Kinetic Analysis of the Effects of Heparin and Lipoproteins on Tissue Plasminogen Activator Mediated Plasminogen Activation†

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ABSTRACT: Heparin sulfate and the less sulfated glycosaminoglycan heparan sulfate enhance human plasminogen (Pg) conversion to plasmin by tissue-type plasminogen activator (t-PA). Kinetic studies indicate that both heparin and heparan increase the k_{cat} of t-PA-mediated Pg activation by 25- and 3.5-fold, respectively. The K_m of plasmin formation is unaltered by the presence of either heparin or heparan. Both heparin and heparan stimulate the activity of t-PA by interacting with the finger domain of t-PA, with association constants of 1 μM and 200 nM, respectively. Additionally, the lipoproteins lipoprotein(a) [Lp(a)] and low-density lipoprotein (LDL) inhibit the heparin enhancement of Pg activation. Lp(a) is a competitive inhibitor and LDL is a mixed inhibitor of t-PA-mediated Pg activation, with inhibition constants of 30 and 70 nM, respectively. The inhibition constants correspond to physiologic concentrations of these lipoproteins. These data suggest that heparin, heparan, and lipoproteins may play an important in vivo role in regulating cell surface associated activation of the fibrinolytic system.

Plasminogen (Pg) is a zymogen which when activated forms plasmin, the enzyme which degrades fibrin clots. Several proteins can mediate this activation, notably tissue-type plasminogen activator (t-PA), urokinase, and streptokinase (Collen, 1980; Castellino, 1981). The rate of plasmin formation catalyzed by t-PA alone is extremely slow (Hoyalerts et al., 1982) but increases manyfold in the presence of fibrinogen, CNBr fibrinogen fragments, or fibrin (Hoyalerts et al., 1982; Ranby, 1982; Nieuwenhuizen et al., 1983; de Serrano et al., 1989). The fibrinolytic system appears to be regulated by assembly on the surfaces of endothelial cells and macrophages (Hajjar et al., 1986; Miles & Plow, 1986). Additionally, several other components can regulate the rate of t-PA-mediated Pg activation including extracellular matrix (Silverstein et al., 1985), chloride (Urano et al., 1987), α_2-antiplasmin (Lijnen & Collen, 1986), Pg activator inhibitor 1 (Sprengers & Kluft, 1987), lipoprotein(a) [Lp(a)] (Edelberg et al., 1990), and heparin sulfate (Markwardt & Klocking, 1977).

Heparin sulfate is a glycosaminoglycan (GAG) well-known for its important pharmacologic anticoagulant properties. Heparin enhances the rate of antithrombin III inhibition of thrombin and factors IXa, Xa, XIa, and XIIa and thereby regulates the coagulation cascade [for a brief review, see Rosenberg et al. (1986)]. Heparin is found on the vascular surface and basement membrane where it exerts its anticoagulant effect (Marcum & Rosenberg, 1984, 1985; Marcum et al., 1986). On the basis of ex vivo studies, Markwardt and Klocking (1977) proposed that heparin increases fibrinolytic activity. In vitro studies have suggested that heparin sulfate may enhance t-PA-mediated plasmin formation (Andrade-Gordon & Strickland, 1986; Paques et al., 1986). This suggests that endothelial cell surface associated heparin plays a role in the regulation of fibrinolysis, in addition to inhibition of coagulation.

This study describes an analysis of the effect of heparin, and the less sulfated, related GAG heparan, on the activation of Pg by t-PA. We kinetically model the interaction between t-PA and both heparin and heparan sulfate. Additionally, we have studied the effect of Lp(a) and the related low-density lipoprotein (LDL) on heparin enhancement of t-PA-mediated plasmin formation.

Materials and Methods

Reagents. The plasmin substrate H-D-Val-L-Lys *p*-nitroanilide dihydrochloride (VLK·pNA or S-2251) and the t-PA substrate H-D-Ile-L-Pro-L-Arg *p*-nitroanilide dihydrochloride (IPR·pNA or S-2288) were purchased from Helena Laboratories, Beaumont, TX. All other reagents were of the best grade commercially available.

Glycosaminoglycans. All GAGs were obtained from Sigma Chemical Co., St. Louis, MO. Heparin sulfate (155 IU/mL) had a molecular weight of 17K. Heparan sulfate, molecular weight 50K, had 10% sulfation w/w. Chondroitins A and C had molecular weights of 17K and 32K, respectively. Molecular weight and sulfation data for these preparations were obtained from Sigma Technical Services.

Proteins. Human Pg isoform 2 was isolated by affinity chromatography as previously described by Deutsch and Mertz (1970) and modified by Brockway and Castellino (1972) and Gonzalez-Gronow and Robbins (1984). t-PA was the generous gift of Dr. Henry Burger, Wellcome Research Laboratories, Research Park, NC. Lp(a) was purified as previously described by Edelberg et al. (1989). LDL was obtained from Sigma Chemical Co., and purity was confirmed by SDS-PAGE, as previously described (Edelberg et al., 1990).

Determination of Heparin and Heparan Kinetic Activation Constants. Pg was activated with t-PA as previously described (Edelberg et al., 1990). Briefly, various concentrations of Pg were incubated *in a buffer of 50 mM Tris-HCl, 0.05% gelatin, and 0.01% Tween 80, pH 7.4*, with 35 IU/mL t-PA, in the presence of the plasmin substrate VLK·pNA (300 μM) and increasing concentrations of either heparin, heparan, or chondroitin A or C. The plasminogen activation velocity was determined by the following equation: $V_i = b(1 + K_E/[S]_0)/\epsilon k_e$, where b is the initial velocity of substrate hydrolysis derived from a plot of the instantaneous rate of substrate cleavage versus time, K_E is the apparent Michaelis constant of VLK·pNA hydrolysis by plasmin (0.3 mM), k_e is the

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catalytic rate constant for plasmin hydrolysis of VLK·pNA determined for fully activated plasminogen 2 [2.3×10^3 M (mol of plasmin)$^{-1}$ s^{-1}], and ϵ is the molar extinction coefficient of the hydrolyzed substrate at A_{405nm} (10^4 M^{-1} cm^{-1}) (Rajagopalan et al., 1985). The kinetic constants for Pg activation were determined by a double-reciprocal plot of initial rates versus Pg concentrations in the absence of any GAGs. In the presence of GAGs, the kinetic activation constants were determined by using the equation $V = V_m([S]/K_m + \beta[A][S]/\alpha K_m K_a)/\{1 + [S]/K_m + [A]/\alpha K_m + [S][A]/\alpha K_m K_a\}$. α and β are the activation constants which modify K_m and k_{cat}, respectively, and K_a is the association constant of the activator for the enzyme (Dixon & Webb, 1979). From a double-reciprocal plot of initial rates of plasmin formation versus Pg concentrations in the presence of increasing concentrations of GAGs, the change (Δ) from control in both abscissa intercepts and slopes was determined for each concentration of GAG. The constants α, β, and K_a were calculated from a double-reciprocal plot of Δ intercept and slope values versus the GAG concentration.

Determination of Lipoprotein(a) and Low-Density Lipoprotein Kinetic Inhibition Constants. Pg was activated in a solution containing 100 nM heparin sulfate as described above, in the presence of increasing concentrations of Lp(a) or LDL. The initial rate of plasmin formation was determined as described above. The inhibition constants were determined by using the equation $1/V = (K_m + [S])/V_m[S] + \{(K_m/K_{ic}) + ([S]/K_{iu})\}[I]/V_m[S]$ in which K_{ic} and K_{iu} represent the competitive and uncompetitive inhibition constants, respectively (Dixon, 1954; Knight, 1986).

Determination of Lipoprotein(a) Inhibition in the Absence of Heparin. Pg (200 nM) was activated as described above, in the presence of increasing concentrations of Lp(a), but with no heparin. The initial rate of plasmin formation was plotted versus Lp(a) concentration to determine the Lp(a) inhibition of Pg activation in the absence of heparin.

Determination of Tissue Plasminogen Activator Activity in the Presence of Heparin and Lipoprotein(a). t-PA activity was determined as described previously (Edelberg et al., 1990). Briefly, t-PA (35 IU/mL) was incubated with IPR·pNA (300 μM) in the presence of 100 nM heparin and increasing concentrations of Lp(a). The initial rate of substrate hydrolysis was plotted versus Lp(a) concentration to determine the effect of Lp(a) on t-PA amidolytic activity. t-PA was also incubated with increasing concentrations of heparin in the absence of Lp(a).

Results

Determination of Heparin and Heparan Kinetic Activation Constants. Activation of Pg by t-PA in the presence of various GAGs resulted in an enhancement of Pg activation (Figures 1 and 2). The addition of heparin sulfate to the reaction (Figure 1A) increased Pg activation by 25-fold. The replot of the data (Figure 1B) indicates that the K_a of the heparin–enzyme complex is 1 μM. Heparan sulfate was less effective as a stimulator of Pg activation. Figure 2A demonstrates that the addition of heparan sulfate resulted in only a 3.5-fold enhancement relative to that of the control. The replot of the heparan activation (Figure 2B) shows that the K_a is 200 nM. Similar experiments with chondroitins A and C demonstrated no significant enhancement of Pg activation (data not shown).

Determination of Lipoprotein(a) and Low-Density Lipoprotein Kinetic Inhibition Constants. Pg was activated by t-PA in the presence of 100 nM heparin and increasing concentrations of Lp(a) or LDL, as described under Materials and Methods. The addition of either lipoprotein inhibited the Pg activation rate (Figures 3 and 4). Figure 3A demonstrates that increasing concentrations of Lp(a) inhibited plasmin formation. The replot of the data (Figure 3B) reveals that Lp(a) is a competitive inhibitor with a K_{ic} of 30 nM. Figure 4A demonstrates that increasing concentrations of LDL also decreased the rate of Pg activation. The replot (Figure 4B) shows that LDL inhibition is noncompetitive with constants, K_{ic} and K_{iu}, both equal to 70 nM.

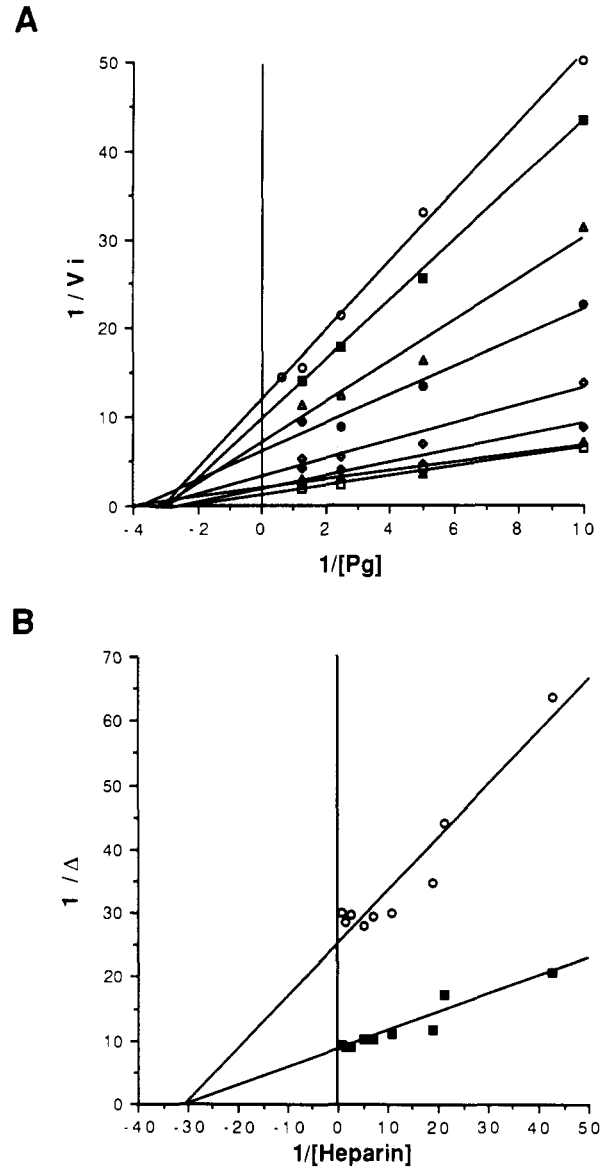


FIGURE 1: Kinetic analysis of the effect of heparin sulfate on plasmin formation. (Panel A) Double-reciprocal plot of plasmin formation (10^{-12} s/mol) versus Pg concentration (μM^{-1}). Increasing concentrations of Pg, 100–400 nM, were activated with 35 IU/mL t-PA with fixed heparin sulfate concentrations, 0 (○), 12 (■), 24 (△), 47 (●), 53 (◇), 94 (◆), 550 (▲), and 1600 nM (□), in a buffer of 50 mM Tris-HCl, pH 7.4, 0.05% gelatin, 0.1% Tween 80, and 300 μM VLK-pNA. Plasmin formation was calculated as described previously under Materials and Methods. (Panel B) Double-reciprocal plot of the change (Δ) from control in both vertical axis intercept (10^{13} s/mol of plasmin) (■) and slope [5×10^6 s (mol of plasmin)$^{-1}$ [Pg]$^{-1}$] (○) versus heparin sulfate concentration (μM^{-1}). The activation constants were calculated as described under Materials and Methods.

Determination of the Lipoprotein(a) Mechanism of Inhibition. The mechanism of Lp(a) inhibition of Pg activation was examined by kinetic studies with heparin absent and synthetic substrates present. In the first study, Pg was activated by t-PA in the presence of increasing concentrations of Lp(a), but with no stimulatory GAG. Lp(a) does not inhibit the formation

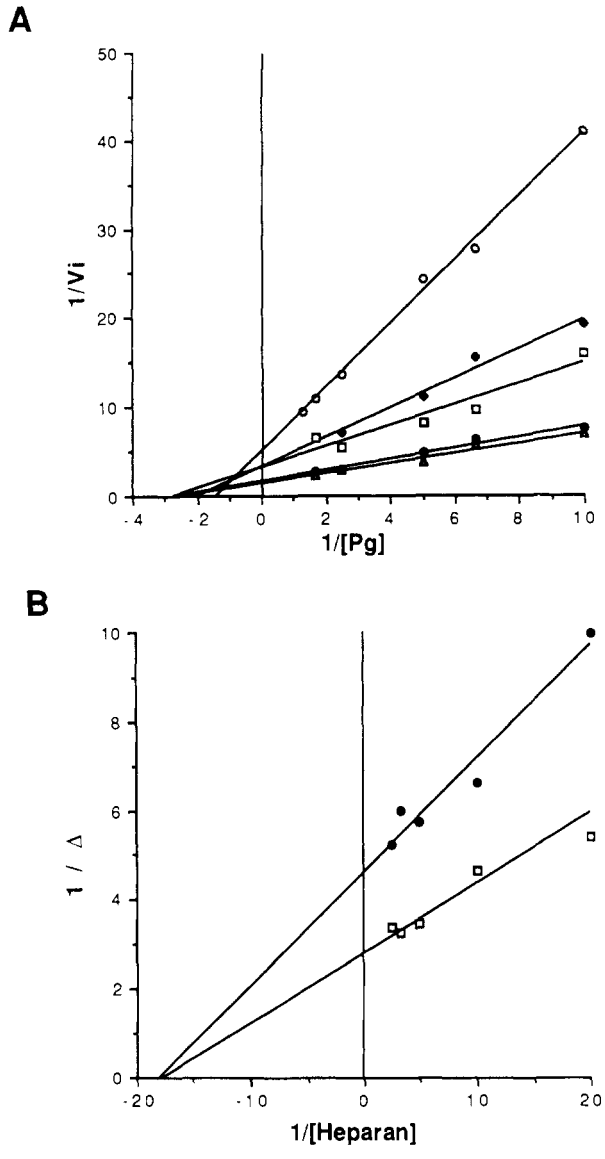


FIGURE 2: Kinetic analysis of the effect of heparan sulfate on plasmin formation. (Panel A) Double-reciprocal plot of plasmin formation (10^{-12} s/mol) versus Pg concentration (μM^{-1}). Increasing concentrations of Pg, 100–400 nM, were activated with 35 IU/mL t-PA with fixed heparan sulfate concentrations, 0 (○), 50 (♦), 100 (□), 200 (●), and 400 nM (△), in a buffer of 50mM Tris-HCl, pH 7.4, 0.05% gelatin, 0.1% Tween 80, and 300 μM VLK·pNA. Plasmin formation was calculated as described previously under Materials and Methods. (Panel B) Double-reciprocal plot of the change (Δ) from control in both vertical axis intercept (10^{13} s/mol of plasmin) (□) and slope [10^{7} s (mol of plasmin)$^{-1}$ [Pg]$^{-1}$] (●) versus heparan sulfate concentration (μM^{-1}). The activation constants were calculated as described under Materials and Methods.

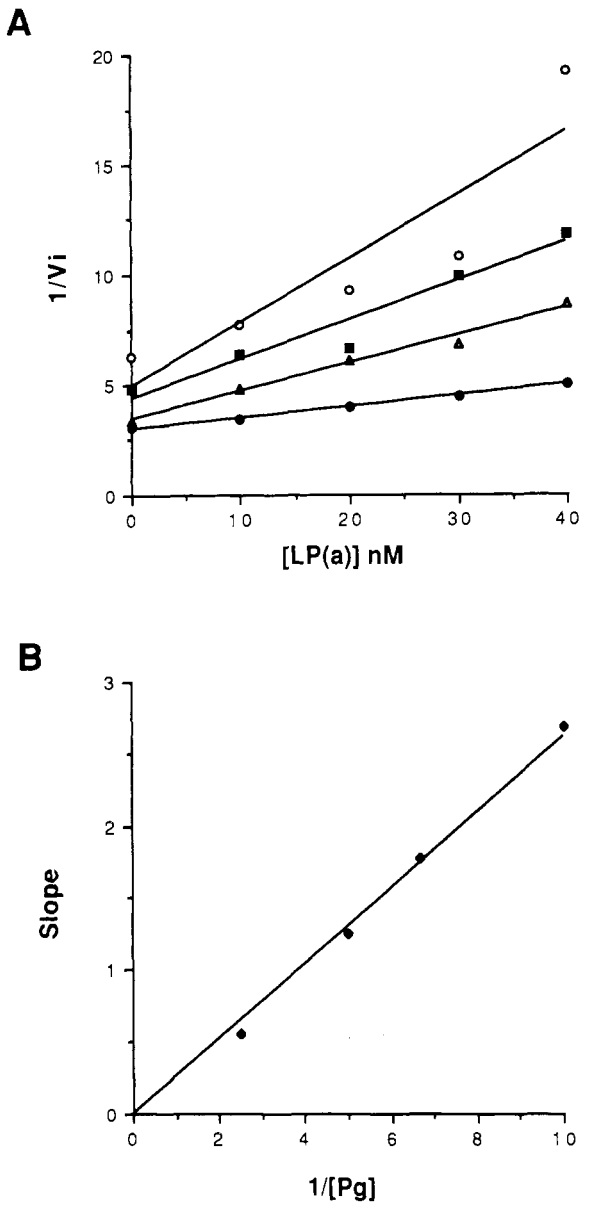


FIGURE 3: Kinetic analysis of the effect of Lp(a) on heparin sulfate enhanced plasmin formation. (Panel A) Dixon plot of the reciprocal velocity of plasmin formation (10^{-11} s/mol of plasmin) versus Lp(a) concentration (nM). Fixed concentrations of Pg, 100 (○), 150 (■), 200 (△), and 400 nM (●), were activated with 35 IU/mL t-PA with 100 nM heparin sulfate in the presence of Lp(a), 0-40 nM, in a buffer of 50 mM Tris-HCl, pH 7.4, 0.05% gelatin, 0.1% Tween 80, and 300 μM VLK·pNA. The velocity was determined as previously described under Materials and Methods. (Panel B) Plot of Dixon slopes [10^{-18} s (mol of plasmin)$^{-1}$ [Lp(a)]$^{-1}$] versus inverse Pg concentration (μM^{-1}). The inhibition constant, K_{ic}, was determined by using the Dixon equation described under Materials and Methods.

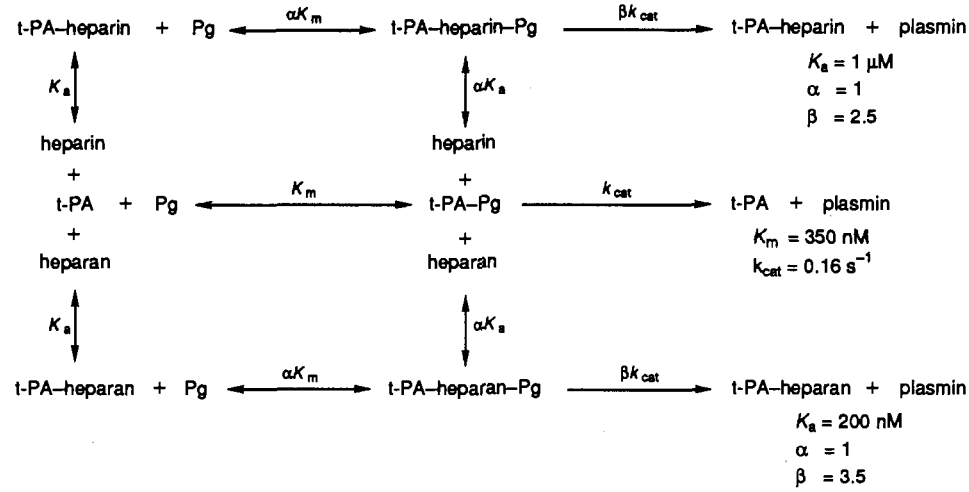
of plasmin in the absence of heparin. Moreover, the effect of Lp(a) on the catlytic activity of t-PA in the presence of heparin was also determined. The Lp(a) does not inhibit the t-PA cleavage rate of IPR·pNA. In addition, the t-PA amidolytic activity was independent of the concentration of heparin present. This result is not surprising in that hydrolysis of a peptide bond in a macromolecular substrate requires a multipoint cooperative process; therefore, the hydrolysis of synthetic tripeptides may not be regulated in a manner similar to the natural substrates (Huseby & Smith, 1980).

Discussion

Heparin and heparan sulfate are important anticoagulant agents. Experiments by Rosenberg and Damus (1973) established that heparin pharmacologically promotes the inhibition of thrombin by antithrombin III. The physiologic basis for regulation of thrombin by both heparin and heparan sulfate, free and conjugated to proteoglycans, based on the fact that they are integral components of the vascular surface and basement membrane (Marcum & Rosenberg, 1984, 1985; Marcum et al., 1986). In addition to their anticoagulant activities, Markwardt and Klocking (1977) proposed a potential role for these GAGs in the promotion of fibrinolysis.

Our results agree with the studies of Andrade-Gordon and Strickland (1986) and Paques et al. (1986) demonstrating that heparin increased Pg activation by t-PA. The previously published reports did not determine the kinetic mechanism of heparin enhancement of t-PA-mediated Pg activation. Studies described here present experiments that quantitate the in-

Scheme I

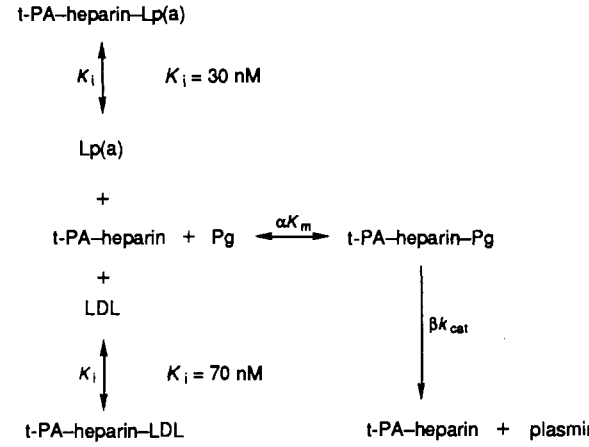


teraction between t-PA and both heparin and the less sulfated GAGs, heparan and chondrointins A and C. Kinetic activation studies show that heparin sulfate stimulation is primarily due to a 25-fold increase in the k_{cat} of t-PA with no affect on the K_m of Pg activation. Heparin has a K_a for t-PA of 1 μM, which agrees well with the heparin–agarose binding data of Andrade-Gordon and Strickland (1986). In addition, Andrade-Gordon and Strickland (1986) studied the interrelationship between heparin and fibrinogen fragments in the enhancement of t-PA activity. Fibrinogen fragments are known stimulants of t-PA-mediated Pg activation (Nieuwenhuizen et al., 1983; de Serrano et al., 1989). Andrade-Gordon and Strickland (1986) demonstrated that the fibrinogen fragment enhancement of t-PA is blocked by heparin sulfate in a dose-dependent manner. At the higher concentrations of heparin, stimulation of t-PA is due exclusively to heparin, with no kinetic enhancement by the fibrinogen fragments. Heparan has much less of an effect on the k_{cat} for the activation of Pg by t-PA (3.5-fold increase), but like heparin has no affect on the K_m of Pg activation. The heparan association constant, 200 nM, is also lower than for heparin, indicating that while the stimulation of t-PA is lower the binding interaction is stronger. Neither heparin nor heparan affects the K_m of Pg activation. The chondroitins did not enhance the rate of t-PA-mediated Pg activation. Scheme I summarizes the kinetic model of t-PA activity enhancement by both heparin and heparan sulfate.

Stern et al. (1989) studied the effect of heparin on the activity of t-PA mutants in which either the finger domain or the kringle 2 domain was deleted. Their data suggested that the t-PA mutant containing the finger domain was 3- to 5-fold more heparin-responsive than either the mutant containing the kringle 2 domain or the native protein. Wojta et al. (1989) employed monoclonal antibodies to probe the catalytic properties of t-PA. Their data suggested that the finger domain was primarily responsible for increases in k_{cat}, while the kringle 2 domain was primarily responsible for decreases in the K_m of Pg activation. Our kinetic studies demonstrate that both heparin and heparan increase Pg activation by increasing the k_{cat} but do not decrease the K_m. Our results, taken with the work of Wojta, et al. (1989), indicate that both heparin and heparan enhance t-PA activity through interaction with the t-PA finger domain, but not the kringle 2 domain.

The enhancement of fibrinolytic activity by heparin and heparan sulfates could be an important aspect in their pharmacologic activity in the prevention of fibrin clot formation. These GAGs may also stimulate fibrinolysis under physiologic conditions since endothelial cells produce both heparin and heparan sulfates (Shimada et al., 1981; Gill et al., 1986; Marcum et al., 1986). The presence of these GAGs on the vascular surface and in basement membranes could regulate the formation of plasmin.

Scheme II



Stimulation of Pg activation by heparin and heparan may be significant in other physiologic and pathophysiologic processes. For example, production of heparin by embryonic fibroblasts (Woods et al., 1985) may regulate plasmin formation which is critical in tissue remodeling (Strickland et al., 1976). Additionally, tumor cells produce both heparin and heparan (Hassell et al., 1985; Furwara et al., 1984) which may stimulate the activation of Pg, and therefore may contribute to the ability of the tumor to metastasize (Reich et al., 1988).

The heparin sulfate enhancement of plasmin formation may be inhibited by lipoproteins. Both Lp(a) and LDL are inhibitors of Pg activation by heparin-stimulated t-PA. Lp(a) acts as a competitive inhibitor of Pg for the heparin-enhanced t-PA-catalyzed plasmin formation as it does in fibrin-stimulated t-PA-mediated Pg activation (Edelberg et al., 1990). Lp(a) inhibition is heparin dependent, since unstimulated t-PA-mediated Pg activation is unaltered. Additionally, as in fibrin-stimulated activation, Lp(a) does not occupy the t-PA active site, which remains able to cleave small substrates. Unlike Lp(a), LDL acts as a mixed inhibitor ($K_{ic} = K_{iu}$) with inhibition constants over twice that of Lp(a). Scheme II depicts the kinetic model of the heparin-enhanced t-PA inhibition by Lp(a) and LDL.

The inhibitory mechanism of the lipoproteins appears to be structurally related. The heparin sulfate binding of both li-

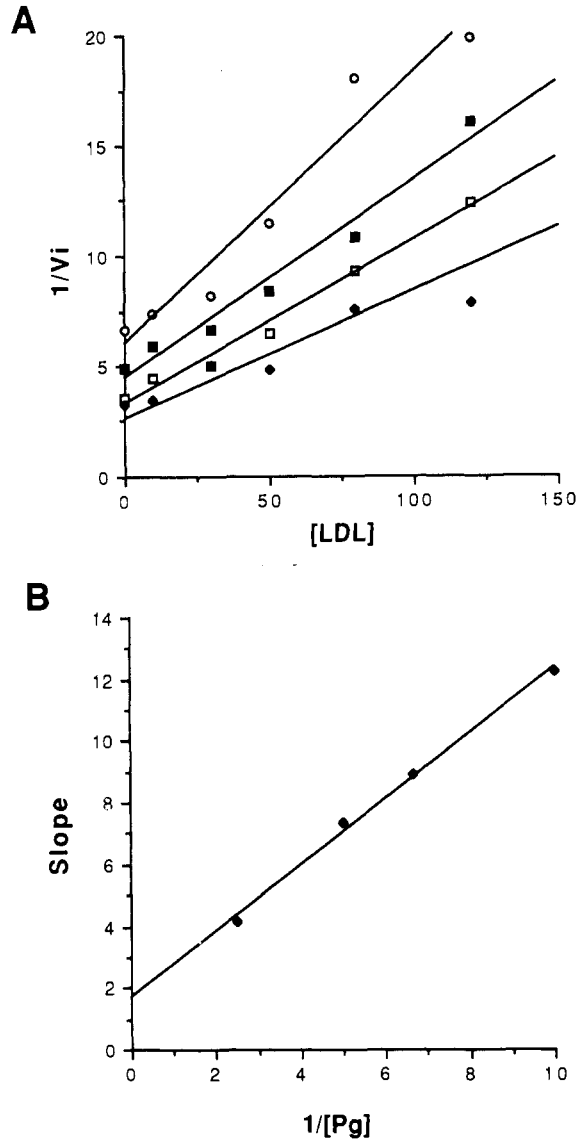


FIGURE 4: Kinetic analysis of the effect of LDL on heparin sulfate enhanced plasmin formation. (Panel A) Dixon plot of the reciprocal velocity of plasmin formation (10^{-11} s/mol of plasmin) versus LDL concentration (nM). Fixed concentrations of Pg, 100 (○), 150 (■), 200 (□), and 400 nM (♦), activated with 35 IU/mL t-PA with 100 nM heparin sulfate in the presence of LDL, 0–120 nM, in a buffer of 50 mM Tris-HCl, pH 7.4, 0.05% gelatin, 0.1% Tween 80, and 300 μM VLK-pNA. The velocity was determined as previously described under Materials and Methods. (Panel B) Plot of Dixon slopes [10^{-18} s (mol of plasmin)$^{-1}$ [LDL]$^{-1}$] versus inverse Pg concentration (μM^{-1}). The inhibition constants, K_{ic} and K_{iu}, were determined by using the Dixon equation as described under Materials and Methods.

poproteins has been well-studied (Berenson et al., 1971; Inverius, 1972; Nakashima et al., 1975), and both bind in a capacity that inhibits Pg activation by heparin-stimulated t-PA. Lp(a), which has a Pg-like apoprotein (a) subunit (Eaton et al., 1987; McLean et al., 1987), competitively inhibits by blocking the Pg access to the t-PA active site but does not occupy the active site itself (Edelberg et al., 1990). With Lp(a), the K_m of t-PA-mediated Pg activation increases but k_{cat} remains unchanged, similar to Lp(a) inhibition of fibrin-stimulated t-PA activation of Pg (Edelberg et al., 1990). The competitive inhibition component of Lp(a) may result from the homology of the apoprotein (a) with Pg. LDL, which lacks the apoprotein (a) subunit, acts as a mixed inhibitor of heparin-enhanced Pg activation by both increasing the K_m and decreasing the k_{cat}. LDL, like Lp(a), also inhibits by blocking Pg access to the t-PA active site. Additionally, LDL can negate the heparin enhancement by decreasing the stimulation of t-PA activity.

The inhibition constants of both Lp(a) and LDL correspond to physiologic concentrations, 12 and 20 mg/dL, respectively. This indicates that both lipoproteins may regulate heparin- and heparan-mediated fibrinolysis. Elevations in lipoprotein concentrations could negate the heparin and heparan enhancement of t-PA-catalyzed Pg activation, and inhibit normal fibrinolysis.

In summary, heparin, heparan, Lp(a), and LDL may all regulate the activation of Pg under physiological and pathophysiological conditions.

Registry No. Heparin, 9005-49-6; plasminogen activator, 105913-11-9; heparin sulfate, 9050-30-0; plasminogen, 9001-91-6; plasmin, 9001-90-5.

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Internal Transcribed Spacer 1 of the Yeast Precursor Ribosomal RNA. Higher Order Structure and Common Structural Motifs†

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ABSTRACT: The higher order structure of the first internal transcribed spacer between the 18S and the 5.8S rRNA sequences in the *Saccharomyces cerevisiae* precursor ribosomal RNA has been investigated. Sites of potential base pairing in the RNA region have been determined by using a combination of enzymatic and chemical structure sensitive probes. Data generated have been used to evaluate secondary structure models predicted by minimum free energy calculations. Several alternative suboptimal structures were also evaluated. The derived model contains several stable hairpins. Theoretical secondary structural models for the corresponding RNA region from *S. carlsbergensis*, *S. pombe*, *N. crassa*, *X. laevis*, and mung bean have also been derived from identical calculations and assumptions. Certain structural motifs appear to be conserved despite extensive divergence in the base sequence. The yeast model should be a useful prototype for investigation of structure and function of precursor ribosomal RNA molecules.

In *Saccharomyces cerevisiae*, the primary rRNA transcription product is a 35S RNA molecule that is processed into three mature rRNA molecules (5.8S, 18S, and 25S). These mature rRNA sequences in the 35S pre-rRNA[1] are separated by spacer sequences. These internal spacer sequences constitute about 10% of the total RNA in length. The necessary structures for the recognition of the proper cleavage sites and the role of the spacer sequences in the regulation of ribosome biogenesis are not clear. Solely on the basis of the terminal nucleotide sequence information of the processing intermediates, Veldman et al. (1981) postulated that small hairpin structures located near the processing sites may be involved in the maturation of the pre-rRNA and that all the sites are brought into one region through long-range RNA-RNA interactions. In an attempt to examine the structural features of the pre-rRNA, we have recently reported the successful cloning of the yeast rRNA gene in an expression vector and production of the 35S pre-rRNA molecule in vitro. Preliminary chemical modification studies with kethoxal and dimethyl sulfate on the internal transcribed spacer 1 (ITS-1) between the 18S and 5.8S rRNA sequences within the pre-rRNA molecule have also been reported (Thweatt & Lee, 1990).

The goal of the present study is to investigate in detail the solution structure of the ITS-1 sequence within the pre-rRNA molecule with a combination of chemical and enzymatic structural probes. By combining the structure probing experimental data and computer theoretical secondary structure modeling, we have constructed a refined secondary structure model. Several suboptimal structures have also been evaluated. They do not agree as well with the experimental data. In

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[1] Abbreviations: DMS, dimethyl sulfate; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; EDTA, ethylenediaminetetraacetic acid; pre-rRNA, precursor ribosomal RNA; ITS, internal transcribed spacer; LETS, left external transcribed spacer; ddATP, ddCTP, and ddTTP, dideoxyadenosine, dideoxycytosine, and dideoxythymidine triphosphates, respectively.

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