Inhibition of Plasminogen Activation by Lipoprotein(a)

**CRITICAL DOMAINS IN APOLIPOPROTEIN(a) AND MECHANISM OF INHIBITION ON FIBRIN AND DEGRADED FIBRIN SURFACES**

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Similarity between the apolipoprotein(a) (apo(a)) moiety of lipoprotein(a) (Lp(a)) and plasminogen suggests a potentially important link between atherosclerosis and thrombosis. Lp(a) may interfere with tissue plasminogen activator (tPA)-mediated plasminogen activation in fibrinolysis, thereby generating a hypercoagulable state in vivo. A fluorescence-based system was employed to study the effect of apo(a) on plasminogen activation in the presence of native fibrin and degraded fibrin cofactors and in the absence of positive feedback catalyzed by plasmin. Human Lp(a) and a physiologically relevant, 17-kringle recombinant apo(a) species exhibited strong inhibition with both cofactors. A variant lacking the protease domain also exhibited strong inhibition, indicating that the apo(a)-plasminogen binding interaction mediated by the apo(a) protease domain does not ultimately inhibit plasminogen activation. A variant in which the strong lysine-binding site in kringle IV type 10 had been abolished exhibited substantially reduced inhibition whereas another lacking the kringle V domain showed no inhibition. Amino-terminal truncation mutants of apo(a) also revealed that additional sequences within kringle IV types 1–4 are required for maximal inhibition. To investigate the inhibition mechanism, the concentrations of plasminogen, cofactor, and a 12-kringle recombinant apo(a) species were systematically varied. Kinetics for both cofactors conformed to a single, equilibrium template model in which apo(a) can interact with all three fibrinolytic components and predicts the formation of ternary (cofactor, tPA, and plasminogen) and quaternary (cofactor, tPA, plasminogen, and apo(a)) catalytic complexes. The latter complex exhibits a reduced turnover number, thereby accounting for inhibition of plasminogen activation in the presence of apo(a)/Lp(a).

Elevated plasma levels of lipoprotein(a) (Lp(a)) have been identified as a risk factor for the development of atherosclerotic disorders including coronary artery disease (1). Originally described by Berg (2), Lp(a) is similar to low density lipoprotein in that it contains a cholesteryl ester core surrounded by a monolayer of unesterified cholesterol and phospholipid in which apolipoprotein B-100 (apoB-100) is embedded. Lp(a) is distinct from low density lipoprotein in that it contains a single molecule of an additional glycoprotein moiety called apolipoprotein(a) (apo(a)), which is covalently linked to apoB-100 by a single disulfide bond (3, 4). The cloning of apo(a) revealed a striking similarity between apo(a) and the fibrinolytic serine proteasezymogen plasminogen (5).

The apo(a) moiety of Lp(a) lacks sequences corresponding to the plasminogen kringle (K) 1, 2, and 3 domains, yet contains multiple repeats of the plasminogen K4-like domain (apo(a) KIV) followed by plasminogen K5-like and protease-like sequences (apo(a) KV and protease-like (P) domains, respectively) (5). There are 10 distinct classes of apo(a) KIV repeats (designated KIV1-KIV10) that differ from one another by only a small number of amino acid substitutions (5). There is a single copy of each KIV repeat except for KIV2, which is known as the major repeat kringle (6, 7). This sequence can be present in 3 to greater than 30 identical copies that gives rise to Lp(a) isoform size heterogeneity in the population (8). Apo(a) KIVn, each contain a weak lysine binding site, which mediate the initial non-covalent interaction between apo(a) and apoB-100 in Lp(a) assembly (9–11). Apo(a) KIVn contains an unpaired cysteine residue that forms the disulfide linkage with apoB-100 in Lp(a) assembly (3, 4). Apo(a) KIVn contains a stronger lysine binding site that is believed to mediate binding of apo(a)/Lp(a) to lysine residues exposed on the surface of biological substrates such as fibrin (12, 13). The apo(a) protease-like domain exhibits no catalytic activity (14).

The similarity of Lp(a) to both low density lipoprotein and plasminogen suggests that the pathogenic mechanism of Lp(a) likely involves both proatherogenic and prothrombotic/antifibrinolytic effects (1). Several in vitro and in vivo studies have shown that apo(a)/Lp(a) can inhibit fibrinolysis (15–17). Moreover, binding interactions have been demonstrated between apo(a)/Lp(a) and fibrinogen, plasmin-modified fibrinogen, plasminogen, and tPA (13, 18–22). Early studies demonstrated that apo(a)/Lp(a) can inhibit the binding of plasminogen and tPA to fibrin (13, 18), but the mechanism by which apo(a) inhibits plasminogen activation remains controversial as both competitive (23) and uncompetitive (18, 24) mechanisms have been reported.
Efficient activation of plasminogen requires the formation of a ternary complex between substrate (plasminogen), enzyme (tPA), and cofactor (fibrin) (25, 26). Kinetic studies of plasminogen activation are complicated by the existence of plasmin-mediated positive feedback reactions. First, limited plasmin cleavage of fibrin results in the exposure of carboxyl-terminal lysine residues in the cofactor that enhance plasminogen binding (27). A nucleotide single strand tPA is cleaved by plasmin to generate the two-chain form. Third, plasmin cleaves native Glu$^1$-plasminogen after Lys$^{37}$ to form Lys$^{38}$-plasminogen, which is a better substrate for tPA (28). Formation of Lys$^{38}$-plasminogen is, in turn, stimulated by partially degraded, but not native, fibrin.

In the present study, we adapted a methodology previously described by Horrovoets and co-workers (29) that allows plasminogen activation to be measured directly in a fibrin clot and in the absence of positive feedback reactions catalyzed by plasmin. Briefly, a recombinant variant of human Glu$^1$-plasminogen, in which the active site serine has been replaced by cysteine, was employed such that the zymogen could be labeled with a thiol-specific fluorescein tag. The labeled protein remains catalytically inactive upon cleavage by tPA. Using a series of recombinant apo(a) (r-apo(a)) variants in this system, we have delineated the domains in apo(a) required for inhibition of plasminogen activation and have described the inhibition mechanism as an equilibrium template model.

EXPERIMENTAL PROCEDURES

Materials—Lysine-Sepharose CL-4B resin was from Amersham Biosciences. 1,5-Dansyl-Glu-Gly-Arg-chloromethylketone dihydrochloride (dEGR-CK), D-Phe-Pro-Arg-chloromethylketone (PPA-CK), and D-Val-Lysine-Sepharose CL-4B resin was from Amersham Biosciences. 1,5-Dansyl-Glu-Gly-Arg-chloromethylketone (dEGR-CK), D-Phe-Pro-Arg-chloromethylketone (PPA-CK), and D-Val-Iodoacetamidofluorescein at 4°C (corrected for Rayleigh scattering) using the following molecular weights and extinction coefficients (21): 17K wild type (wt) (M$_r$ = 278,719; $\varepsilon_{280 nm}$ = 20.7); 17KDS6A (M$_r$ = 278,719; $\varepsilon_{280 nm}$ = 20.7); 17K-V (M$_r$ = 269,978; $\varepsilon_{280 nm}$ = 21.1); 17K-Pi (M$_r$ = 249,244; $\varepsilon_{280 nm}$ = 22.0); 12K (M$_r$ = 207,674; $\varepsilon_{280 nm}$ = 32.7); 10K (M$_r$ = 180,000; $\varepsilon_{280 nm}$ = 20.7); 6K (M$_r$ = 122,542; $\varepsilon_{280 nm}$ = 25.6); KIV$_{50}$ (M$_r$ = 84,802; $\varepsilon_{280 nm}$ = 20.2); KIV$_{10}$ (M$_r$ = 70,000; $\varepsilon_{280 nm}$ = 21.0); KIV$_{7}$ (M$_r$ = 108,269; $\varepsilon_{280 nm}$ = 24.4); KIV$_{3}$ (M$_r$ = 94,676; $\varepsilon_{280 nm}$ = 24.1); KIV$_{5}$ (M$_r$ = 80,022; $\varepsilon_{280 nm}$ = 23.4); KIV$_{17}$ (M$_r$ = 66,117; $\varepsilon_{280 nm}$ = 22.8); KIV$_{6}$ (M$_r$ = 52,040; $\varepsilon_{280 nm}$ = 21.6). The proteins were assessed for purity by SDS-PAGE under non-reduced and reduced conditions and migrated in 10 mM diithiothreitol conditions, followed by Coomasie Blue staining. All purified r-apo(a) preparations exhibited single bands under both non-reduced and reduced conditions and migrated at the expected molecular weights.

Preparation of Fluorescently Labeled Recombinant Plasminogen—The construction and expression of a variant of native Glu$^1$-plasminogen, containing an active site serine to cysteine mutation (r-plasminogenS741C), has been described previously (29). Likewise, r-plasminogenS741C protein was purified from the conditioned medium of stably expressing human embryonic kidney (HEK 293) cell lines by affinity chromatography as described previously (11, 21). Briefly, conditioned medium (Opti-MEM) harvested every 3 days from the stably transfected cells was loaded over a 50-ml lysine-Sepharose column pre-equilibrated in PBS. The column was washed extensively with PBS containing 500 mM NaCl and r-apo(a) was eluted with 200 mM e-ACA in the same buffer. Protein-containing fractions were pooled, dialyzed extensively against HBS, and concentrated using 300 KDa PEG 20,000 A. All purified r-apo(a) protein concentrations were determined spectrophotometrically (corrected for Rayleigh scattering) using the following molecular weights and extinction coefficients (21): 17K wild type (wt) (M$_r$ = 278,719; $\varepsilon_{280 nm}$ = 20.7); 17KDS6A (M$_r$ = 278,719; $\varepsilon_{280 nm}$ = 20.7); 17K-V (M$_r$ = 269,978; $\varepsilon_{280 nm}$ = 21.1); 17K-Pi (M$_r$ = 249,244; $\varepsilon_{280 nm}$ = 22.0); 12K (M$_r$ = 207,674; $\varepsilon_{280 nm}$ = 32.7); 10K (M$_r$ = 180,000; $\varepsilon_{280 nm}$ = 20.7); 6K (M$_r$ = 122,542; $\varepsilon_{280 nm}$ = 25.6); KIV$_{50}$ (M$_r$ = 84,802; $\varepsilon_{280 nm}$ = 20.2); KIV$_{10}$ (M$_r$ = 70,000; $\varepsilon_{280 nm}$ = 21.0); KIV$_{7}$ (M$_r$ = 108,269; $\varepsilon_{280 nm}$ = 24.4); KIV$_{3}$ (M$_r$ = 94,676; $\varepsilon_{280 nm}$ = 24.1); KIV$_{5}$ (M$_r$ = 80,022; $\varepsilon_{280 nm}$ = 23.4); KIV$_{17}$ (M$_r$ = 66,117; $\varepsilon_{280 nm}$ = 22.8); KIV$_{6}$ (M$_r$ = 52,040; $\varepsilon_{280 nm}$ = 21.6). The proteins were assessed for purity by SDS-PAGE under non-reduced and reduced conditions and migrated at the expected molecular weights.

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The amount of fluorescein incorporated was determined spectrophotometrically as previously described (29); labeling efficiency was typically greater than 80%. The Flu-plasminogen protein was assessed for purity by SDS-PAGE under non-reduced and reduced conditions and migrated at 10 mM diithiothreitol conditions, followed by Coomasie Blue staining. All purified Flu-plasminogen preparations exhibited single bands under both non-reduced and reduced conditions and migrated at the expected molecular weight.

Purification of Human Fibrinogen and Non-cross-linked FDPs—Fibrinogen was purified from units of citrated, fresh frozen, human plasma as described previously (35), but with modifications. Briefly, all steps were performed at room temperature with the exception of the final dialysis performed at 4°C. Initially, 80 ml of 1.0 M BaCl$_2$ was added to 1.0 liter of plasma over 5 min with stirring. The solution was stirred for an additional 30 min and then centrifuged at 5000 g for 30 min. the 1% stock of diithiothreitol was added to 1% stock of diithiothreitol (1 mM final) and stirred for 10 min. A 4.0 mM $\beta$-alanine stock in TSC buffer (50 mM sodium citrate, pH 6.5, 150 mM NaCl) was then added to the solution over 5 min with stirring (to 1.0 mM final), stirred an additional 30 min, and centrifuged at 5000 g for 30 min.

A. R. Belczewski and M. L. Koschinsky, unpublished results.
Apo(a) Inhibition of Plasminogen Activation

β-Alanine was added to the supernatant again (to 2.0 M final), stirred, and centrifuged as before. The pellet was dissolved in 1.0 liter of TSC buffer and β-alanine added again (to 2.0 M final), stirred, and centrifuged as before. The pellet was dissolved in 250 mL of TSC buffer and PEG 8000 (40% w/v stock in water) was added to the solution over 15 min with stirring (to 1.2% final), stirred an additional 30 min, and centrifuged at 5000 × g for 30 min. PEG 8000 was added to the supernatant again over 15 min with stirring (to 5.0% final), stirred an additional 30 min, and centrifuged at 5000 × g for 30 min. The pellet was dissolved in 100 mL of 20 mM HEPES, pH 7.4, 500 mM NaCl by rapid agitation on an orbital shaker table for 2 h. Subsequently, the 100-μL solution was diluted to 20 μL HEPES, pH 7.4 (to 2.5 mM NaCl final), before passage over 5-ml lysine-Sepharose and 100-ml DEAE-cellulose fibrinogen flow column forms. Both columns were washed with 300 μL HEPES, pH 7.4, 25 mM NaCl and were linked in tandem. The columns were washed with 50 mL of the same equilibration buffer and the DEAE column was disconnected and washed further with 300 mL of the same buffer. Fibrinogen was eluted from the DEAE column using 20 mM HEPES, pH 7.4, 100 mM NaCl and further fractionated to yield high molecular weight fibrinogen (containing intact α-chains) by precipitation with 19% ammonium sulfate as described previously (36). The purified fibrinogen was dialyzed extensively against HBS, passed through a 5-μm syringe filter, and aliquots were stored at −70°C.

Non-cross-linked FDPs were prepared by plasmin-mediated lysis of fibrin clots as described previously (37), but with modifications. Briefly, all steps were performed at room temperature with the exception of the final dialysis performed at 4°C. In HBS containing 0.02% (v/v) Tween 80 (HBST), 50 mg of fibrinogen was clotted with 5 mM CaCl2 and 20 nM thrombin in the presence of 20 nM plasmin. Clot formation (occurred within 1 min) and subsequent fibrinolysis were monitored spectrophotometrically at 600 nm. The reaction was allowed to proceed to 75% total lysis and then terminated by the addition of VFK-CK (5 mM final) and PPA-CK (1 mM final). Remaining aggregates were removed by centrifugation at 13,000 rpm for 5 min. The resultant FDPs were finally dialyzed extensively against 20 mM HEPES, pH 7.4, 500 mM NaCl and further fractionated to yield high molecular weight fibrinogen (containing intact α-chains) by precipitation with 19% ammonium sulfate as described previously (36). The purified fibrinogen was dialyzed extensively against HBS, passed through a 5-μm syringe filter, and aliquots were stored at −70°C.

Fluorescence measurements were converted to corrected values according to Equation 2, where $I_{corr}$ is the corrected fluorescence intensity, $I_{meas}$ is the measured fluorescence intensity, $a$ is the exponential coefficient, and $i$ is the amount of fluorescence per mole of Flu-plasminogen.

Initial rates of fluorescence decrease were then determined by linear regression analysis and converted to rates of plasminogen activation according to Equation 3:

\[ d[P]/dt = (d[P]/dt)/[a] = (d[P]/dt)/[r] + (d[P]/dt)/[I] = (d[P]/dt)/[P][f][A][r][a][i] \] (Eq. 3)

where $d[P]/dt$ is the rate of plasmin formation per mole of TPA (s⁻¹), $d[P]/dt$ is the initial rate of fluorescence decrease, $r$ is the maximum change in fluorescence intensity (0.5 for fibrin surface (29) and 0.4–0.5 as determined empirically for FDP solution), $I_0$ is the initial fluorescence intensity, $A$ is the initial concentration, and $[R]$ is the initial TPA (activator) concentration. In all cases, rates of plasminogen activation reported represent the average of duplicate measurements.

For apo(a) domain studies with fibrin as the cofactor, 80-μL volumes containing Flu-plasminogen (0.3–1.8 μM final), fibrinogen (3.0 μM final), and Lp(a) or r-apo(a) (0–1.4 μM final) in HBS containing 0.02% (v/v) Tween 80 (HBST) were added to wells already containing 20-μL volumes of CaCl2 (10 mM final), thrombin (6 nM final), and TPA (5 nM final) in HBST. For apo(a) domain studies with FDP as the cofactor, 80-μL volumes containing Flu-plasminogen (0.1–0.6 μM final), FDP (0.6 μM final), and Lp(a) or r-apo(a) (0–1.4 μM final) in HBS containing 0.02% (v/v) Tween 80 (HBST) were added to wells already containing 20-μL volumes of TPA (3 μM final) in HBST. Compared with Flu-plasminogen cleavage in the absence of Lp(a)/apo(a), percent inhibition (of plasminogen activation) values were determined for each data set at 1.4 μM Lp(a)/r-apo(a).

Mechanism studies with 12K r-apo(a) were also performed in a similar fashion to the domain studies described above. With the fibrin cofactor, the final assay concentrations were as follows: Flu-plasminogen, 0.3–1.8 μM; fibrinogen, 0.02–3.0 μM; 12K r-apo(a), 0–3.0 μM; CaCl2, 10 mM; thrombin, 6 nM; TPA, 5 nM. With the FDP cofactor, the final assay concentrations were as follows: Flu-plasminogen, 0.1–0.6 μM; FDP, 0.05–0.8 μM; 12K r-apo(a), 0–3.0 μM; TPA, 3 μM. The mechanism initial fits were analyzed by non-linear regression according to the equilibrium model template of plasminogen activation described below.

A Model for the Modulation by Apo(a) of Fibrin-dependent Plasminogen Activation—Our data indicate that apo(a) both inhibits and promotes plasminogen activation, depending on the concentrations of plasminogen, fibrin, and apo(a) (see “Results”). At most concentrations studied, inhibition was observed; however, very low concentrations of clotted fibrinogen, stimulation occurred. Efforts were made to rationalize these observations by taking into account the known interactions between the various components and the template mechanism for fibrin-dependent plasminogen activation (26). Among the known interactions are fibrin/plasminogen, fibrin/TPA, fibrin/apo(a), apo(a/plasminogen, fibrin/apo(a)/plasminogen, fibrin/plasminogen/apo(a), and apo(a)/TPA. Numerous models were constructed that included only binary interactions of apo(a) with fibrin, TPA, or plasminogen. None of these could adequately account for all of the experimental observations, particularly the stimulation of plasminogen activation by apo(a) at very low concentrations of clotted fibrinogen. Eventually, however, a model was found that accounts for all of the experimental observations. In the absence of apo(a), the model comprises the template model described by Horrevoets and co-workers (26). In this model, either the plasminogen substrate (P) or the TPA activator (A) can bind to the fibrin cofactor (F) to form the binary FP or FA complexes. These can then bind the third component to form the ternary FAP complex, from which plasmin is generated with a first-order rate constant $k_2$. When the inhibitory ligand (I) is included, the first assumption is made that I can bind F to form an IF complex, and this can function as a template analogous to F, such that P can bind IF to form IFP, and A can bind IF to form IFPA. These ternary complexes can then bind the fourth component to form the quaternary complex, IFAP, from which plasmin is generated with first-order rate constant $k_2$. These concepts, along with the equilibrium binding expressions and conservation equations for each of the species involved in the process, allow derivation of the rate equation for the model as follows:

\[ r = k_2[FAP] + k_3[IFP] \] (Eq. 4)

The binding interactions and equilibrium expressions associated with assembly of FAP and IFAP are defined as follows.

\[ 1 + F = [IF] + [FAP] \] (Eq. 5)
where \( A_0 \) is the total concentration of the activator. The equilibrium definitions Equations 6, 8, 10, 12, and 14 allow Equation 17 to be expressed in rate per unit \( A_0 \) in terms of constants.

The conservation equation for the activator is given by Equation 17, Equation 19.

Equations 5–15 can be used to express \([FAP]\) and \([IFAP]\) in Equation 4 in terms of the free species and the equilibrium binding constants. The result is given in Equation 16.

\[
 r = k_1[F][A][P]/K_K + k_2[A][F][P]/K_KK
\]

The conservation equation for the activator is given by Equation 17, where \([A]_b\) is the total concentration of the activator. The equilibrium Equations 6, 8, 10, 12, and 14 allow Equation 17 to be expressed in terms of free components. The result is given in Equation 18.

\[
 [A]_b = [A] + [FA] + [FAP] + [FA] + [IFAP] + [IFAP]
\]

Division of Equations 16 by 18 eliminates free \([A]\) and expresses the rate per unit \([A]_b\) in terms of \( k_1, k_2, \) the equilibrium binding constants, and the free concentrations of \( F, P, \) and \( I. \) The result is given in Equation 19.

\[
 r[A]_b = k_1[F][P]/K + k_2[A][F][P]/K
\]

As \([F] \rightarrow \infty\), Equation 20 becomes Equation 21.

\[
 r[A]_b = k_1[F][P]/K
\]

Under these conditions, the reaction rate with respect to free \([F]\) is no longer dependent on the fibrin concentration and the equation is formally identical to the Michaelis-Menten equation. This invites the definitions \( k_1 = k_{cat1} \) and \( K_a = K_{cat1}. \) Similarly, as \([I] \rightarrow \infty, \) plasmin is generated solely from IFAP, and Equation 19 becomes Equation 22.

\[
 r[A]_b = k_2[A][P]/K
\]

As \([F] \rightarrow \infty\), this equation becomes Equation 23.

\[
 r[A]_b = k_2[A][P]/K
\]

Again, under these conditions, the rate with respect to free \([F]\) is no longer dependent on the fibrin concentration and the equation is formally identical to the Michaelis-Menten equation. Accordingly, the definitions \( k_2 = k_{cat2} \) and \( K_a = K_{cat2} \) are assigned. Equation 19 is expressed again as Equation 24 using more intuitive names for the constants.

\[
 r[A]_b = \left( \frac{k_{cat1}[K_a][K_a + ([I]/[A])/[I]_0]}{1 + ([F]/[P]) + ([I]/[A])/[I]_0} \right)\left( \frac{k_{cat2}[K_a][K_a + ([I]/[A])/[I]_0]}{1 + ([F]/[P]) + ([I]/[A])/[I]_0} \right)
\]

In this equation \( k_{cat1}, K_{cat1}, \) and \( K_a \) are as defined above. \( K_a \) is the dissociation constant for the binding of tPA to fibrin, and \( K_{cat1} \) is the dissociation constant for the binding of tPA to the fibrin-apo(a) complex \((K_{cat1} = K_aK_K/K_{cat1}).\)

To evaluate Equation 24, the free concentrations of \( F, P, \) and \( I \) are needed. These are found from the equilibrium binding expressions and conservation equations for total \( F, P, \) and \( I. \) The conservation equations, except for terms containing \( A, \) which are negligibly small because \( A \) was used only in trace concentrations, are Equations 25–27.

\[
 [F]_b = [F] + [IF] + [IFP] + [IFP]
\]

\[
 [P]_b = [P] + [IFP] + [IFP] + [IFP]
\]

\[
 [I]_b = [I] + [IFP] + [IFP] + [IFP]
\]

These can be expressed in terms of free concentrations of each of the respective species as follows.

\[
 [F]_b = [F] + [IF] + [IFP] + [IFP]
\]

\[
 [P]_b = [P] + [IFP] + [IFP] + [IFP]
\]

\[
 [I]_b = [I] + [IFP] + [IFP] + [IFP]
\]

After inserting published values for \( K_a, K_{cat1}, K_{cat2}, \) and \( K_{cat3} \) into Equations 31–33, initial values for \([F]_b, \) \([P]_b, \) and \([I]_b \) were calculated. If the calculated values were different from assigned values by one part in 10\(^3\) or more, new assigned values were provided that were equal to the average of the calculated value and the previously assigned value. This process was repeated until the calculated and assigned values differed by no more than 1 part in 10\(^3\). This typically required 10 to 20 iterations and provided solutions to all three equations accurate to 1 part in 10\(^3\). Convergence was always rapid and robust because if an assigned value were larger than the “true” value, the calculated value was smaller, and vice versa. Thus, averaging guaranteed rapid convergence. The value of \( K_a \) (binding of apo(a) to fibrin) was 1.4 µM (20). The value for \( K_{cat2} \) (binding plasminogen to fibrin) was 30 µM when intact fibrin was used (29) and 0.225 µM when fibrin degradation products were used (40). The value for \( K_{cat1} \) (the binding of apo(a) to plasminogen) was 20 nM (21). The value for \( K_F \) (the binding of plasminogen to the apo(a)-fibrin complex) was 25-fold greater than the value for binding of plasminogen to fibrin or fibrin degradation products, which reflects the observation the apo(a) negatively influences the binding of plasminogen to fibrin (21). For any given set of experimental conditions, values for \([F]_b, \) \([P]_b, \) \([I]_b, \) \( [F]_b, [P]_b, \) and \([I]_b \) were known, and values for \([F]_b, [P]_b, \) and \([I]_b \) were calculated as described above. The data were then fit by non-linear regression (SYSTAT, Evanston, IL) to Equation 24. The input data were \( r[A]_b, [F]_b, [P]_b, \) and \([I]_b \) and the best fit parameters were \( k_{cat1}, K_{cat1}, K_{cat2}, K_{cat3}, K_a, \) and \( K_{cat1} \) (the dissociation constant for the solution-phase tPA/apo(a) interaction). The regression algorithm returned best fit values for the parameters, along with their asymptotic standard errors, estimated values, and residuals.

**RESULTS**

Identification of Apo(a) Domains Mediating Inhibition of Plasminogen Activation—Quantitative assays of tPA-mediated Glu-plasminogen activation were performed using a catalytically inactive, fluorescently labeled form of recombinant human plasminogen (Flu-plasminogen) in the presence of intact fibrin (clots) or soluble FDPs as cofactors. We utilized a series of recombinant apo(a) variants and systematically varied the...
concentrations of Flu-plasminogen and r-apo(a) to delineate the domains in apo(a) that are required for inhibition of plasminogen activation. In the absence of apo(a)/Lp(a), rates of plasminogen activation were maximal in the presence of fibrin (Fig. 1) or soluble FDPs (Fig. 2) over all Flu-plasminogen concentrations. Native Lp(a) and the 17K, 12K, 10K, and 17K(-P) r-apo(a) variants inhibited plasminogen activation in a dose-dependent manner and to similar extents over all Flu-plasminogen concentrations in the presence of both cofactors (Figs. 1A and 2A). Abolishing the strong lysine-binding site in the apo(a) KIV10 domain (17K(D56A) variant) substantially decreased the extent of inhibition observed (Figs. 1B and 2B) as compared with wild type 17K. Deletion of the apo(a) KV domain (17K(-V) variant) abolished the inhibitory effect seen with wild type 17K in the presence of both cofactors (Figs. 1C and 2C). Finally, truncated r-apo(a) proteins lacking the KIV10 and/or KV domains (KIV5–9 and KIV5–10 variants) or possessing intact KIV10 and KV domains (6K, KIV6–P, KIV7–P, KIV8–P, KIV9–P, and KIV10–P variants) also resulted in substantially decreased or no inhibition of plasminogen activation (Figs. 1D and 2D) compared with wild type 17K.

The effects of the different r-apo(a) variants on tPA-mediated plasminogen activation, in the presence of fibrin or FDPs, is summarized in Fig. 3. For the most part, there was little difference in the magnitudes of the inhibitory effects in the presence of the respective cofactors. Notable differences included a minor inhibitory effect of 6K in the presence of fibrin but none in the presence of FDPs, a greater inhibitory effect of 12K in the presence of FDPs than in the presence of fibrin, and a greater inhibitory effect of Lp(a) in the presence of fibrin than in the presence of FDPs.

Analysis of the Mechanism by Which Apo(a) Inhibits Plasminogen Activation—The kinetics of plasminogen activation are complicated by the role of the fibrin/FDP cofactor. Therefore, kinetic analysis of inhibition of plasminogen activation by apo(a) required us to perform initial rate experiments in which the concentrations of cofactor, plasminogen, and r-apo(a) were systematically varied. In these studies, apo(a) was titrated over a higher concentration range (0–3.0 μM) than that employed in the domain studies (0–1.4 μM). At fixed Flu-plasminogen concentrations, apo(a) was then titrated over variable fibrin and FDP concentration ranges. Interestingly, 12K r-apo(a) inhibited plasminogen activation in a dose-dependent manner at fibrin concentrations of 0.56–3.0 μM (Fig. 4B) while below 0.5 μM fibrin 12K r-apo(a) in fact stimulated plasminogen activation in a dose-dependent manner. On the other hand, 12K r-apo(a) inhibited plasminogen activation in a dose-dependent manner over all FDP concentrations tested (Fig. 5B).
The data (Figs. 4 and 5) were first analyzed by non-linear regression according to Michaelis-Menten kinetics (data not shown). This approach did not provide good global fits to any of the four standard types of inhibition (competitive, non-competitive, uncompetitive, or mixed). Thus, non-linear regression of the rate data to an equilibrium template model of plasminogen activation (Equation 24) was performed. In this model (Fig. 6), all three fibrinolytic components (cofactor, tPA, and plasminogen) are allowed to interact with the apo(a) inhibitor. Global fits of the fibrin and FDP data to this model resulted in regression lines that correlated well with the respective experimental data (Figs. 4 and 5). Of special note, both cofactor series fit equally well to the same model and the model predicted the profibrinolytic behavior of apo(a) that was observed at low fibrin concentrations (Fig. 4B).

Examination of the kinetic parameters predicted by the equilibrium template model (Table I) reveals several notable findings. Michaelis constants ($K_m$) for the apo(a)-containing quaternary catalytic complexes (IFAP) were significantly lower than those for the normal fibrinolytic ternary (FAP) complexes in the presence of fibrin (0.003 μM versus 1.35 μM, respectively) and FDPs (0.001 versus 0.034 μM, respectively). Turnover numbers ($k_{cat}$) for the IFAP complexes were also lower than those for the FAP complexes in the presence of fibrin (0.050 s$^{-1}$ versus 0.112 s$^{-1}$, respectively) and FDPs (0.038 s$^{-1}$ versus 0.045 s$^{-1}$, respectively). Dissociation constants for the interaction of tPA with fibrin or FDPs were similar in the presence ($K_I$) or absence ($K_A$) of apo(a). Specifically, the dissociation constants for the interactions of tPA with fibrin or FDPs ($K_A$) were 0.055 and 1.93 μM, respectively. The first value is in reasonable agreement with the value reported by Horrevoets and co-workers (26) and the latter agrees with values reported by Walker and Nesheim (40) for FDPs with relatively lower molecular weights below 1 × 10$^6$. Finally, the dissociation constants for the solution-phase apo(a)-tPA interaction ($K_{10}$) predicted by the model were 3.65 μM with fibrin and 4.55 μM with FDPs.

**DISCUSSION**

It is generally accepted that apo(a)/Lp(a) is capable of inhibiting fibrinolysis, but the mechanism by which this inhibition occurs remains controversial. In addition, the domains in apo(a) that mediate inhibition of fibrinolysis are not known. We addressed these issues in the current study using a series of recombinant apo(a) variants and a system to measure tPA-mediated Glu$^1$-plasminogen activation kinetics in the absence of plasmin-mediated positive feedback reactions. In the absence of apo(a), the observed rates of plasminogen activation agreed well with values reported in the literature. For example, a rate of 0.06 s$^{-1}$ for 1.8 μM Flu-plasminogen activated with 3 μM fibrin and 5 nM tPA agreed well with previous work by Horrevoets and co-workers (29). Likewise, a
rate of 0.03 s⁻¹ for 0.6 μM Flu-plasmin activated in the presence of 0.6 μM FDP and 3 nM tPA agreed well with previous work by Walker and Nesheim (40). The latter finding is significant as it indicates that our preparation of non-cross-linked, soluble FDPs possesses similar intrinsic cofactor activity to the cross-linked, soluble FDPs described by Walker and Nesheim (40). These latter species display similar tPA cofactor activities over a range of FDP sizes (weight-average molecular weight 0.48 × 10⁶ to 4.94 × 10⁶). In agreement with the findings of Walker and Nesheim (40), we found that the FDPs show a similar kcat but a substantially reduced (≈10-fold) Km relative to fibrin, which accounts for the enhanced cofactor activity of the FDPs. As such, our non-cross-linked FDPs likely represent an excellent surrogate for the large soluble FDPs as well as for insoluble, partially degraded, fibrin.

The carboxyl-terminal lysine residues present in partially degraded fibrin serve to mediate positive feedback in the fibrinolytic cascade by (i) promoting the binding and activation of plasminogen, (ii) promoting the plasmin-mediated conversion of Glu1-plasminogen to Lys 78-plasminogen, the latter of which is a better substrate for plasminogen activators, and (iii) by binding plasmin and thus protecting it from consumption by

| r-apo(a) inhibitor | fibrin cofactor % inhibition | FDP cofactor % inhibition |
|--------------------|-----------------------------|---------------------------|
| 17K wt             | 25.4                        | 24.5                      |
| 17K(D56A)          | 10.7                        | 16.4                      |
| 17K(-V)            | 1.1                         | 8.3                       |
| 17K(-P)            | 29.7                        | 26.8                      |
| 12K                | 25.0                        | 44.7                      |
| 10K                | 27.9                        | 22.3                      |
| 6K                 | 13.5                        | 0.0                       |
| KIV₅:₁₀            | 0.4                         | 0.0                       |
| KIV₅:₅             | 2.0                         | 7.5                       |
| KIV₆:₉             | 0.0                         | 1.1                       |
| KIV₇:₉             | 3.8                         | 0.0                       |
| KIV₈:₉             | 4.0                         | 3.7                       |
| KIV₉:₉             | 0.0                         | 0.0                       |
| KIV₁₀:₉            | 3.3                         | 4.7                       |
| h-Lp(a)            | 45.4                        | 30.0                      |

**FIG. 3.** Summary of tPA-mediated Flu-plasminogen activation with fibrin and FDP cofactors in the presence of 1.4 μM Lp(a)/r-apo(a). Percent inhibition values were calculated by dividing the rates of Flu-plasminogen activation in the presence of 1.4 μM Lp(a)/r-apo(a) by those in the absence of Lp(a)/r-apo(a) and represent the average of n independent assays. Shown to the left are schematic representations of the r-apo(a) variants employed.

**FIG. 4.** Kinetics of Flu-plasminogen activation with fibrin cofactor and 5 nM tPA in the presence of increasing 12K r-apo(a) concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μM). Panel A, varied Flu-plasminogen concentrations (0–1.8 μM) and fixed fibrin cofactor concentrations (3.0 μM). Panel B, fixed Flu-plasminogen concentrations (0.3 μM) and varied fibrin cofactor concentrations (0.02–3.0 μM). The symbols represent experimental data points and the lines represent non-linear regression according to equilibrium template model (Equation 24). Data corresponding to fibrin cofactor concentrations greater than 1.0 μM are not shown for clarity.
FDPs, respectively) were fit globally to Equation 24 by non-linear regression according to equilibrium template model (Equation 24).

FIG. 6. Proposed equilibrium template model to describe the inhibitory effect of Lp(a)/apo(a) on tPA-mediated plasminogen activation with fibrin or FDP cofactors. F, cofactor (fibrin or FDPs); A, tPA (enzyme); P, plasminogen (substrate); Pn, plasmin (product); I, apo(a) (inhibitor); kcat, turnover number; Kd, tPA-cofactor dissociation constant; KdA, tPA-cofactor dissociation constant in the presence of apo(a); km, Michaelis constant; Kf, apo(a)-cofactor dissociation constant; K10, apo(a)-tPA dissociation constant; and K11, apo(a)-plasminogen dissociation constant.

Table I
Parameter values: equilibrium template model
Data from Figs. 4 and 5 (performed in the presence of fibrin and FDPs, respectively) were fit globally to Equation 24 by non-linear regression. km is the turnover number for the ternary catalytic complex in the absence of apo(a); kcat is the turnover number for the quaternary catalytic complex in the presence of apo(a); Kd and Km are the dissociation constants for binding of tPA to the cofactor in the absence or presence of apo(a), respectively; Kf is the Michaelis constant for the formation of the ternary catalytic complex; KdA is the Michaelis constant for the formation of the quaternary catalytic complex; and K11 is the dissociation constant for the solution-phase apo(a)-tPA interaction. Results are presented as best fit values ± the asymptotic standard error returned by the regression algorithm.

| Kinetic constant | Value |
|------------------|-------|
| kcat (s⁻¹)       | 0.1211 ± 0.0083 0.04558 ± 0.00356 |
| Km (μM)          | 0.05454 ± 0.01081 1.940 ± 0.3283 |
| Kd (μM)          | 1.352 ± 0.173 0.03405 ± 0.00583 |
| kcat (s⁻¹)       | 0.05028 ± 0.00193 0.03801 ± 0.00266 |
| Km1 (μM)         | 0.05867 ± 0.01677 0.6153 ± 0.2814 |
| Km2 (μM)         | 0.00129 ± 0.000124 0.001141 ± 0.000422 |
| K10 (μM)         | 3.651 ± 1.099 4.553 ± 0.742 |

its major plasma inhibitor α2-antiplasmin (41). Activated thrombin-activated fibrinolysis inhibitor is a recently described enzyme that removes the carboxyl-terminal lysines from partially degraded fibrin and thus attenuates fibrinolysis through suppression of positive feedback in the fibrinolytic cascade (reviewed in Ref. 42). It has been shown that partial plasmin digestion of fibrin increases the capacity of fibrin for Lp(a) and plasminogen, but not its affinity for these molecules (13). Thus, we would predict that the presence of activated thrombin-activable fibrinolysis inhibitor in our system would decrease the amount of both apo(a) and plasminogen bound to fibrin. Further experimentation will be required to determine the impact of the activated thrombin-activable fibrinolysis inhibitor pathway on inhibition of plasminogen activation by apo(a).

Our data do not indicate that there is direct competition of plasminogen and apo(a) for binding to fibrin or FDPs. This is in keeping with the results of Sangrar and co-workers (21), who found that plasminogen and apo(a) bind to distinct sites on partially degraded fibrinogen and proposed that the solution-phase interaction between plasminogen and apo(a) results in a complex that binds less avidly to plasminogen binding sites. Our data also indicate that apo(a) would not compete with plasmin for fibrin binding and thus would not potentiate plasmin consumption by α2-antiplasmin. However, we cannot rule out that the presumptive solution-phase interaction between apo(a) and plasmin might influence α2-antiplasmin interaction with plasmin.

It was found that the apo(a) domains required for inhibition of plasminogen activation with fibrin were identical to those required for inhibition with FDPs. We discovered that the lysine-binding KIV10 domain and an intact KV domain were both required for maximal inhibition. Decreased inhibition upon mutation (17K(D56A)) or deletion (17K(−)) of the respective domains supports the notion that the strong lysine-binding site in apo(a) KIV10 alone cannot account for the interaction of Lp(a) with fibrinogen; this is in keeping with reports by Edelstein and co-workers (19, 43) that provide evidence for a fibrinogen-binding domain of apo(a) that is outside the lysine-binding site in KIV10. A variant lacking the protease domain (17K(−)) was also a potent inhibitor-like wild type 17K indicating that the high affinity, solution-phase, apo(a)-plasminogen binding interaction mediated by this domain (21) is not ultimately responsible for inhibition of plasminogen activation in the presence of fibrin or FDPs. Our model, however, does take this solution-phase interaction into account as a determinant of the concentration of free plasminogen.

Interestingly, KV is a kringle domain that is missing in apo(a) from some species including rhesus monkey (44) and baboon. The kringle V-protease domains have also been recently identified as a fibrinogen-binding region within apo(a) because a KV-P fusion construct was shown to compete for the binding of 125I-labeled Lp(a) to plasmin-modified fibrinogen (45). These studies, along with our identification of the critical role for KV in inhibition of plasminogen activation, suggest...
that rhesus monkey and baboon may possess a less pathogenic, less antifibrinolytic form of Lp(a) in the absence of the KV domain. Indeed, rhesus monkeys normally exhibit almost no atherosclerosis unless challenged with a high fat and high cholesterol diet (46, 47), and such animal studies have detected apo(a) in atherosclerotic plaques but without co-localization with fibrinogen (48, 49).

Because 17K, 12K, and 10K r-apo(a) all inhibit plasminogen activation to similar extents in our study, the number of KIV_{2} repeats present (i.e. apo(a) isoform size heterogeneity) did not appear to be critical to the inhibition process. Because smaller apo(a) isoforms have been shown to have a greater inhibitory effect on plasin generation (50, 51), we speculated that 10K r-apo(a) might be a more potent inhibitor than either 12K or 17K r-apo(a). Although this was not observed in our study, it should be noted that all of the r-apo(a) variants that we examined correspond to small apo(a) isoforms (M_{r} < 580,000) in the study of Falco and co-workers (50). In addition, it has been demonstrated that the isoform size dependence of fibrin binding is a property of Lp(a) but not apo(a) (52).

Despite the presence of intact KIV_{10} and KV domains, deletion of the amino-terminal apo(a) KIV_{1} domains (especially KIV_{2} and KIV_{3}) resulted in dramatically decreased inhibition with either the fibrin or FDP cofactors (compare 17K to 6K r-apo(a); Fig. 3). Whereas no functional lysine-binding sites are predicted to exist in the KIV_{1} domains (53), other non-lysine-dependent interactions involving these domains may help stabilize or orient apo(a) at the site of a fibrin clot to elicit inhibition of plasminogen activation through the KIV_{10} and KV domains. Likewise, the present study has shown that the high affinity, solution-phase, apo(a)-plasminogen binding interaction (mediated by the apo(a) protease domain and originally hypothesized to result in decreased ternary complex formation and decreased plasmin generation (21)) is not sufficient to inhibit plasminogen activation alone. Therefore, apo(a)-mediated inhibition of plasminogen activation in the presence of fibrin/FDPs is likely a complex function of contributions from several apo(a) structural domains. Although our kinetic model does not specifically identify the components in the quaternary catalytic complex that are involved in direct binding interactions with apo(a), it is conceivable that the requirement for multiple domains in apo(a) for inhibition reflects the interaction of apo(a) with multiple components in the quaternary complex.

Analysis according to Michaelis-Menten kinetics failed to provide good global fits for the experiments in which the concentrations of plasminogen, fibrin/FDPs, and 12K r-apo(a) were systematically varied (Figs. 4 and 5). This is probably because of the large number of components and interactions involved in plasminogen activation and its inhibition. The traditional Michaelis-Menten model does not strictly apply to a three-component template mechanism. Accordingly, earlier attempts to describe apo(a)/Lp(a)-mediated inhibition of plasminogen activation by Michaelis-Menten kinetics may account for the apparent variable modes of inhibition (i.e. competitive, uncompetitive) reported in the literature to date (18, 23, 24). However, the uncompetitive inhibition kinetics described previously (18, 24) are compatible with the interaction of apo(a) with the catalytic complex that our equilibrium template model implies.

Because plasminogen activation can be adequately described by a template mechanism, the data were analyzed according to the model of Horrevoets and co-workers (26). In this model (Fig. 6), activator (A) and substrate (P) can bind to the cofactor surface (F) in either order; subsequent ternary complex formation (FAP) leads to efficient plasmin generation. Accordingly, the template model was revised in the present work to allow apo(a) to interact with all three fibrinolytic components simultaneously. Plasminogen (P) and tPA (A) are still envisaged to bind to the apo(a)-bound cofactor surface (IF) in either order; subsequent quaternary complex formation (IFAP) still results in plasmin generation.

We developed a rate equation (Equation 24) to describe the revised, equilibrium template model. Non-linear regression of the data to this equation resulted in very good global fits for both the fibrin and FDP data (Figs. 4 and 5). Interestingly, Michaelis constants (K_{m}) for the apo(a)-bound quaternary (IFAP) complexes were significantly lower than those for the corresponding ternary (FAP) complexes lacking apo(a) with the fibrin and FDP cofactors. These very low values are likely because of the very high affinity interaction of apo(a) with plasminogen and the fact that K_{m} reflects the free rather than the total concentration of plasminogen. On the other hand, turnover numbers (k_{cat}) for the IFAP complexes were lower than those for the FAP complexes on the fibrin and FDP surfaces. Essentially, more stable but less catalytically robust catalytic complexes are formed in the presence of apo(a). Despite the nominal increase in catalytic efficiency of apo(a)-containing catalytic complexes, inhibition of plasminogen activation in the presence of apo(a) is observed at physiological concentrations of fibrinogen because of the lower k_{cat} value. Conversely, the apparent stimulation of plasminogen activation by apo(a) at low fibrinogen concentrations would be explained by the lower K_{m} for IFAP complexes than FAP complexes. Interestingly, one study reported that Lp(a) increased the rate of plasma clot lysis (54); however, in this work the plasma was significantly diluted (to 13.3% of the final volume) which, in the context of our findings of enhanced plasminogen activation at low fibrin concentrations (Fig. 4B), might account for these apparently paradoxical results. Dissociation constants for the binding of tPA to fibrin or FDPs remained the same in the presence (K_{A}) or absence (K_{A}^*) of apo(a), indicating that tPA binding was unaltered by the presence of apo(a) bound to the cofactor. This might imply that there is no direct interaction between apo(a) and tPA in the quaternary complex. Finally, relatively large dissociation constants were predicted by the model for the apo(a)-tPA interaction (K_{Ap}) in solution, indicative of a relatively weak interaction between apo(a) and tPA in these settings.

In summary, we have defined, for the first time, the structural domains in apo(a) that mediate its ability to inhibit plasminogen activation. Furthermore, our studies are the first to investigate the mechanism of apo(a) inhibition of plasminogen activation in the presence of native, intact fibrin and soluble FDPs derived from plasmin digestion of fibrin. We have developed an equilibrium template kinetic model that, unlike models based on Michaelis-Menten kinetics, holds at all concentrations of substrate, cofactor, and inhibitor concentrations tested. Our results imply a common mechanism for apo(a) inhibition of plasminogen activation in the presence of native fibrin or degraded fibrin (FDPs) cofactors. In addition, our findings demonstrate that the ability of apo(a) to inhibit plasminogen activation is a complex function of the contribution of numerous apo(a) structural domains. Indeed, our kinetic analysis implies that interactions between apo(a) and potentially each of plasminogen, fibrin/FDPs, and tPA determine the functional outcome.

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