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Bovine Plasminogen Activator Inhibitor 1: Specificity Determinations and Comparison of the Active, Latent, and Guanidine-Activated Forms†

Carla M. Hekman‡,§ and David J. Loskutoff*,‡

Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037, and Department of Chemistry, University of California, San Diego, La Jolla, California 92093

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ABSTRACT: The plasminogen activator inhibitor 1 (PAI-1) synthesized and released by cultured bovine aortic endothelial cells is present in conditioned medium in a latent form that can be activated by guanidine hydrochloride [Hekman, C. M., & Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581–11587]. The purified, guanidine-activated PAI-1 was shown to inhibit both plasmin and trypsin in a dose- and time-dependent manner. Second-order rate constants for these interactions were calculated to be 6.6×10^5 and 7.0×10^6 M^{-1} s^{-1} for plasmin and trypsin, respectively. Experiments were conducted to compare the inherently active and the guanidine-activated forms of PAI-1. The two active forms had similar kinetic parameters for interaction with urokinase (K_d, 0.3 pM; k_{assoc}, 1.5×10^8 M^{-1} s^{-1}) and were both inactivated upon treatment with acid or base and by incubation at 37 °C. The latent form was relatively stable when incubated under similar conditions. The decrease in PAI-1 activity upon incubation at 37 °C was partially restored by a second treatment with guanidine hydrochloride. However, the degree of recovery decreased as a function of incubation time at 37 °C. These data suggest that active and guanidine-activated PAI-1 represent a single form of PAI-1. Incubation of this form at 37 °C yields two distinct populations of inactive PAI-1, one capable of reactivation and another that appears to be irreversibly inactivated.

The specific lysis of fibrin is catalyzed by the protease plasmin that exists in plasma as the inactive zymogen, plasminogen (Collen, 1980). The generation of plasmin occurs through the limited cleavage of plasminogen by the plasminogen activators, urokinase (UK),[1] and tissue plasminogen activator (tPA). In addition to their role in fibrinolysis, the plasminogen activators (PA's) have been implicated in various other biological processes including ovulation (Beers et al., 1975; Reich et al., 1985; Ny et al., 1985), cell migration (Ossowski et al., 1975), epithelial differentiation (Henrickson & Astrup, 1967), and tumor invasion (Dano et al., 1985). Thus, precise regulation of PA activity is essential to the maintenance not only of hemostasis but also of other biological processes. This control is accomplished through several different mechanisms including the formation and resolution of fibrin (Collen, 1980; Korninger & Collen, 1981), the controlled synthesis and secretion of the PA's (Loskutoff, 1986), and the presence of specific PA inhibitors (PAI's). These recently discovered PAI's have been detected in tissues (Holmberg et al., 1978), cells (Loskutoff & Edgington, 1977; Dosne et al., 1978; Coleman et al., 1982; Emeis et al., 1983; Levin, 1983; Loskutoff et al., 1983; Badenoch-Jones et al., 1985; Laug, 1985; Kruithof et al., 1986; Vassalli et al., 1984), and plasma and serum (Chmielewska et al., 1983; Kruithof et al., 1984; Verheijen et al., 1984a; Thorsen et al., 1984; Erickson et al., 1986). Recent reports correlating high plasma PAI levels with increased incidence of deep vein thrombosis and myocardial

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* Address correspondence to this author at the Department of Immunology (IMM14), Scripps Clinic and Research Foundation, 10666 N. Torrey Pines Road, La Jolla, CA 92037.
‡ Scripps Clinic and Research Foundation.
§ University of California, San Diego.

[1] Abbreviations: PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; UK, urokinase; PBS, phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; p-APMSF, *p*-amidinophenylmethanesulfonyl fluoride; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G.

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infarction (Haggroth et al., 1986; Verheijen et al., 1984b; Nilsson et al., 1985; Hamsten et al., 1985) support the hypothesis that these inhibitors play an important role in PA regulation. At least four immunologically distinct PAI's have been identified (Collen, 1986). These include PAI-1, originally referred to as the endothelial cell PAI (Loskutoff et al., 1983; van Mourik et al., 1984); PAI-2, previously termed the placental-type PAI (Kopitar et al., 1985; Vassalli et al., 1987; Holmberg et al., 1978); PAI-3, recently discovered in urine (Stump et al., 1986); and protease nexin, an inhibitor of thrombin that also inhibits PA's, plasmin, and trypsin (Baker et al., 1980).

The PAI-1 synthesized by cultured bovine aortic endothelial cells is present in the conditioned medium in at least two forms, one active and the other latent. The latent PAI-1 can be converted to an active form by treatment with denaturants including sodium dodecyl sulfate (SDS) and guanidine hydrochloride (Hekman & Loskutoff, 1985). The objective of the current work was to study the specificity of PAI-1 and to compare the biochemical properties of its active, latent, and guanidine-activated forms. We demonstrate that purified, guanidine-activated PAI-1 inhibits both plasmin and trypsin and that the active and guanidine-activated PAI-1 have similar kinetic parameters and biochemical properties. In addition, we provide evidence to suggest that incubation of the active form of PAI-1 at 37 °C results in its conversion to the latent form.

Materials and Methods

Chemicals, proteins, and other materials were obtained as follows: 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), bovine trypsin [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated], and bovine serum albumin from Sigma; Z-Lys-S-Bzl ester from Peninsula Labs; Bio-Gel P-60 from Bio-Rad; concanavalin A–Sepharose and Sephacryl S-200 from Pharmacia; Blue B agarose and ultrafiltration cells and membranes from Amicon; and microtitration plates (Linbro) from Flow Laboratories Inc. Human α_2-antiplasmin was from American Diagnostica Inc. Human two-chain urokinase (M_r 55 000) was either purchased from Sterling-Winthrop (Winkinase) or supplied by the National Institute for Biological Standards and Controls (London, England; WHO reference 66/46). Human plasminogen was prepared according to the method of Deutsch and Mertz (1970). Plasmin was prepared by incubating 3.6 mg of plasminogen with 3250 international units of UK in the presence of soybean trypsin inhibitor covalently bound to Sepharose beads (23.7 mg bound to 5 mL of Sepharose 4B). After a 17-h incubation at 37 °C, the beads were packed into a column, washed with 0.1 M Tris-HCl, pH 7.6, containing 0.6 M NaCl and 0.01% Tween 80 to remove the UK, and eluted with 0.1 M acetic acid, pH 3.0. The eluted plasmin was stored in this buffer at −70 °C and adjusted to pH 7.2 with 1.0 M Tris base, pH 10, immediately prior to assay.

The molar concentrations of trypsin and UK were determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (NPGB) (Chase & Shaw, 1969). The concentration of plasmin, which was too low to be determined by NPGB titration, was determined by back titration with known concentrations of both α_2-antiplasmin and PAI-1 (Scott et al., 1985). Briefly, plasmin was incubated with increasing concentrations of PAI-1 (60 min, 37 °C) or α_2-antiplasmin (45 min, 37 °C), and the amount of plasmin activity remaining was determined by analysis in the Z-Lys-S-Bzl assay (see below). The concentration of plasmin was then determined by extrapolation to 100% inhibition on a plot of percent inhibition vs concentration of PAI-1 or α_2-antiplasmin.

Purification of PAI-1. Conditioned media (1800 mL) from cultured bovine aortic endothelial cells was prepared and passed over a concanavalin A–Sepharose 4B column (1.6 × 7.0 cm) at a flow rate of 10 mL/h at 4 °C essentially as described (van Mourik et al., 1984). The column was washed with phosphate-buffered saline (PBS) containing 0.01% Tween 80 (PBS/Tween) and 1.0 M NaCl and then eluted with the same buffer containing 0.5 M methyl α-D-mannoside. Fractions were assayed for inhibitory activity in the tPA binding assay (Schleef et al., 1985) and by reverse fibrin autography (Erickson et al., 1984). Inhibitor-containing fractions were pooled (12 mL), treated with diisopropyl fluorophosphate (8 mM final concentration) for approximately 20 min at room temperature, and loaded directly onto a Sephacryl S-200 column (1.6 × 115 cm) equilibrated in PBS/Tween containing 0.5 M NaCl. The column was eluted with the same buffer at 9.5 mL/h, and 1.9-mL fractions were collected. Inhibitor-containing fractions were pooled (39 mL), diluted 1:5 with cold, deionized, distilled water containing 0.01% Tween 80, and passed over a column of Blue B agarose (2.5 × 8.0 cm) previously equilibrated in PBS/Tween. The column was washed at 40 mL/h with 300 mL of PBS/Tween and eluted at 13 mL/h with a gradient (150 mL) of 0–1.1 M NaCl in PBS/Tween. Fractions of 2.2 mL were collected, and the inhibitor-containing fractions were pooled (62 mL) and concentrated to 8.6 mL by ultrafiltration on a YM-10 membrane. This material was loaded onto a column of Bio-Gel P-60 (1.0 × 220 cm) equilibrated in 10 mM Tris-HCl, pH 7.6, containing 0.01% Tween 80 and 3.0 M NaCl. The column was eluted with the same buffer at 6.0 mL/h, and 1.0-mL fractions were collected, diluted 1:3 with water, and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in order to assess their purity. The PAI-1 eluted as a single peak. Those fractions free of contaminants (the ascending limb) were pooled (8.4 mL), dialyzed into 10 mM Tris-HCl, pH 7.6, 0.01% Tween 80, and 0.15 M NaCl, and stored at −70 °C. The fractions corresponding to the descending limb of the PAI-1 peak were contaminated by a single protein migrating with a M_r of 26 000 when analyzed by SDS–PAGE. These fractions were pooled (6.3 mL) and rechromatographed on the Bio-Gel P-60 column. Again, fractions free of detectable contaminants were pooled, dialyzed, and frozen. The purity of the final preparation was assessed by SDS–PAGE. After electrophoresis, the gels were analyzed by staining with silver (Morrissey, 1981), by immunoblotting with antiserum directed against the SDS–PAGE-purified PAI-1 (Erickson et al., 1986; Towbin et al., 1979), or by reverse fibrin autography. In all cases only a single band (M_r 51 000) was evident, indicating that the preparation was free of both contaminating proteins and either degraded or aggregated forms of the PAI-1 (data not shown). The protein concentrations of both the final, purified preparation and aliquots from each step of the purification procedure were determined by the method of Lowry et al. (1951). Activation with 4 M guanidine hydrochloride was performed as previously described (Hekman & Loskutoff, 1985). The concentration of active PAI-1 in both the nonactivated and the guanidine-activated preparations was determined by incubating various amounts of PAI-1 with UK (final concentration 2.4 pM as determined by titration with NPGB) for 45 min at 37 °C. The concentration of active UK remaining was then determined by analysis in the Z-Lys-S-Bzl assay described below. Control experiments (not shown) demonstrated that the concentration of free UK did not vary with incubation times between 35 and 55 min, indicating that

the UK/PAI-1 interaction is essentially complete within the 45-min incubation used. The linear portion of a plot of percent inhibition vs volume of PAI-1 added (0–60% inhibition) was then extrapolated to 100% inhibition. The volume of PAI giving 100% inhibition (by extrapolation) was assumed to contain an amount of PAI-1 equal to the amount of UK used. This method is essentially the same as that used by others (Christensen et al., 1982; Wiman & Collen, 1978) and is based on a previous demonstration that UK and PAI-1 form a 1:1 stoichiometric complex (Hekman & Loskutoff, 1985).

Assays. Activity measurements to establish kinetic parameters and to determine the effect of various treatments on the ability of PAI-1 to inhibit UK were conducted by using purified PAI-1 and purified UK in a modification of the assay method of Coleman and Green (1981). The assay was performed in microtitration plates previously treated with 3% bovine serum albumin to block the protein-binding sites. Various concentrations of UK and PAI-1, diluted in PBS/Tween, were incubated in the wells of the plate (preincubation volume 20 μL) at 37 °C for 45 min in a humidified chamber. Plasminogen (5 μL; final concentration between 0.8 and 4.0 μM) was added, and the samples were incubated for an additional 60 min. Finally, 200 μL of the Z-Lys-S-Bzl ester plasmin substrate (200 μM Z-Lys-S-Bzl ester, 220 μM DTNB in 200 mM phosphate, pH 7.5, 200 mM NaCl, 0.01% Triton X-100) was added, and the absorbance was determined every 15 min by use of a Titertek Multiscan with a 414-nm filter. In all instances the absorbance remained linear for at least 3 h, indicating that the addition of the plasmin substrate mixture had effectively terminated the turnover of plasminogen by the UK (Coleman & Green, 1981). The concentration of plasmin generated was calculated from a previous determination that 1.0 nM plasmin (25 μL) produced an absorbance change of 0.030 A_{414} units/h.

The rate of interaction of the UK with PAI-1 was studied by utilizing an immunoradiometric assay specific for UK/PAI-1 complexes (Schleef & Guthrie, 1985). Briefly, microtitration wells were coated with the IgG fraction of goat antiserum to human UK, washed, and blocked with 3% bovine serum albumin. Samples containing UK/PAI-1 complexes were then incubated in the wells for 60 min at 37 °C. Unbound PAI-1 was removed by washing, and UK/PAI-1 complexes were detected by incubating the wells first with rabbit antiserum to bovine aortic endothelial cell PAI-1 (van Mourik et al., 1984) and then with ^{125}I-labeled goat anti-rabbit IgG. Stock solutions of UK/PAI-1 complexes were prepared by incubating UK (approximately 100 ng/mL final concentration) with PAI-1 (approximately 1.0 μg/mL final concentration) for 1 h at 37 °C and used as standards for this assay. The actual concentration of UK/PAI-1 complex present in the standard was determined by analysis of duplicate samples in the immunoradiometric assay for UK/PAI-1 complexes and in the Z-Lys-S-Bzl assay as described above to quantitate residual UK activity. All standards and samples were brought to a final *p*-amidinophenylmethanesulfonyl fluoride (p-APMSF) concentration of 10 mM before analysis in the immunoradiometric assay (see below).

Determination of Kinetic Parameters for the Interaction of UK with Active and Guanidine-Activated PAI-1. UK (2.4 pM final concentration) was incubated with various concentrations of PAI-1 (2.5–9.0 pM final concentration as determined by titration against UK) for 45 min at 37 °C. As stated previously, the UK/PAI-1 interaction appears to be essentially complete in 45 min at 37 °C (within the limits of our ability to quantitate small changes at this time). The amount of free UK was then determined by using the Z-Lys-S-Bzl assay as described above. The concentrations of free PAI-1 and UK/PAI-1 complex were determined by difference. The $K_{d(45')}$'s[2] were calculated from the slope of a plot of concentration of UK/PAI-1 complex ([complex]) vs the ratio of concentration of UK/PAI-1 complex to concentration of free PAI-1 ([complex]/[PAI]) according to

$$[\text{complex}] = -K_{d(45')}([\text{complex}]/[\text{PAI}]) + [\text{PA}]_0 \quad (1)$$

where $[\text{PA}]_0$ represents the initial concentration of UK.

Rate constants for the UK/PAI-1 interaction were determined by incubating UK (30, 42, or 60 pM final concentration) and PAI-1 (69 pM final concentration) at 35 °C for various time periods. The reaction was terminated by the addition of p-APMSF (10 mM final concentration). The concentration of UK/PAI-1 complex formed in each time period was then determined by analysis of the samples in the immunoradiometric assay specific for UK/PAI-1 complexes (see above). In control experiments in which the p-APMSF was added to the UK either together with or immediately after the addition of PAI-1, no significant quantities of UK/PAI-1 complex (less than 2 pM) were detected by the immunoradiometric assay used in these studies (see Figure 2 at $t = 0$). These results together with experiments demonstrating that p-APMSF prevented the binding of PAI-1 to immobilized tPA (data not shown) in the tPA binding assay (Schleef et al., 1985) demonstrate that the addition of p-APMSF both immediately and completely terminates not only the UK/PAI-1 interaction but also the tPA/PAI-1 interaction. The residual free UK and PAI-1 concentrations were then calculated by difference. The second-order rate constant, k_{assoc}, was determined by linear regression of a plot of log $([\text{PA}][\text{PAI}]_0/[\text{PA}]_0[\text{PAI}])$ vs time for times ranging from 0 to 5 min according to the second-order rate equation (Daniels & Alberty, 1975)

$$(k_{assoc})t = 2.303[1/([\text{PA}]_0 - [\text{PAI}]_0)] \log ([\text{PA}] \times [\text{PAI}]_0/[\text{PA}]_0[\text{PAI}]) \quad (2)$$

where $[\text{PA}]_0$ and $[\text{PAI}]_0$ represent initial concentrations of UK and PAI-1, respectively, and [PA] and [PAI] represent the concentrations of free UK and PAI-1 present at time t.

Specificity Determinations. Equimolar concentrations (either 320 or 145 pM final concentrations) of trypsin and purified, guanidine-activated PAI-1 were incubated together at 37 °C in the wells of a microtitration plate (50 μL final volume) for periods of time ranging from 5 to 60 min. The Z-Lys-S-Bzl substrate mixture (200 μL) was then added, and the absorbances were determined periodically over a 30-min interval. The rate of change in the absorbance was used to determine the concentration of remaining free trypsin. The concentration of free PAI-1 was then determined by difference. The second-order rate constants were determined by linear regression of a plot of 1/[trypsin] vs time (Daniels & Alberty, 1975) according to

$$(k_{assoc})t = 1/[\text{trypsin}] - 1/[\text{trypsin}]_0 \quad (3)$$

where $[\text{trypsin}]_0$ represents the initial concentration of trypsin and [trypsin] represents the concentration of trypsin present at time t. Similar analyses were conducted with plasmin (300 or 115 pM final concentrations) and PAI-1 (2500 or 1300 pM final concentrations) for periods of time ranging from 4 to 45

[2] To stress the fact that the dissociation constants determined here represent the progress of the reaction at 45 min, they are referred to as $K_{d(45')}$ rather than K_d. Insofar as the initial concentrations of UK and PAI-1 were the same in both the study of inherently active and guanidine-activated PAI-1, these constants represent a valid comparison of the two forms of PAI-1.

Table I: Second-Order Rate Constants for the Interaction of Various Inhibitors with Trypsin and Plasmin[a]

inhibitor	trypsin (M^{-1} s^{-1})	plasmin (M^{-1} s^{-1})
PAI-1	(7.0 ● 1.4) × 10^6	(6.6 ± 2.9) × 10^5
α_2-antiplasmin[b]	1.8 × 10^6	3.8 × 10^7
PN[c]	4.2 × 10^6	1.3 × 10^5
α_2-macroglobulin[d]	2 × 10^7	5 × 10^5
PTI[e]	1.1 × 10^6	

[a] Trypsin (320 or 145 pM) or plasmin (300 or 115 pM) and guanidine-activated PAI-1 (320 or 145 pM for trypsin studies; 2500 or 1300 pM for plasmin studies) were incubated at pH 7.2, 37 °C, for various time intervals. Following the addition of the Z-Lys-S-Bzl substrate, the residual enzyme activity was measured. The rate constants were calculated as described under Materials and Methods and are presented as the mean ± standard deviation for three to five separate determinations. [b] pH 7.3, 25 °C (Wiman & Collen, 1978). [c] Protease nexin, pH 8.0, 37 °C (Scott et al., 1985). [d] pH 7.6, 25 °C (Christensen & Sottrup-Jensen, 1984). [e] Pancreatic trypsin inhibitor, pH 8.0, 25 °C (Vincent & Lazdunski, 1972).

min. Since unequal concentrations of enzyme and inhibitor were utilized in this case, second-order rate constants were calculated as described for UK (eq 2). Control experiments in which the PAI-1 was added to the enzyme after addition of the Z-Lys-S-Bzl substrate mixture were conducted in all cases and demonstrated that addition of the substrate mixture prevented the interaction of the enzyme with PAI-1. The concentrations of trypsin and plasmin were chosen so as to give an absorbance large enough to quantitatively measure 90% inhibition of the enzyme's activity within 10–20 min after addition of the substrate. The concentrations of PAI-1 were chosen such that inhibition was complete in a period of time that was negligible with respect to the rate of decay of the PAI-1 activity (see Figure 4). All dilutions were made in PBS/Tween, pH 7.2, containing 0.25% gelatin (autoclaved).

Stability Studies. In order to determine the effect of acid and base treatment on PAI-1 activity, 20 μL of purified, nonactivated PAI-1 or of purified, guanidine-activated PAI-1 were brought to the indicated pH by the addition of 10 μL of either 0.1 M sodium acetate (pH's between 1 and 7) or 0.1 M Tris base (pH's between 7 and 12). Because of the technical difficulties involved in measuring the pH of such small volumes, the actual pH of the samples was determined by measuring the pH of a 2-mL sample of PBS/Tween to which 1 mL of either Tris or acetate had been added. After a 60-min incubation at 25 °C, the pH of the samples was adjusted to 7.2 by dilution with PBS/Tween. Aliquots of the nonactivated PAI-1 samples were then activated with guanidine hydrochloride. All samples (e.g., nonactivated, activated before acid or base treatment, and activated after acid or base treatment) were then assayed for inhibitory activity in the Z-Lys-S-Bzl assay.

In order to determine the effect of incubation at 37 °C on PAI-1 activity, purified PAI-1, either nonactivated or guanidine activated, was incubated at 37 °C for various periods of time followed by cooling on ice. Aliquots of the nonactivated PAI-1 samples were then activated with guanidine hydrochloride, and all samples (e.g., nonactivated, activated before incubation, and activated after incubation) were assayed for inhibitory activity in the Z-Lys-S-Bzl assay.

Results

Inhibition of Plasmin and Trypsin by PAI-1. In order to study the specificity of PAI-1, the ability of guanidine-activated PAI-1 to inhibit the turnover of Z-Lys-S-Bzl by plasmin and trypsin was studied. The data reveal that both plasmin and trypsin (Figure 1) are inhibited by PAI-1 in a dose- and time-dependent manner. Second-order rate constants were

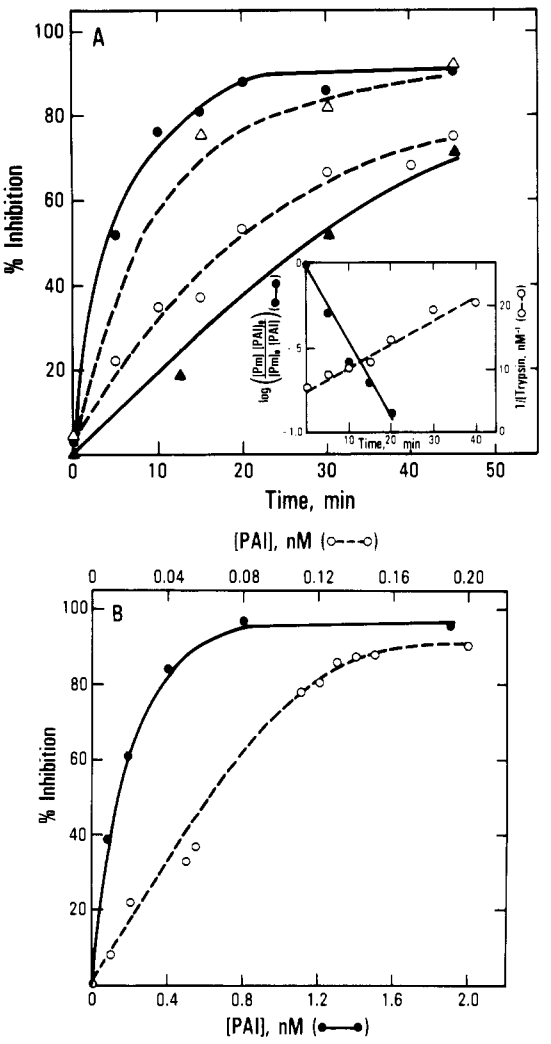

FIGURE 1: Time and dose dependence of plasmin and trypsin inhibition by PAI-1. (Panel A) Plasmin (solid lines) or trypsin (broken lines) was incubated with guanidine-activated PAI-1 at 37 °C, pH 7.2, for various periods of time (●, 2500 pM PAI-1 and 300 pM plasmin; ▲, 1300 pM PAI-1 and 115 pM plasmin; △, 320 pM PAI-1 and 320 pM Trypsin; ○, 145 pM PAI-1 and 145 pM trypsin). The Z-Lys-S-Bzl substrate mixture was then added, and residual enzyme activity was determined as described under Materials and Methods. The second-order rate constants were calculated from the slope of the inset plots [inset: ● 300 pM plasmin (Pm) and 2500 pM PAI-1; ○, 145 pM trypsin and 145 pM PAI-1] as described under Materials and Methods. (Panel B) Plasmin (●, 300 pM) or trypsin (○, 100 pM) was incubated with various concentrations of PAI-1 at 37 °C, pH 7.2, for 60 min. The Z-Lys-S-Bzl substrate mixture was added, and the residual enzyme activity determined as described under Materials and Methods. The percent of inhibition is plotted as a function of incubation time (panel A) or concentration of PAI-1 (panel B).

determined for these interactions (Figure 1A, inset) and are listed in Table I together with the reported rate constants for the inhibition of these enzymes by α_2-antiplasmin, protease nexin, α_2-macroglobulin, and pancreatic trypsin inhibitor.

Comparison of the Active, Latent, and Guanidine-Activated Forms of PAI-1. The experiments described above, as well as other work described previously (Hekman & Loskutoff, 1986a,b), were conducted with guanidine-activated PAI-1. Experiments were therefore performed to demonstrate that the guanidine-activated PAI-1 does not differ from the inherently active PAI-1 in its biochemical properties. As an initial approach, the dissociation (see footnote 2) and second-order rate constants (Figure 2) were determined for the interaction of UK with both the inherently active PAI-1 (i.e., that present in the nonactivated preparation) and the guani-

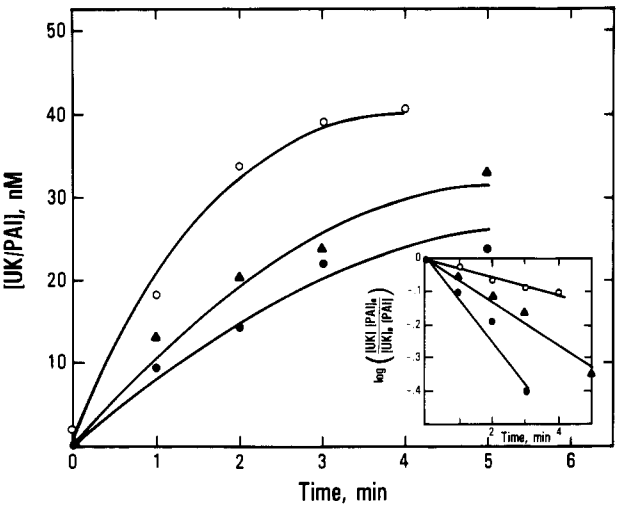

FIGURE 2: Time dependence of UK/PAI-1 complex formation. UK (○, 60 pM; ▲, 42 pM; ●, 30 pM) and nonactivated PAI-1 (69 pM) were incubated at 35 °C, pH 7.2, for various periods of time. The reaction was terminated by the addition of p-APMSF, and the concentration of UK/PAI-1 complex formed in each time period was determined by analysis of the samples in the immunoradiometric assay specific for these complexes. The concentration of UK/PAI-1 complex is plotted as a function of time. The second-order rate constants were calculated from the slope of the inset plot as described under Materials and Methods.

Table II: Dissociation (K_d) and Rate (k_{assoc}) Constants for the Interaction of the Active and the Guanidine-Activated PAI-1 with UK[a]

PAI-I	$K_{d(45')}$ (pM)	k_{assoc} (M^{-1} s^{-1})
inherently active	0.3 ± 0.1	(1.5 ± 0.1) × 10^8
guanidine activated	0.23 ± 0.05	(1.6 ● 0.1) × 10^8

[a] $K_{d(45')}$'s were determined by a Scatchard analysis of the data obtained as described under Materials and Methods. Rate constants were determined by measuring UK/PAI-1 complex formation as a function of time using the immunoradiometric assay specific for UK/PAI-1 complexes. The constants are presented as the mean ± standard deviation for four separate determinations.

dine-activated PAI-1. These data (Table II) illustrate that both the active and the guanidine-activated PAI-1 inhibit UK with similar affinities and rates.

Experiments to compare the pH stability of the active and guanidine-activated forms were also conducted. Purified PAI-1, either nonactivated or guanidine-activated, was incubated with buffer solutions varying in pH from 1 to 12. After the pH was restored to 7.2, the amount of PAI-1 activity remaining in each of the samples was determined and plotted as a function of pH (Figure 3). The data illustrate that acid or base treatment (i.e., pH's below 4 or above 8) of both the active PAI-1 and of the guanidine-activated PAI-1 resulted in a significant loss of PAI-1 activity. Figure 3 also shows the effect of acid or base treatment on the ability of the latent PAI-1 to be activated. In these experiments, aliquots of the nonactivated PAI-1 samples were first treated with acid or base and then activated with guanidine hydrochloride and assayed as before. Exposure of the latent PAI-1 to acid or base did not alter the amount of PAI-1 activity that could be induced by activation.

To determine the effect of incubation at 37 °C on the three forms of PAI-1, aliquots of either nonactivated PAI-1 or of guanidine-activated PAI-1 were incubated at 37 °C for various periods of time. The amount of PAI-1 activity remaining in each sample was then determined (Figure 4). These data indicate that 37 °C incubation resulted in a significant decrease in PAI activity in both the active and the guanidine-activated

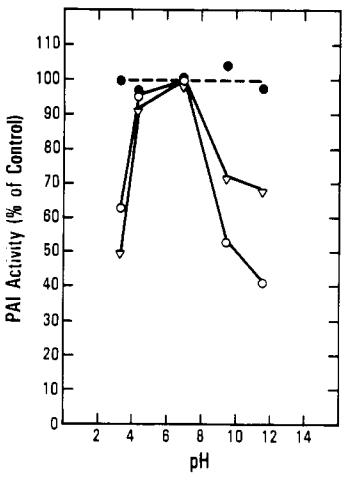

FIGURE 3: Effect of pH on the inhibitory activity of the active, latent, and guanidine-activated forms of PAI-1. Purified PAI-1 (20 μL), either nonactivated (▽) or previously activated with guanidine hydrochloride (○), was incubated with 10 μL of buffer solution (pH 1–12) as described under Materials and Methods. The pH of the samples was then adjusted to 7.2, and the samples were assayed for inhibitory activity in the Z-Lys-S-Bzl assay. Aliquots of nonactivated PAI-1 samples were activated with guanidine hydrochloride after exposure to acid and base and tested for inhibitory activity as before (●). The amount of PAI activity remaining in the sample, when compared to a control sample that had not been exposed to acid or base, is plotted as a function of pH.

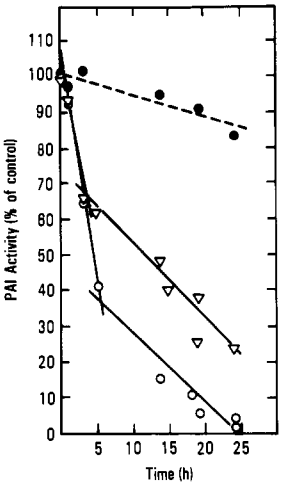

FIGURE 4: Effect of incubation at 37 °C on the inhibitory activity of the active, latent, and guanidine-activated forms of PAI-1. Purified PAI-1, either nonactivated (▽) or previously activated with guanidine hydrochloride (○), was incubated at 37 °C for various periods of time and assayed for inhibitory activity in the Z-Lys-S-Bzl assay as described under Materials and Methods. Aliquots of the nonactivated PAI-1 samples were activated with guanidine hydrochloride after incubation at 37 °C and assayed for inhibitory activity as before (●). The percent of PAI-1 activity remaining in the sample, when compared to a control sample that had not been incubated at 37 °C, is plotted as a function of time at 37 °C.

PAI-1 samples. In order to assess the effect of 37 °C incubation on the ability of the latent PAI-1 to be activated, aliquots of the nonactivated PAI-1 samples were first incubated at 37 °C and then activated with guanidine hydrochloride. The samples were then assayed as before. The latent PAI-1 lost only 10% of its potential activity after 24 h at 37 °C (Figure 4).

Experiments were performed to test the possibility that the decrease of PAI-1 activity during incubation at 37 °C resulted from a conversion of the active form of PAI-1 to the latent form. Guanidine-activated PAI-1 was inactivated by incubation at 37 °C and then treated a second time with guanidine

Table III: Effect of Guanidine Treatment on PAI-1 Previously Inactivated by Incubation at 37 °C[a]

sample	treatment	% PAI activity
37 °C, 18 h	none	11.3
	PBS/Tween	18.6
	guanidine hydrochloride	66.0
37 °C, 24 h	none	3.7
	PBS/Tween	8.7
	guanidine hydrochloride	44.6

[a] Purified, guanidine-activated PAI-1 was incubated at 37 °C for the indicated time periods. Aliquots of the samples were then treated with either PBS/Tween (as a control) or guanidine hydrochloride, and all samples were assayed for inhibitory activity in the Z-Lys-S-Bzl assay. The data are presented as the percent of PAI activity remaining when compared to guanidine-activated samples that had not been incubated at 37 °C.

hydrochloride. The results (Table III) demonstrate that the PAI-1 activity which is lost upon incubation at 37 °C can be partially recovered by a second treatment with guanidine hydrochloride. However, the amount of activity that can be recovered decreases as a function of the time the sample was incubated at 37 °C. Thus, while 66% of the original activity could be recovered by guanidine activation of a sample incubated at 37 °C for 18 h, only 45% of the original activity could be restored when the sample was incubated at 37 °C for 24 h.

Discussion

The primary structure of the human PAI-1 was recently elucidated through the use of DNA sequencing techniques (Ny et al., 1986; Pannekoek et al., 1986; Ginsburg et al., 1986). Comparison of the PAI-1 sequence with known sequences of other proteins indicate that PAI-1 is a member of the serpin (serine protease inhibitor) family (Carrell & Travis, 1985). Other members of this family include α-1-proteinase inhibitor and α_2-antiplasmin (Holmes et al., 1987). Because of this homology, experiments were conducted to determine the ability of PAI-1 to inhibit trypsin and plasmin. Similar studies have been reported previously (Andreasen et al., 1986; Coleman et al., 1982; Badenoch-Jones et al., 1985). However, because of the low concentration of active PAI-1 available in the crude mixtures previously employed, most investigations have been unable to demonstrate inhibition of these enzymes by the PAI-1. In the studies reported here, we utilized a purified, concentrated preparation of guanidine-activated PAI-1 that allowed us to overcome these difficulties. Under the conditions employed, PAI-1 was shown to inhibit trypsin and plasmin in both a dose- and time-dependent manner (Figure 1; Table I). This inhibition was observed to be second order with rate constants calculated to be 7.0×10^6 and 6.6×10^5 M^{-1} s^{-1} for trypsin and plasmin, respectively (Table I). These rate constants are between 20 and 200 times lower than those observed for either the UK/PAI-1 (Table II) or the tPA/PAI-1 interaction (Chmielewska et al., 1983; Kruifhof et al., 1986; Colucci et al., 1986; Coleman et al., 1986). In fact, the second-order rate constant calculated for the UK/PAI-1 interaction is approximately 4 times greater than that either determined by us for the single-chain tPA/PAI-1 interaction (4×10^7 M^{-1} s^{-1}, data not shown) or reported by others (Coleman et al., 1986; Chmielewska et al., 1983; Kruithof et al., 1986; Colucci et al., 1986). The UK/PAI-1 rate constant is one of the highest reported for protease–protease inhibitor interactions (Travis & Salvesen, 1983) and approaches the diffusion-controlled limit (Fersht, 1985). These data clearly illustrate that the PAI-1 is indeed a more efficient inhibitor of both UK and tPA than it is of either trypsin or plasmin. The trypsin/PAI-1 interaction is also slightly slower than the interaction between trypsin and α_2-macroglobulin (Table I) (Christensen & Sottrup-Jensen, 1984), although it occurs at approximately the same rate as the interaction between trypsin and either α_2-antiplasmin (Wiman & Collen, 1978), protease nexin (Scott et al., 1985), or pancreatic trypsin inhibitor (Kunitz) (Vincent & Lazdunski, 1972). Similarly, the plasmin/PAI-1 interaction is slow by comparison with the plasmin/α_2-antiplasmin reaction (Table I) (Wiman & Collen, 1978) but occurs at approximately the same rate as the interaction between plasmin and either protease nexin (Scott et al., 1985) or α_2-macroglobulin (Christensen & Sottrup-Jensen, 1984). These data coupled with the low concentrations of PAI-1 in plasma (Chmielewska et al., 1983; Verheijen et al., 1984a; Korninger et al., 1985) suggest that PAI-1 probably does not play a significant role in the in vivo inhibition of these enzymes in blood plasma (Travis & Salvesen, 1983).

In this and previous studies (Hekman & Loskutoff, 1986a,b) the interaction between various enzymes and the guanidine-activated PAI-1 was characterized. It therefore appeared necessary to demonstrate that the guanidine-activated PAI-1 and the inherently active PAI-1 have similar functional and biochemical properties. Our results illustrate that both the active and the guanidine-activated PAI-1 have similar kinetic parameters for inhibition of UK (Figure 2; Table II), similar apparent M_r's as determined by gel filtration (data not shown), and similar sensitivities to pH (Figure 3) and incubation at 37 °C (Figure 4).

Further studies, in which the properties of the active and guanidine-activated PAI-1's were compared with those of the latent PAI-1, revealed that treatments that inactivate the active forms (i.e., extremes of pH and incubation at 37 °C) do not significantly alter the ability of the latent form to be activated (Figures 3 and 4). In addition, the loss of PAI-1 activity observed when the guanidine-activated PAI-1 was incubated at 37 °C was partially reversed by a second treatment with guanidine hydrochloride. Our results agree with a recent paper by Levin (Levin, 1986) demonstrating that a crude preparation of active PAI-1 from human endothelial cell extracts was inactivated upon incubation at 37 °C. The same report also illustrated that the PAI-1 activity that was lost during 37 °C incubation could be recovered by a subsequent activation with SDS. Together these observations suggest that incubation at 37 °C of either the active or the guanidine-activated PAI-1 causes a conversion of this relatively unstable form of PAI-1 to the latent form from which it can be recovered by treatment with denaturants. However, the data outlined in Table III demonstrate that the PAI-1 activity lost during incubation at 37 °C can be only partially recovered by a second activation with guanidine hydrochloride. Additional studies have revealed that with each repeated cycle of 37 °C incubation followed by guanidine activation, less PAI-1 activity can be recovered until, after five cycles, no PAI-1 activity can be induced by guanidine treatment (data not shown). These observations suggest that in addition to the active and the latent forms, a third, irreversibly inactivated, form of the PAI-1 also exists. The presence of irreversibly inactivated PAI-1 may explain the low specific activities observed by ourselves and others (Hekman & Loskutoff, 1986a; Wagner & Binder, 1986; Neilsen et al., 1986) for purified preparations of PAI-1. Our current hypothesis is that PAI-1 is synthesized and secreted in an active form. However, the majority of it is irreversibly inactivated during the preparation of conditioned media (24 h, 37 °C) and subsequent purification. A small amount of PAI-1 activity can, however, be recovered from these samples

by treatment with denaturants.

The physiological significance of the latent and the irreversibly inactivated forms of PAI-1 remains to be determined. The report that both platelets (Sprengers et al., 1986) and whole blood serum (Erickson et al., 1986) contain latent PAI-1 indicates that the latent form of PAI-1 is not unique to endothelial cells but may, in fact, be common to a variety of biological samples. It therefore seems possible that a mechanism may exist in vivo to convert latent PAI-1 into the active form. In spite of these considerations, no physiological meaningful activator of latent PAI-1 has been identified, leaving open the possibility that the latent and the irreversibly inactivated forms of PAI-1 merely represent a clearance mechanism.

In conclusion, this paper illustrates that the guanidine-activated PAI-1 inhibits both plasmin and trypsin. The active and the guanidine-activated PAI-1 behave similarly with respect to both activity and stability and appear to represent a single form of the PAI-1. Inactivation of the active form by mild thermal denaturation yields two populations of inactive PAI-1, one form capable of reactivation (latent) and another form that appears to be irreversibly inactivated.

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Registry No. PAI, 105844-41-5; UK, 9039-53-6; plasmin, 9001-90-5; trypsin, 9002-07-7; guanidine hydrochloride, 50-01-1.

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Chicken Liver Sulfite Oxidase. Kinetics of Reduction by Laser-Photoreduced Flavins and Intramolecular Electron Transfer†

Cary A. Kipke,‡ Michael A. Cusanovich,*,§ Gordon Tollin,*,§ Roger A. Sunde,‖ and John H. Enemark‡
Departments of Chemistry, Biochemistry, and Nutrition and Food Science, University of Arizona, Tucson, Arizona 85721
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ABSTRACT: Laser flash photolysis was used to study the reaction of photoproduced 5-deazariboflavin (dRFH·), lumiflavin (LFH·), and riboflavin (RFH·) semiquinone radicals with the redox centers of purified chicken liver sulfite oxidase. Kinetic studies of the native enzyme with dRFH· yielded a second-order rate constant of 4.0×10^8 M^{-1} s^{-1} for direct reduction of the heme and a first-order rate constant of 310 s^{-1} for intramolecular electron transfer from the Mo center to the heme. The reaction with LFH· gave a second-order rate constant of 2.9×10^7 M^{-1} s^{-1} for heme reduction. Reoxidation of the reduced heme due to intramolecular electron transfer to the Mo center gave a first-order rate constant of 155 s^{-1}. The direction of intramolecular electron transfer using dRFH· and LFH· was independent of the buffer used for the experiment. The different first-order rate constants observed for intramolecular electron transfer using dRFH· and LFH· are proposed to result from chemical differences at the Mo site. Flash photolysis studies with cyanide-inactivated sulfite oxidase using dRFH· and LFH· resulted in second-order reduction of the heme center with rate constants identical with those obtained with the native enzyme, whereas the first-order intramolecular electron-transfer processes seen with the native enzyme were absent. The isolated heme peptide of sulfite oxidase gave only second-order kinetics upon laser photolysis and confirmed that the first-order processes observed with the native enzyme involve the Mo site. The flash-induced difference spectrum of native sulfite oxidase using dRFH· and LFH· resulted in absorbance increases in the 530-570-nm region of the spectrum that were not present in the static difference spectrum of the enzyme. These absorbances are proposed to be associated with the Mo center.

Sulfite oxidase is a dimeric molybdenum-containing protein that catalyzes the oxidation of sulfite to sulfate (Rajagopalan, 1980). The enzyme contains two molybdenum–pterin centers and two cytochrome b_5 type hemes per molecule (Cohen et al., 1971; Cohen & Fridovich, 1971b; Johnson et al., 1977), and catalysis occurs at the molybdenum site (Johnson et al., 1974a,b). Although sulfite oxidase has considerably fewer redox sites than the kinetically well-studied molybdenum-containing protein xanthine oxidase [cf. Hille and Massey (1986)], the transient kinetics of the redox chemistry of the enzyme have yet to be reported.

The proposed catalytic cycle of sulfite oxidase requires the fully reduced Mo site to undergo two, one-electron, intramo-

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‡ Department of Chemistry.
§ Department of Biochemistry.
‖ Department of Nutrition and Food Science.

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