23 μM) was found, but five binding sites were present, suggesting that plasminogen bound to fibrin-bound Lp(a) rather than to D-dimer. Consistent with this explanation was the finding that when D-dimer-coated plates were first precoated with Lp(a) before plasminogen was added, similar lower affinity plasminogen binding was found. This binding to Lp(a) was fibrin-dependent since, in its absence, plasminogen failed to bind to Lp(a). Therefore, a conformational change in Lp(a) appeared to be required for plasminogen binding to occur. This finding of two types of binding sites of different affinities helps to explain why Lp(a) has been reported to inhibit plasminogen binding to fibrin in studies in which only low concentrations of plasminogen (<0.4 μM) were used. At these concentrations, few of the low-affinity binding sites on fibrin-bound Lp(a) will be occupied by plasminogen, an effect that was found to be exaggerated by the omission of NaCl. By contrast, at physiological concentrations of plasminogen (2.0 μM), Lp(a) promotes plasminogen binding and plasmin generation by t-PA due to the larger number of plasminogen binding sites becoming occupied on the fibrin-bound Lp(a). This Lp(a)-bound plasmin was, however, relatively ineffective at lysing the fibrin monolayer. In conclusion, these findings indicate that Lp(a) contains at least five plasminogen binding sites, which are fibrin-induced and which complicate the mechanism by which Lp(a) may influence fibrinolysis.

Lipoprotein(a) [Lp(a)][1] is a plasma low-density lipoprotein made up of lipid and apoprotein B-100. Its distinguishing feature is an additional glycoprotein subunit, apoprotein(a), which is linked by a disulfide bridge to apoprotein B-100 (Fless *et al.*, 1986; Scanu, 1988). This apoprotein(a) component is highly homologous to plasminogen by virtue of the fact that it contains multiple repeats of plasminogen kringle IV (McLean *et al.*, 1987; Eaton *et al.*, 1987), a fibrin or lysine binding domain. Like plasminogen, Lp(a) binds to both lysine–Sepharose and immobilized fibrin (Harpel *et al.*, 1989), leading to the proposition that Lp(a) may be a biological competitor for the plasminogen binding sites on fibrin and thereby inhibit fibrinolysis since, unlike plasminogen, it cannot be converted to an active proteinase (Eaton *et al.*, 1987).

An inverse correlation between the fibrinolytic activity of blood and cardiovascular disease has been reported frequently (Chakrabati *et al.*, 1986; Hamster *et al.*, 1985; Juhan-Vague & Collen, 1988; Prins & Hirsch, 1991). Therefore, it has been postulated that the well-documented atherogenic effect of Lp(a) (Alberts *et al.*, 1977; Seed *et al.*, 1990; Dahlen *et al.*, 1986; Rhoads *et al.*, 1986; Muria *et al.*, 1986) may be mediated by its inhibitory effect on fibrinolysis.

A number of investigations have supported this hypothesis, since they have shown that Lp(a) inhibits plasminogen activation by urokinase (UK) (Edelberg *et al.*, 1991), streptokinase (Edelberg *et al.*, 1989), and tissue plasminogen activator (t-PA) (Edelberg *et al.*, 1990). However, these studies used very low concentrations of plasminogen and were performed under salt-free conditions. In other studies, no inhibition of fibrinolysis by Lp(a) was found in the presence of physiological concentrations of plasminogen (Kluft *et al.*, 1989; Eaton *et al.*, 1990; Lu *et al.*, 1990; Halvorsen *et al.*, 1992). Furthermore, although competitive fibrin binding by Lp(a) at low concentrations of plasminogen has been shown (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990), uncompetitive inhibition of plasminogen activation by Lp(a) was found by most investigators (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991; Liu *et al.*, 1993). However, competitive inhibition has also been reported (Edelberg *et al.*, 1990; Edelberg & Pizzo, 1990).

These inconsistencies have not been explained. A reconciliation of these disparate reports is provided by the present findings, which indicate that Lp(a) inhibits plasminogen binding and activation only at very low concentrations of plasminogen and that this inhibition is exaggerated if NaCl is omitted. An explanation is provided by the discovery of lower affinity plasminogen binding sites on fibrin-bound Lp(a).

† This study was supported in part by a grant from Farmitalia Carlo Erba (V.G.), by USPH Grant HL-18828, and by the Council for Tobacco Research—U.S.A., Inc. (P.C.H.).

* Author to whom correspondence should be addressed at the Vascular Research Laboratory, Burlington Bldg., Rm 554G, New England Deaconess Hospital, 185 Pilgrim Rd., Boston, MA 02215.

‡ New England Deaconess Hospital, Harvard Medical School.

§ Mount Sinai Medical Center.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1994.

[1] Abbreviations: Lp(a), lipoprotein(a); pro-UK, prourokinase; ELISA, enzyme-linked immunosorbant assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; t-PA, tissue plasminogen activator; K_D, binding constant; B_{max}, the maximum of binding; EACA, ϵ-aminocaproic acid; mA, miniabsorbance.

0006-2960/94/0433-2554$04.50/0

MATERIALS AND METHODS

(1) Preparation of Lipoprotein(a). Lp(a) was purified from a donor with an elevated Lp(a) concentration by ultracentrifugation and gel filtration according to the procedure previously described (Harpel *et al.*, 1989); the molecular weight of Lp(a) was 923 000. On the basis of molecular weight, this represents the isoform containing 37 kringle IV-like copies and one kringle V-like copy plus the protease domain.

(2) Preparation of Fibrin D-dimer. D-dimer was prepared by a modification of the method of Varadi and Patthy (1983) as follows: A plasmin digest of fibrin was prepared by treatment of a solution of human fibrinogen (10 mg/mL) in 0.05 M Tris·HCl, 0.10 M NaCl, and 0.01 M $CaCl_2$ (pH 7.4). The solution was equilibrated to 37 °C, and Glu-plasminogen (25 μg/mL) and streptokinase (SK) (Hoechst Pharmaceuticals, Somerville, NJ) (7.5 IU/mL) were added immediately before the addition of highly purified thrombin (8 NIH units/mL). The reaction mixture was incubated (37 °C) for 3 h. Digestion was arrested by the addition of aprotinin (Trasylol, 100 KIU/mL, Miles Inc., West Haven, CT), EACA (0.02 M), and EDTA (0.02 M). The digest was then applied to a Sephacryl S-200 (Pharmacia, Piscataway, NJ) column (2.5 × 160 cm) equilibrated with 0.3 M NH_4HCO_3 and eluted with the same buffer. Fragments of E were obtained from pool B, whereas pool A contained D-dimer–E. The latter pool was lyophilized and dissolved in 0.05 M sodium citrate and 3 M urea (pH 5.5) and incubated (37 °C) for 3 h as described by Varadi and Patthy (1994). D-dimer was separated from D-dimer–E by gel filtration on a Sephacryl S-300 column (2.5 × 160 cm) equilibrated with 0.05 M sodium citrate and 3 M urea. The purified D-dimer preparation was desalted and lyophilized.

To eliminate any traces of residual plasmin, the preparation was treated with diisopropyl phosphorofluoridate (5 mM) for 1 h (37 °C) and then dialyzed exhaustively against 0.05 M Tris·HCl and 0.10 M NaCl (pH 7.4).

The concentration of D-dimer was determined from the absorbance using the extinction coefficients of fragment D ($E^{1\%}_{280nm} = 20.0$). Protein was also measured using a Bio-Rad protein assay kit. The final preparation was examined by gradient (5–15%) SDS–PAGE and shown to be pure (Liu & Gurewich, 1991).

(3) Binding of [^{125}I]Glu-plasminogen to a D-dimer-Immobilized Surface in the Presence and Absence of Lp(a). (a) D-dimer Immobilization. D-dimer (100 μL, 0.025 mg/mL) in 0.05 M Tris·HCl and 0.01 M NaCl (pH 7.4) was added to wells of a 96-well plate (Imminolog II, Dynatech, Chantilly, VA) and incubated at 4 °C overnight. After the wells were emptied, 200 μL of 20 mg/mL BSA in a Tris buffer containing 0.05 M Tris·HCl, 0.10 M NaCl, 0.2% BSA, and 0.01% Tween 80 (pH 7.4) was added and incubated at 4 °C overnight for blocking. Tris buffer instead of D-dimer was used in the first incubation in some wells to serve as a control for the assay of nonspecific binding. The plate was readied for use after washing four times with cold washing buffer containing 0.05 M Tris·HCl, 0.10 M NaCl, 2% BSA, and 0.02% Tween 80 (pH 7.4). The amount of immobilized D-dimer per well was measured by ELISA using rabbit anti-D-dimer antibody, as previously described (Liu *et al.*, 1993).

(b) Binding of [^{125}I]Glu-plasminogen to D-dimer-Immobilized Plates in the Presence or Absence of Lp(a). Glu-plasminogen (0.5 mg) was labeled with Na^{125}I (DuPont-New England Nuclear, Boston, MA) using Bio-Rad Enzymobeads (Sigma, St. Louis, MO). Its specific activity was 6.4 μCi/μg. Glu-plasminogen (0.02–16 μM) containing 0.5% of the radiolabeled form was added to D-dimer-immobilized wells or control wells in the presence or absence of 0.2 μM Lp(a) and incubated in 100 μL of the Tris buffer (pH 7.4) at 37 °C for 2 h. The plate was then washed with the cold washing buffer four times.

Thereafter, t-PA (0.1 nM) and S2251 (1.5 mM) in 100 μL of Tris·HCl buffer were added to the wells and incubated (37 °C) for 1 h in order to determine whether the bound Glu-plasminogen could be activated by t-PA. Plasmin generation was monitored by measuring the OD increase with time at 410 nm against a reference wavelength of 490 nm (410/490 nm) on a microtiter plate reader. The initial reaction rate was then determined directly by curve fitting using the integrated rate equation as previously described (Liu & Gurewich, 1991). Curve fitting was performed on early time points, before the depletion of substrates became significant.

Finally, 20 μL of 30% SDS was added and incubated (70 °C) for 30 min to solubilize the bound plasminogen/plasmin. The bound plasminogen/plasmin was measured by counting the radioactivity in the SDS samples removed from the plate. EACA (0.2 M) was coincubated with plasminogen and Lp(a) in some experiments in order to distinguish specific from nonspecific binding. The specific binding was obtained by subtracting nonspecific binding from total binding. Binding curves were drawn and analyzed by Scatchard plots.

In other experiments, instead of coincubating Lp(a) with plasminogen, the D-dimer plate was precoated with Lp(a) as follows: The D-dimer plate was incubated with 100 μL per well of Lp(a) (0.2 μM) in 0.05 M Tris·HCl and 0.10 M NaCl (pH 7.4) overnight. After the wells were emptied, the plate was washed with cold washing buffer four times. The amount of bound Lp(a) per well was determined by ELISA using rabbit anti-Lp(a) antibody as described (Harpel *et al.*, 1989). There was no significant difference in plasminogen binding between the coincubation with Lp(a) and the precoating with Lp(a).

(c) Binding of [^{125}I]Glu-plasminogen to Lp(a)-Immobilized Plates. In order to evaluate the binding of plasminogen to Lp(a) in the absence of fibrin, plates were incubated (37 °C) with 100 μL per well of Lp(a) (0.2 μM) in 0.05 M Tris·HCl and 0.10 M NaCl (pH 7.4) for 2 h. After the wells were emptied, 200 μL of 20 mg/mL BSA in a Tris buffer was added and incubated (4 °C) overnight in order to occupy extra protein binding sites. The amount of immobilized Lp(a) per well was determined by ELISA, again using rabbit anti-Lp(a) antibody as described (Harpel *et al.*, 1989). Glu-plasminogen (0.02–16 μM) containing 0.5% of the radiolabeled plasminogen was added to the wells containing immobilized Lp(a) and incubated (37 °C) in Tris buffer (pH 7.4) for 2 h. After the plate was washed with cold washing buffer, bound plasminogen was measured as described above. No binding of Glu-plasminogen to immobilized Lp(a) was observed.

(4) Inhibition of Lysis of Immobilized Fibrin by Lp(a). (a) [^{125}I]Fibrin Immobilization. Fibrinogen was labeled with Na^{125}I using Bio-Rad Enzymobeads. Its specific activity was 1.6 μCi/μg and its clottability was 96%. [^{125}I]Fibrinogen (100 μL, 0.5 μM) was added to the wells of a 96-well plate and incubated (4 °C) overnight. After the wells were emptied, 200 μL of 20 mg/mL BSA in the Tris·HCl buffer was added and incubated at 4 °C overnight for blocking. The plate was washed with cold washing buffer four times. Then 100 μL

of thrombin (2 units/mL) in 0.05 M Tris·HCl, 0.10 M NaCl, and 0.01 M $CaCl_2$ (pH 7.4) was added and incubated (37 °C) for 30 min. The plate was used after washing four times with cold washing buffer.

(b) Inhibition of Degradation of Immobilized Fibrin by Lp(a). Glu-plasminogen (2 μM) in the Tris·HCl buffer was added to the [^{125}I]fibrin-immobilized wells in the presence or absence of 0.2 μM Lp(a) and incubated in the Tris buffer (pH 7.4) at 37 °C for 1 h. The plate was then washed with cold washing buffer two times. Then 100 μL of t-PA (0.1 nM) in the Tris·HCl buffer was added in the presence or absence of 0.1 μM α_2-antiplasmin and incubated (37 °C) for 1 h. The reaction was terminated by discharging the wells. To the wells was added 100 μL of 4× SDS sample buffer, and they were sealed and incubated (70 °C) overnight in order to detach fibrin and fibrin degradation products (FDPs) from the wells. The SDS samples were applied to nonreduced 7.5% SDS–PAGE to separate the moieties of fibrin and FDPs. After radioautography, the [^{125}I]fibrin bands were determined to cut for τ-counting.

(5) Effect of Salt Concentration on the Binding of Plasminogen and Lp(a) to Fibrin. In order to evaluate the effect of salt on plasminogen binding to fibrin D-dimer, the binding of Glu-plasminogen (0.1, 1.0, and 2.0 μM) in the presence of 0.2 μM Lp(a) was studied in a 0.01 M sodium phosphate buffer (pH 7.4), with or without 0.15 M NaCl.

Each experiment in sections 3–5 was performed in quadruplicate.

RESULTS

(1) Effect of Lp(a) on the Binding of [^{125}I]Glu-plasminogen to Immobilized D-dimer (Figure 1A,B and Table 1). (a) Physiological Concentrations of Plasminogen (2.0 μM). Glu-plasminogen specifically bound to D-dimer in a saturatable manner (Figure 1A), with a K_D of 1.01 ± 0.18 μM and a B_{max} of 3.10 ± 0.23 pmol/well (Table 1). Approximately 1.56 ± 0.28 pmol of D-dimer, as determined by ELISA, was present in each well. Therefore, there were two binding sites per D-dimer for Glu-plasminogen, as also previously shown by Lucas *et al.* (1983) for fibrin monomer.

Since the K_D of Lp(a) binding to fibrin has been shown to be 0.02–0.03 μM (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990), a saturating concentration (0.2 μM) of Lp(a) was used to maximally occupy its binding sites on D-dimer. As determined by ELISA, 1.43 ± 0.44 pmol of Lp(a) was present per well. Therefore, there was about one bound Lp(a) per D-dimer (1.43:1.56).

When 0.2 μM Lp(a) was included in the incubation mixture or was precoated onto the D-dimer before the addition of plasminogen, the amount of Glu-plasminogen bound significantly increased (Figure 1A) and the affinity decreased (Figure 1B). The B_{max} was increased to 7.90 ± 0.39 pmol/well and the K_D increased to 3.16 ± 0.35 μM (Table 1). Therefore, the binding sites for Glu-plasminogen per molecule of D-dimer increased from two to five or 5.52 binding sites per bound Lp(a). At physiological concentration (2.0 μM) of Glu-plasminogen, a 1.5-fold promotion of binding was observed in the presence of 0.2 μM Lp(a).

Glu-plasminogen binding to either D-dimer or D-dimer-bound Lp(a) was abolished by EACA (0.2 M).

(b) Low Concentrations of Plasminogen (<0.4 μM). By contrast to the higher concentrations, Lp(a) inhibited Glu-plasminogen binding to fibrin D-dimer at the lower range of concentrations of Glu-plasminogen (Figure 1B). The two Scatchard plots crossed at the point at which the concentrations

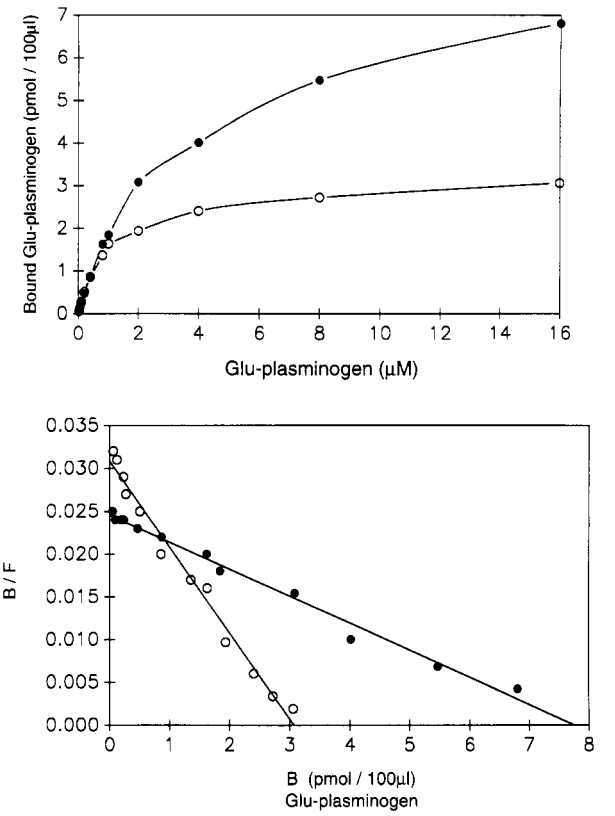

FIGURE 1: Binding of plasminogen to immobilized fibrin D-dimer in the absence (○) and presence (●) of 0.2 μM Lp(a) (A, top). Twelve different concentrations of plasminogen were used for incubation (from left to right: 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 8.0, and 16.0 μM). Scatchard plots of binding of Glu-plasminogen to fibrin D-dimer in the absence (○) and presence (●) of 0.2 μM Lp(a) (B, bottom). Twelve different concentrations of plasminogen were used for incubation (from left to right: 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 8.0, and 16.0 μM).

Table 1: The Binding Analysis: Plasminogen Binding to Fibrin ± Lp(a)

	fibrin D-dimer – Lp(a)	fibrin D-dimer + Lp(a)
K_D (μM)	1.01 ± 0.18	3.16 ± 0.35
B_{max} (pmol/well)	3.10 ± 0.23	7.90 ± 0.39
n (sites/D-dimer)[a]	2	5
occupied binding sites (by 2 μM incubated plasminogen)	1.33 (66.5%)	1.94 (38.8%)

[a] 1.56 ± 0.28 pmol of D-dimer/well.

of plasminogen used were both 0.4 μM. On the left side of the crossing point, the plots showed that there was more binding observed in the absence of Lp(a).

Since it is well-established that Lp(a) binds to fibrin with a high affinity (K_D = 20–30 nM) (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990), the change in binding constants of Glu-plasminogen (higher K_D and B_{max}) in the presence of Lp(a) suggests that whereas Lp(a) blocked the plasminogen binding sites on D-dimer, it provided new, lower affinity (K_D = 3.16 μM) binding sites that were more numerous. These binding sites were lysine-dependent since binding was abolished by EACA. Furthermore, the binding was fibrin-dependent, since Glu-plasminogen did not bind to Lp(a) directly when it was coated onto a plate.

Glu-plasminogen, which was bound in either the absence or presence of Lp(a), was activatable by t-PA (0.1 nM). Plasmin generation after 1 h of incubation was little influenced

Table 2: Preincubation at a Plasminogen Concentration of 2.0 μM

	−Lp(a)	+Lp(a)
plasminogen bound (μM)[a]	0.02	0.03
actual rate of activation (mA/min²)	0.080 ± 0.010	0.089 ± 0.011
calculated rate of activation (mA/min²)[b]	0.0809	0.113
inhibition (%)	1.11	21.27

[a] The volume of the reaction mixture is 0.1 mL. [b] Calculated from the kinetic constants of t-PA-induced Glu-plasminogen activation in the presence of 1.0 μM D-dimer (Liu & Gurewich, 1993a).

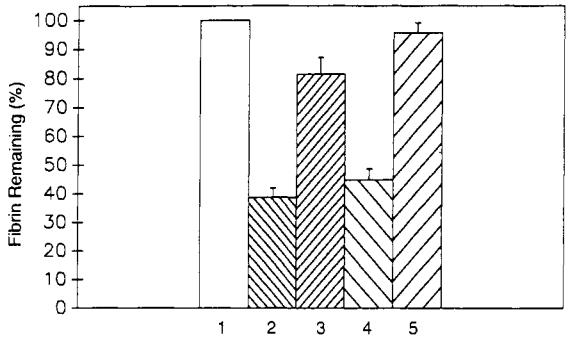

FIGURE 2: Effect of Lp(a) on fibrin degradation by surface-bound plasmin: bar 1, control (no plasmin, fibrin alone); bar 2, plasmin without Lp(a); bar 3, plasmin with Lp(a); bar 4, plasmin without Lp(a), plus α_2-antiplasmin; and bar 5, plasmin with Lp(a), plus α_2-antiplasmin.

by Lp(a). At a physiological concentration (2 μM) of Glu-plasminogen, the initial rate of plasminogen activation in the presence of Lp(a) was only 1.1-fold higher than that in the absence of Lp(a) (0.089 ± 0.011 vs 0.080 ± 0.010 mA/min²). However, since there was 1.5-fold more substrate in the presence of Lp(a) (Figure 1A, Table 1), the findings suggested that Lp(a) reduced the catalytic efficiency of t-PA against plasminogen.

As shown in Table 2, a 21.27% inhibition of plasminogen activation was obtained in the presence of Lp(a). However, relative to the reaction rate of t-PA-induced plasminogen activation in the absence of fibrin under these conditions, as calculated from previously published kinetic constants (Liu & Gurewich, 1993a), there was still a 73.5-fold promotion: 0.00121 vs 0.089 mA/min² (Table 2).

(2) Inhibition of Lysis of Immobilized Fibrin by Lp(a) (Figure 2). About 60% of the immobilized fibrin was degraded by the plasmin generated during 1 h incubation with t-PA, as assessed by counting undegraded radiolabeled fibrin, which was isolated from the degradation mixture by SDS–PAGE. By contrast, in the presence of fibrin-bound Lp(a), less than 20% fibrinolysis was induced by t-PA, even though a slightly higher (1.1-fold) amount of plasmin generation occurred in the presence of Lp(a). This suggested that Lp(a)-bound plasmin did not effectively access fibrin cleavage sites. When α_2-antiplasmin was included in the incubation mixture in the absence of Lp(a), fibrinolysis by fibrin-bound plasmin was not significantly inhibited. This is consistent with the known protection of fibrin-bound plasmin from inhibition by α_2-antiplasmin (Wiman & Collen, 1979). However, in the presence of fibrin-bound Lp(a), α_2-antiplasmin inhibited fibrinolysis almost completely, indicating that the plasmin previously mediating fibrinolysis was unprotected (Figure 2). Since α_2-antiplasmin efficiently inactivates only free plasmin, the findings suggest that Lp(a)-bound plasmin must first dissociate from the Lp(a) in order to lyse the fibrin monolayer. This step could be blocked by α_2-antiplasmin.

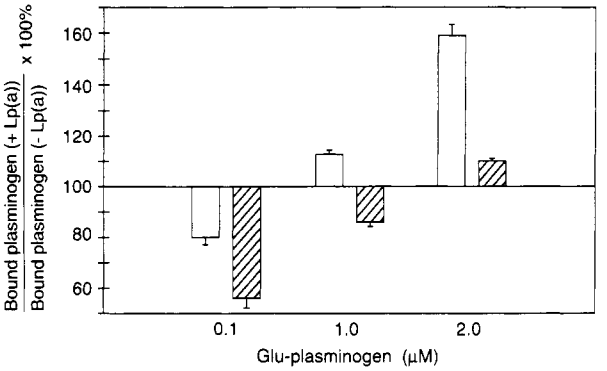

FIGURE 3: Effect of NaCl on Lp(a)-induced promotion or inhibition of plasminogen binding to fibrin D-dimer.

(3) Effect of Salt Concentration on the Binding of Plasminogen to Immobilized Fibrin in the Presence or Absence of Lp(a) (Figure 3). Glu-plasminogen (0.1, 1.0, or 2.0 μM) binding to immobilized D-dimer was measured in the presence or absence of Lp(a) and in the presence or absence of 0.15 M NaCl. The results were expressed as a ratio of the binding ± Lp(a). In the absence of physiological salt, the Lp(a)-induced inhibition of binding at low concentrations of plasminogen was about 2-fold greater than that in the presence of NaCl. At higher concentrations of plasminogen, the absence of salt greatly attenuated the promotion of plasminogen binding by Lp(a) observed under physiological salt conditions. Therefore, the absence of NaCl tended to exaggerate the inhibitory effect of Lp(a) on plasminogen binding (Figure 3).

These findings are consistent with reports in the literature in which substantial Lp(a)-induced inhibition of plasminogen binding was observed when little or no salt was included in the incubation mixture and low concentrations of plasminogen were used (Rouy *et al.*, 1991; Edelberg *et al.*, 1990). Under these unphysiological conditions, competitive inhibition was found.

DISCUSSION

In the present study, fibrin D-dimer was physically immobilized onto plastic plates by simple incubation. Fibrin D-dimer was selected for the immobilization since it is the fibrin domain shown to specifically bind Lp(a) (Liu *et al.*, 1993). The findings showed that the effect of Lp(a) on plasminogen binding was dependent on the concentration of plasminogen. At very low concentrations of plasminogen, binding to fibrin was inhibited by Lp(a) (Figure 1B), as reported previously by others (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990). However, at higher plasminogen concentrations (>0.4 μM), Lp(a) significantly promoted plasminogen binding. At the physiological concentration (2.0 μM) of plasminogen, Lp(a) increased the number of plasminogen binding sites from 1.32 to 1.94. At higher concentrations of plasminogen, the number of binding sites on D-dimer was two, whereas on D-dimer-bound Lp(a) it was five (Table 1).

This biphasic effect of Lp(a) suggested that whereas it inhibited plasminogen binding to the two sites on D-dimer, it also exposed five new binding sites that had a 2–3-fold lower affinity (Table 1). These binding sites were found only after Lp(a) bound to fibrin, since Lp(a) bound directly to the plate failed to bind plasminogen. Therefore, it appeared that these binding sites were masked on Lp(a) and required a fibrin-induced conformational change in the molecule to be exposed. These observations are consistent with the report of Smith and Crosbie (1991) showing colocalization of Lp(a) and

Table 3: Sequence Alignment of Kringles from t-PA and Lp(a)[a]

>TPAK2	-	C	LPWNSMILIGKVY	TAQ	NPSAQALGLG	K	HNY	C	RNPDGDA	KPW	C	HVLKNRRLTWEY		C
>HLK1	-	C	QAWSSMTPHQHNR	TTE	NYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK2	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK3	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK4	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK5	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK6	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK7	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK8	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK9	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK10	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK11	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK12	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK13	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK14	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK15	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK16	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK17	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NY	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK18	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK19	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK20	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK21	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK22	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK23	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK24	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK25	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK26	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK27	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK28	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK29	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDAVAA	PY	C	YTRDPG	VRWEY	C
>HLK30	-	C	QAWSSMTPHSHSR	TPE	YYPNAGLI	M	NP	C	RNPDPVAA	PY	C	YTRDPS	VRWEY	C
>HLK31	-	C	QAWSSMTPHSHSR	TPA	YYPNAGLI	K	NY	C	RNPDPVAA	PW	C	YTTDPS	VRWEY	C
>HLK32	-	C	QAWSSMTPHQHSR	TPE	NYPNAGLT	R	NY	C	RNPDAEIR	PW	C	YTMDPS	VRWEY	C
>HLK33	-	C	QSWSSMTPHWHQR	TTE	YYPNGGLT	R	NY	C	RNPDAEIS	PW	C	YTMDPN	VRWEY	C
>HLK34	-	C	QSWSSMTPHWHQR	TTE	YYPNGGLT	R	NY	C	RNPDAEIR	PW	C	YTMDPS	VRWEY	C
>HLK35	-	C	QSWSSMTPHWHRR	IPL	YYPNAGLT	R	NY	C	RNPDAEIR	PW	C	YTMDPS	VRWEY	C
>HLK36	-	C	QSWSSMIPHWHQR	TPE	NYPNAGLT	E	NY	C	RNPDSGKQ	PW	C	YTTDPC	VRWEY	C
>HLK37	-	C	QSWSSMTPHRHQR	TPE	NYPNDGLT	M	NY	C	RNPDADTG	PW	C	FTMDPS	IRWEY	C
>HLK38	-	C	QEWAAQEPHRHST	FIP	GTNKWAGLE	K	NY	C	RNPDGDINGPW		C	YTMNPR	KLFDY	C

[a] TPAK2: t-PA kringle 2. HLK1–38: human Lp(a) kringle 1–38.

plasminogen in atherosclerotic lesions and a weak positive correlation between the two in intravascular thrombi.

Plasminogen bound to the fibrin-bound Lp(a) was found to be activatable by t-PA, but at a somewhat lower efficiency. Specifically, t-PA-induced plasmin generation was similar in the absence or presence of Lp(a). However, under the conditions used (Figure 1A, Table 2), there was 1.5-fold more substrate in the presence of Lp(a). Therefore, about 21% inhibition of plasminogen activation by Lp(a) was obtained.

The findings (Table 2) indicated that Lp(a) did not reduce plasmin generation at physiological concentrations of plasminogen. However, the Lp(a)-bound plasmin was found to be relatively ineffective in lysing the fibrin monolayer, suggesting that the Lp(a)-bound plasmin could not access fibrin cleavage sites on the fibrin monolayer but had to dissociate to induce fibrinolysis. This conclusion was supported by the finding that α_2-antiplasmin inhibited fibrinolysis under these conditions. By contrast, α_2-antiplasmin had little effect in the absence of Lp(a), since fibrin-bound plasmin is protected from inhibition (Wiman & Collen, 1979) (Figure 3). The Lp(a)-bound plasmin should have been similarly protected since its binding was lysine binding site mediated (abolished by EACA). Since it was not, the findings suggest that dissociated plasmin was responsible for the lysis.

Therefore, the observations are consistent with the proposition that Lp(a) may induce inhibition of fibrinolysis. However, the mechanism responsible for inhibition was not related to an inhibition of plasminogen binding or activation. Moreover, the inhibition of fibrinolysis that occurred under the present experimental conditions may be far less efficient in a fibrin clot where fibrin cleavage sites may be more accessible to Lp(a)-bound plasmin than on the fibrin monolayer. This could explain reports that Lp(a) does not inhibit clot lysis in a plasma milieu (Kluft *et al.*, 1989; Eaton *et al.*, 1990; Lu *et al.*, 1990; Halvorsen *et al.*, 1992; Mao & Tucci, 1990).

The effect of NaCl was investigated because low or no salt conditions were used in a number of published studies (Edelberg *et al.*, 1989, 1990; Edelberg & Pizzo, 1990; Leerink *et al.*, 1991; Rouy *et al.*, 1991, 1992). In the absence of physiological concentrations of NaCl, the Lp(a)-mediated inhibition of plasminogen binding to fibrin was found to be promoted by about 2-fold. At the same time, the absence of NaCl greatly attenuated the promotion of plasminogen binding

by Lp(a) (Figure 3). Therefore, when salt was omitted, inhibition by Lp(a) was exaggerated whereas promotion by Lp(a) was attenuated. The results obtained under these unphysiological conditions may explain why it was concluded that Lp(a) is a competitive inhibitor of fibrin-mediated plasminogen binding and activation.

The current data showing that the effect of Lp(a) on fibrinolysis is not related to direct competition for plasminogen binding sites on fibrin are consistent with the reports that Lp(a) induced uncompetitive, rather than competitive, inhibition of plasminogen activation by t-PA (Loscalzo *et al.*, 1990; Leerink *et al.*, 1991; Liu *et al.*, 1993). Moreover, the finding of plasminogen binding sites on fibrin-bound Lp(a) is also consistent with the mechanism by which uncompetitive inhibition occurs. Plasminogen binding to Lp(a) was inhibited by EACA, indicating lysine binding sites, and these binding sites are probably lysine/arginine residues on certain Lp(a) kringles. In the absence of the fibrin-induced conformational change in Lp(a), these residues may have been occupied by other kringles of Lp(a) itself. A comparable kringle–kringle interaction was previously demonstrated in the crystal structure of t-PA by de Vos *et al.* (1992). In these studies, the lysine[47] of kringle II was implicated. Examination of the primary structure of the kringles of Lp(a) showed that a comparable lysine/arginine residue is found in six of the Lp(a) kringles (Table 3). The number of these sites available in Lp(a) for a kringle interaction with plasminogen is, therefore, consistent with our finding that there were five binding sites for plasminogen on Lp(a) (Table 1).

It has previously been shown by Rejante *et al.* (1991) and by us (Liu & Gurewich, 1993b) that certain arginyl residues bind to plasminogen kringle IV. Furthermore, a kringle-mediated interaction between Lp(a) and plasminogen is also consistent with the promoting effect of NaCl that was found (Figure 3), since Cl^- has been shown to be required for kringle–kringle interactions (de Vos *et al.*, 1992) and to affect Glu-plasminogen activation (Urano *et al.*, 1987a,b). In the absence of Cl^-, the kringles of Lp(a) may not interact readily and therefore may be more ready for fibrin binding, which was also found under these conditions. On the other hand, the hypothesis that a fibrin-mediated conformational change in Lp(a) exposes the essential kringle domains is not supported by the electron microscopic appearance of *rec*-Apo(a) as an open-coiled structure on an EM grid (Phillips *et al.*, 1993). However, the conformation of *rec*-Apo(a) on an EM grid may not be the same as that of native Lp(a) in solution.

In conclusion, the present findings show that the competition between Lp(a) and plasminogen for binding sites on fibrin is complicated by the exposure of plasminogen binding sites on fibrin-bound Lp(a). These sites are occupied by plasminogen at physiological concentrations of plasminogen and NaCl, but not at low concentrations of plasminogen, especially in the absence of salt. These findings help to explain many of the inconsistencies in the literature. According to the model based on the present study (Figure 4), Lp(a) binds with high affinity (K_D = 0.02–0.03 μM) to fibrin (Harpel *et al.*, 1989; Loscalzo *et al.*, 1990), inhibiting direct binding of plasminogen to its two binding sites on D-dimer (K_D = 1.01 μM). Concomitantly, five new plasminogen binding sites of lower affinity (K_D = 3.16 μM) are exposed on Lp(a) after it binds to fibrin, thereby enhancing plasminogen binding and maintaining t-PA-mediated plasmin generation at physiological concentrations of plasminogen. However, the resultant Lp(a)-bound, in contrast to fibrin-bound, plasmin was a poor mediator of lysis of the fibrin monolayer, since it had to dissociate from Lp(a) to access the fibrin cleavage site. This was evidenced by the fact that α_2-antiplasmin could effectively inhibit plasminogen activation.

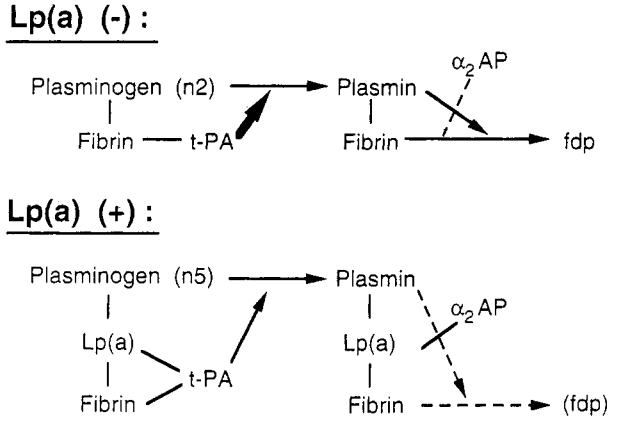

FIGURE 4: Mechanism of the effects of Lp(a) on plasminogen binding to fibrin and its activation: Lp(a) binds with high affinity (K_D = 0.02–0.03 μM) to fibrin, inhibiting direct binding of plasminogen to its two binding sites on D-dimer (K_D = 1.01 μM). Concomitantly, five new plasminogen binding sites of lower affinity (K_D = 3.16 μM) are exposed on Lp(a) after it binds to fibrin, thereby enhancing plasminogen binding and maintaining t-PA-mediated plasmin generation at physiological concentrations of plasminogen. However, the resultant Lp(a)-bound, in contrast to fibrin-bound, plasmin was a poor mediator of lysis of the fibrin monolayer since it had to dissociate from Lp(a) to access the fibrin cleavage site. This was evidenced by the fact that α_2-antiplasmin could effectively inhibit plasminogen activation.

ACKNOWLEDGMENT

We acknowledge the excellent technical assistance of Wendy Kung and the help of Joyce J. Lloyd and Barbara-Jean Fox in the preparation of the manuscript.

REFERENCES

Albers, J. J., Adolphson, J. L., & Hazzard, W. D. (1977) *J. Lipid Res. 18*, 331–338.
Chakrabati, R., Hocking, Fearnley, G. R., Mann, R. D., Attwell, T. N., & Jackson, D. (1968) *Lancet 1*, 7550–7559.
Dahlen, G. H., Guyton, J. R., & Attar, M. (1986) *Circulation 74*, 758–765.
de Vos, A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L., & Kossiakoff, A. A. (1992) *Biochemistry 32*, 270–279.
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.
Eaton, D. L., Tomlinson, J. E., Higgins, D. H., Kochinsky, M. L., & Zioncheck, T. F. (1990) *Fibrinolysis 4* (Suppl. 3), 16.
Edelberg, J. M., & Pizzo, S. V. (1990) *Biochemistry 28*, 2370–2374.
Edelberg, J. M., Gonzalez-Gronow, M., & Pizzo, S. V. (1989) *Biochemistry 28*, 2370–2374.
Edelberg, J. M., Gonzalez-Gronow, M., & Pizzo, S. V. (1990) *Thromb. Res. 57*, 155–162.
Edelberg, J. M., Weisler, M., & Pizzo, S. V. (1991) *Biochem. J. 276*, 785–791.
Fless, G. M., Zum Mallen, M. E., & Scanu, A. M. (1986) *J. Biol. Chem. 261*, 8712– 8718.
Halvorsen, S., Skønsberg, O. H., Berg, K., Rutyer, R., & Godal, H. C. (1992) *Thromb. Res. 68*, 223–232.
Hamsten, A., Wiman, B., De Faire, U., & Blombäck, M. (1985) *New Engl. J. Med. 313*, 1257–1263.
Harpel, P. C., Gordon, B. R., & Parker, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3847–3851.

Juhan-Vague, I., & Collen, D. (1988) *Hypofibrin. Atherothromb. 14*, 479–483.
Kluft, C., Jie, A. F. H., Los, P., de Wit, E., & Havekes, L. (1989) *Biochem. Biophys. Res. Commun. 161*, 427–433.
Leerink, C. B., Gimpel, J. A., Korlandt, W. Bouma, B. N., & van Rijn, H. J. M. (1991) *Fibrinolysis 5*, 233–238.
Liu, J., & Gurewich, V. (1991) *J. Clin. Invest. 88*, 12257–12259.
Liu, J., & Gurewich, V. (1992) *Biochemistry 31*, 6311–6317.
Liu, J., & Gurewich, V. (1993a) *J. Biol. Chem. 268*, 12257–12259.
Liu, J., & Gurewich, V. (1993b) *Blood 81*, 980–987.
Liu, J., Harpel, P. C., Pannell, R., & Gurewich, V. (1993) *Biochemistry 32*, 9694–9700.
Loscalzo, J., Weinfeld, M., Fless, G. M., & Scanu, A. M. (1990) *Atherosclerosis 10*, 240–245.
Lu, H., Bruckert, J., Soria, J., Li, H., de Gennes, J. L., Legrand, A., Peynet, J., & Soria, C. (1990) *Fibrinolysis 4*, 513–516.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258*, 4249–4256.
Mao, S. J., & Tucci, M. A. (1990) *FEBS Lett. 267*, 131–134.
McLean, J. W., Tomlinson, J. E., Kuang, E., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M., & Lawn, R. M. (1987) *Nature 330*, 132–137.
Muria, A., Miyahara, T., & Fujimoto, N. (1986) *Atherosclerosis 59*, 199–204.
Phillips, M. L., Lembertas, A. V., Schumaker, V. N., Lawn, R. M., Shire, S. J., & Zioncheck, T. F. (1993) *Biochemistry 32*, 3722–3728.
Prins, M. H., & Hirsh, J. (1991) *Am. Heart J. 122*, 545–551.
Rejante, M. R., Byeon, I. J. L., & Linás, M. (1991) *Biochemistry 30*, 11081–11092.
Rhoads, G. G., Dahlen, G., & Berg, K. (1986) *J. Am. Med. Assoc. 256*, 2540–2544.
Rouy, D., Grailhe, P., Nigon, P., Chapman, J., & Angles-Cano, E. (1991) *Arterioscl. Thromb. 11*, 629–637.
Rouy, D., Koschinsky, M. L., Fleury, V., Chapman, J., & Angles-Cano, E. (1992) *Biochemistry 31*, 6333–6339.
Scanu, A. M. (1988) *Arch. Pathol. Lab. Med. 112*, 1045–1047.
Seed, M., Hoppichler, F., & Reaveley, D. (1990) *New Engl. J. Med. 322*, 1494–1499.
Smith, E. B., & Crosbie, L. (1991) *Atherosclerosis 89*, 127–136.
Urano, T., Chibber, B. A. K., & Castellino, F. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031–4036.
Urano, T., deSerrano, V. S., Chibber, B. A. K., & Castellino, F. J. (1987b) *J. Biol. Chem. 262*, 15959–15964.
Varadi, A., & Patthy, L. (1983) *Biochemistry 22*, 2440–2446.
Varadi, A., & Patthy, L. (1984) *Biochemistry 23*, 2108–2112.
Wiman, B., & Collen, D. (1979) *J. Biol. Chem. 254*, 9291–9297.