Characterization of the Binding of Different Conformational Forms of Plasminogen Activator Inhibitor-1 to Vitronectin

IMPLICATIONS FOR THE REGULATION OF PERICELLULAR PROTEOLYSIS

(Received for publication, May 13, 1996, and in revised form, November 29, 1996)

Daniel A. Lawrence‡, Selvi Palaniappan, Steingrimur Stefansson, Steven T. Olson¶,
Ann Marie Francis-Chmura††, Joseph D. Shore†††, and David Ginsburg§§§

From the ‡Department of Biochemistry, American Red Cross Holland Laboratory, Rockville, Maryland 20855,
¶Departments of Internal Medicine and Human Genetics and Howard Hughes Medical Institute, University of Michigan,
Ann Arbor, Michigan, 48109-0650, **Center for Molecular Biology of Oral Diseases, University of Illinois, Chicago,
Illinois 60612–7213, and $$$Henry Ford Health System, Detroit, Michigan 48202–3450

Plasminogen activator inhibitor type 1 (PAI-1), the primary physiologic inhibitor of plasminogen activation, is associated with the adhesive glycoprotein vitronectin (Vn) in plasma and the extracellular matrix. In this study we examined the binding of different conformational forms of PAI-1 to both native and urea-purified vitronectin using a solid-phase binding assay. These results demonstrate that active PAI-1 binds to urea-purified Vn with approximately 6-fold higher affinity than to native Vn. In contrast, inactive forms of PAI-1 (latent, elastase-cleaved, synthetic reactive center loop peptide-annexed, or complexed to plasminogen activators) display greatly reduced affinities for both forms of adsorbed Vn, with relative affinities reduced by more than 2 orders of magnitude. Structurally, these inactive conformations all differ from active PAI-1 by insertion of an additional strand into β-sheet A, suggesting that it is the rearrangement of sheet A that results in reduced Vn affinity. This is supported by the observation that PAI-1 associated with β-anhydrotrypsin, which does not undergo rearrangement of β-sheet A, shows no such decrease in affinity, whereas PAI-1 complexed to β-trypsin, which does undergo sheet A rearrangement, displays reduced affinity for Vn similar to PAI-1-plasminogen activator complexes. Together these data demonstrate that the interaction between PAI-1 and Vn depends on the conformational state of both proteins and suggest that the Vn binding site on PAI-1 is sensitive to structural changes associated with loss of inhibitory activity.

Plasminogen activators (PAs)† are specific serine proteinases

† This work was supported in part by National Institutes of Health Grants HL 55374 (to D. A. L.), HL 28888 (to S. T. O.), HL 49590 (to J. D. S.), and HL 49184 (to D. G.) and by American Heart Association Grant-in-aid 96007350 (to D. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Biochemistry, American Red Cross Holland Laboratory, 15601 Crabbs Branch Way, Rockville, MD 20855. Tel.: 301-517-0536; Fax: 301-738-0794; E-mail: lawrencd@usa.redcross.org.
§ Supported in part by a research fellowship from the American Heart Association, Maryland affiliate.
¶ Investigator of the Howard Hughes Medical Institute.
† The abbreviations used are: PA, plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; Vn, vitronectin; nVn, vitronectin purified under native conditions; uVn, urea-purified vitronectin; RCL, reactive center loop; tPA, tissue-type plasminogen activator; TBS, Tris-buffered saline, pH 7.5; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin that activate the proenzyme plasminogen to the broad specificity enzyme plasmin (1). There are only two known physiologic activators of plasminogen, tissue-type PA (tPA) and urokinase-type PA (uPA) (2). In addition to their role in vascular fibrinolysis (3), PAs are thought to critically influence many other biological processes involving cell migration or tissue remodeling (4). These include ovulation (5), inflammation (6), tumor metastasis (7), and angiogenesis (8). The serpin PAI-1, the most efficient inhibitor known of both tPA and uPA, is thus a critical regulator of the PA system (9). PAI-1 is present in plasma at nM concentrations (10) and in platelets at a much higher concentration (11). This latter pool has been shown to contribute to clot stabilization in vivo (12). In plasma and the extracellular matrix, PAI-1 is associated with Vn (13–15), and this association may be involved in maintaining the integrity of the cell substratum in vivo. PAI-1 exists in at least three distinct conformational forms: active, latent, and cleaved (16, 17). Active PAI-1 decays to the latent form with a half-life of approximately 1–2 h at 37 °C (18). After treatment with denaturants, latent PAI-1 can be partially returned to the active form (19). Although the biologic significance of the latent conformation remains unknown, it may contribute to the regulation of PAI-1 activity (20). Cleaved PAI-1 can be generated either by the slow deacylation of the enzyme inhibitor complex (21, 22) or by reaction of PAI-1 with a nontarget protease such as elastase, which cleaves the reactive center loop (RCL) at a site other than the P1-P1 reactive center bond (17). The latent and cleaved forms of PAI-1 are inactive due to full insertion of the RCL into β-sheet A of the inhibitor and therefore are unavailable for reaction with proteinases (16, 23). In contrast, the active form of PAI-1 is thought to have its RCL fully exposed and thus available for interaction with proteinases (21). Complex formation with a target proteinase is associated with a rapid conformational change in the inhibitor that results in RCL insertion (21, 24).

The adhesive glycoprotein, Vn, is present in plasma at μM concentrations and is also associated with the extracellular matrix of many tissues. Like PAI-1, Vn can also exist in multiple conformational states (25, 26). In addition to stabilizing PAI-1, Vn has been shown to alter the PAI-1 specificity, converting it to an efficient inhibitor of thrombin (27, 28). Vitronectin-bound PAI-1 has a 270-fold greater second order rate constant toward thrombin than does free PAI-1. However, this increase depends upon the conformational form of Vn (28). Vitronectin also promotes the clearance of PAI-1-thrombin min; wtPAI-1, wild-type plasminogen activator inhibitor type 1; serpin, serine protease inhibitor.
complexes by the low density lipoprotein receptor-related protein (29). Recently, PAI-1 bound to vitronectin in the extracellular matrix has been shown to block the binding of integrins (30) and the uPA receptor (31) to vitronectin, and this interaction was shown to inhibit cell adhesion and migration on vitronectin. The precise nature of the PAI-1-Vn interaction has been the subject of considerable debate and was recently reviewed (32). Using solid-phase binding assays to quantitate this interaction, several studies have suggested that only active PAI-1 binds Vn (13, 33, 34); however, other investigators have reported no apparent difference in the binding of active and latent PAI-1 (35, 36). In addition, the reported dissociation constant for PAI-1 binding to immobilized Vn ranges from 0.3 to 190 nM (34, 35, 37). The Vn binding domain within PAI-1 was recently localized to a region on the surface of PAI-1 that includes β-strand 1A (37–39). The Vn binding site for PAI-1 appears to be localized to the somatomedin B domain at the N terminus of Vn (40–42). However, other reports suggest that PAI-1 binds to the C terminus of Vn between residues 348 and 370 (36) or to a site near the center of Vn between amino acids 115 and 121 (43).

A critical dependence of the PAI-1/Vn interaction on the PAI-1 and/or Vn conformation could explain these conflicting reports. To test this hypothesis we examined the binding of PAI-1 in six different conformations to both native and urea-purified immobilized Vn. Our results indicate that the two forms of Vn bind to PAI-1 with markedly different affinities and that the Vn binding domain on PAI-1 is very sensitive to the PAI-1 conformation. We suggest that there may have been an evolutionary selection of the PAI-1 structure to permit efficient removal of inactive PAI-1 at sites of subcellular attachment.

EXPERIMENTAL PROCEDURES

Materials—Purified PAI-1 (44), either active (>95%) or latent (>95%), were obtained from Molecular Innovations (Royal Oak, MI). To eliminate any active PAI-1 present in the latent preparations, latent PAI-1 was treated with a 0.01 molar eq of elastase for 30 min at 23 °C in Tris-buffered saline, pH 7.5 (TBS), followed by inactivation of residual enzyme by 1 mM (final concentration) phenylmethylsulfonyl fluoride. PAI-1 complexes with uPA and tPA were formed by incubation of 1.5 molar eq of either enzyme with 4.6 μM active PAI-1 and/or trypsin in 25 mM sodium phosphate, 125 mM NaCl, 0.5 mM EDTA, 10 mM CaCl₂, pH 6.6, for 30 min at 23 °C, after which the remaining active PAI-1 was removed by chromatography on uPA-agarose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that the complexes contained no detectable uncleaved PAI-1 and 20% free cleaved PAI-1. The PAI-1-peptide complex was produced by incubating 6.4 μM active PAI-1 with 200 μM peptide in 0.1 M HEPES, 0.1 M NaCl, 0.1% PEG-8000, 0.1% Tween 80, pH 7.4, at 25 °C until no detectable PAI-1 inhibitory activity remained (approximately 20 h). The free peptide was then removed by chromatography on heparin-Sepharose. PAI-1-peptide complex formation was confirmed by chromatography on heparin-Sepharose (18). SDS-polyacrylamide gel electrophoresis (47), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis with or without tPA (48). This latter analysis indicated that the peptide-annulled PAI-1 was a substrate for tPA and contained approximately 15% latent PAI-1, consistent with previous studies (48).

Assays for PAI-1 Binding to Vitronectin—PAI-1 binding to immobilized Vn was determined either functionally (49) or in a vitronectin-specific ELISA, as described previously (37). Briefly, Vn at 1 μg/ml in phosphate-buffered saline was coated overnight onto Immulon 2 (Dynatech Laboratories Inc.) microtiter plates in a volume of 100 μl at 4 °C, and all subsequent steps were performed at room temperature. The plates were washed with phosphate-buffered saline followed by distilled H₂O, allowed to air dry for 15 min, and then blocked with 200 μl of 3% bovine serum albumin in phosphate-buffered saline for 30 min. Next, PAI-1 samples in TBS containing 100 μg/ml BSA and 0.01% Tween 80 were added in a final volume of 100 μl, and incubation continued for 1 h, after which the unbound PAI-1 was washed away. During this incubation period <15% of the active PAI-1 should have converted to the latent form since we have determined the t₁/₂ for this conversion to be ~8 h at 23 °C in the absence of vitronectin (data not shown). In the functional assay, PAI-1 binding was determined by reacting the bound PAI-1 with 0.7 nM uPA for 30 min followed by the addition of the chromogenic substrate S-2444 (Kabi Pharmacia/Chromogenix) as described (49). The bound PAI-1 was then calculated from the loss of uPA amidolytic activity. Kᵣ values for the solid-phase binding of PAI-1 to immobilized Vn were calculated using the following form of the standard binding equation from the GrafIt program (Erithacus Software),

\[
y = \frac{[L] \cdot K_r + [L]}{K_r}
\]

where \( y \) is the amount of bound PAI-1, \( L \) is free PAI-1, and \( C \) is the Vn capacity for PAI-1 binding.

The Vn-dependent ELISA assay was performed as above except that bound PAI-1 was detected with affinity-purified, biotinylated, rabbit anti-PAI-1 antibodies (50) and streptavidin conjugated to alkaline phosphatase using the substrate p-nitrophenyl phosphate, disodium hexahydrate (Sigma) at a concentration of 4 mg/ml in 100 mM Tris-HCl, pH 9.5, 5 mM MgCl₂. To control for nonspecific binding, all assays were simultaneously analyzed on plates coated with BSA alone and processed in parallel. The background binding to BSA was subtracted from all samples before data analysis. For examination of the PAI-1-anhydrotrypsin complex binding to Vn, 1 μM (final concentration) anhydrotrypsin was included in all wells during the PAI-1 incubation step. This concentration of anhydrotrypsin was 20-fold higher than the highest concentration of PAI-1 tested and 10-fold higher than the reported \( K_r \) for the interaction of PAI-1 and anhydrotrypsin (17). For data analysis of the experiments, \( K_r \) was estimated for active PAI-1 with Equation 1 above by assuming that PAI-1 bound as a percent of the maximal binding was proportional to the actual PAI-1 bound and that free PAI-1 was approx. equal to PAI-1 added. For the inactive PAI-1 samples examined, no value for \( K_r \) could be established since none of these samples achieved saturation at the concentrations tested.

Competitive Inhibition of PAI-1 Binding to Immobilized Vitronectin by Solution-phase Vitronectin—Microtiter plates were coated with Vn and blocked with BSA as above. Next, either native or urea-purified Vn was added to the plates and serially diluted 3-fold in TBS containing 100 μg/ml BSA and 0.01% Tween 80 after which active PAI-1 was added to a final concentration of 2 nM (final volume, 100 μl). The samples were allowed to react for 1 h at 23 °C and washed, and bound PAI-1 was determined as in the ELISA assay as above. IgG values for the inhibition by solution-phase Vn were calculated using a four-parameter logistic fit (51) from the GrafIt program (Erithacus Software).

The Binding of PAI-1 to Vitronectin 7677
RESULTS AND DISCUSSION

Tomasini and Mosher (26) and Deng et al. (32) suggest that the controversy surrounding the interaction between Vn and PAI-1 results from the conformational variability of both proteins. In the current study we directly examined the binding of alternative conformations of PAI-1 to both native and urea-purified Vn. Previously we described a functional assay for PAI-1 binding to Vn in which active PAI-1 was shown to bind specifically to surface-adsorbed nVn in a dose-dependent and saturable manner (49). This assay was used to compare the binding of active wtPAI-1 with both forms of immobilized Vn (Fig. 1). These data demonstrate that both urea-purified and native Vn have a similar binding capacity for active PAI-1 and that active PAI-1 binds to both forms of Vn with high affinity. However, the calculated $K_d$ for the immobilized uVn is approximately 6-fold lower than for immobilized nVn ($127 \pm 20$ pM compared with $825 \pm 190$ pM). This difference may reflect the different conformational states of the two Vn preparations since nVn is predominately monomeric (53), whereas uVn is a disulfide-linked multimer (54). The observation that PAI-1 has a higher affinity for immobilized multimeric Vn than for immobilized monomeric Vn is consistent with the result that PAI-1 isolated from plasma is predominately in complex with a high molecular weight form of Vn (13) even though the majority of Vn in plasma is monomeric (55). Therefore, to see if solution-phase multimeric Vn also bound PAI-1 with higher affinity than solution-phase monomeric Vn, competitive inhibition assays were performed with both nVn and uVn competing for PAI-1 binding to immobilized nVn. These data are shown in Fig. 2 and demonstrate that both nVn and uVn compete for PAI-1 binding to immobilized nVn. This suggests that PAI-1 is binding to the same site on both nVn and uVn either when the Vn is in solution or immobilized. Furthermore, solution-phase uVn is a more efficient competitor for PAI-1 binding ($IC_{50}$ 65 nM) than is solution-phase nVn ($IC_{50}$ 375 nM). This approximately 6-fold difference is similar to that observed in Fig. 1 and suggests that either in solution or when immobilized uVn has a higher affinity for PAI-1 than does nVn. $K_d$ values of $20 \pm 1.4$ nM and $125 \pm 12$ nM for the interaction of PAI-1 with solution forms of uVn and nVn, respectively, were calculated from these data (see “Experimental Procedures”). This indicates that PAI-1 binds to immobilized Vn with a significantly higher affinity than to solution-phase Vn and has an approximately 150-fold higher $K_d$ for the solution-phase interaction with either form of Vn. This enhanced binding to immobilized Vn may result from the different conformation that Vn is known to assume when it adsorbs to a surface (56, 57).

To examine the binding of alternative conformational forms of PAI-1, an ELISA-based assay was performed. This assay is similar to the solid-phase assay shown in Fig. 1 except that PAI-1 is detected with an anti-PAI-1 antibody, permitting analysis of inactive conformations of PAI-1. Fig. 3 shows the binding of both active and latent PAI-1 to surface-adsorbed urea-purified and native Vn. Analysis of the binding of active PAI-1 to the two forms of immobilized Vn yields calculated $K_d$ values of $150 \pm 16$ pM with uVn and $1300 \pm 200$ pM with nVn. These values are similar to those calculated using the PAI-1 functional assay (Fig. 1), indicating that the indirect antibody assay is also suitable for evaluating PAI-1 binding to immobilized Vn. In contrast to active PAI-1, latent PAI-1 binds to both forms of immobilized vitronectin with much lower affinity. In this case no $K_d$ could be determined since saturable PAI-1 binding was not obtained at the concentrations tested. However, if we assume that latent PAI-1 is binding with the same stoichiometry as active PAI-1, then we can estimate a minimum value for $K_d$ of $>225$ nM (the highest concentration tested) in both cases (Fig. 3). These data are consistent with previous reports that only active PAI-1 binds to vitronectin with high affinity (13, 33, 34) and contradict the suggestion that both forms of PAI-1 bind to vitronectin with equal affinity (35, 36). The $K_d$ values calculated for active PAI-1 binding to immobilized Vn are also similar to previously reported values (127 pM (this study) versus $300$ pM (34) with uVn and $825$ pM (this study) versus $4.4$ nM (37) with nVn). An earlier report that calculated a lower affinity $K_d$ of $55$–$190$ nM for these interactions using a similar assay failed to account for the presence of both active and latent PAI-1 in the preparation and may have been measuring primarily the binding of latent PAI-1 (35). Consistent with this interpretation, the reported $K_d$ of $190$ nM is similar to our estimated minimum $K_d$ for latent PAI-1 binding to either native or uVn ($K_d > 225$ nM) (Fig. 3). These authors also noted a high affinity, “low capacity” binding site ($K_d < 100$ pM) that may have represented the active PAI-1 in their preparation (35).

The observation that latent PAI-1 binds to Vn with a much lower affinity than active PAI-1 suggests that the conformational change associated with conversion to the latent form may be responsible for the reduced affinity. In a previous study (37) we suggested that the stabilization of PAI-1 by Vn occurs when Vn binding to strand 1 of $\beta$-sheet A limits the mobility of $\beta$-sheet A necessary for insertion of the PAI-1 RCL during transformation to the latent conformation. This model is com-
The binding of PAI-1 to vitronectin.

Recent studies of the serpin mechanism of inhibition indicate that it is a complex process that requires an exposed RCL (21, 22, 24, 60). Upon association with a target proteinase, the serpin RCL is cleaved at its P$_1$-P$_1’$ bond, and the RCL is inserted into $\beta$-sheet A, yielding the stable serpin-proteinase complex. In the present study we demonstrate that the PAI-1 Vn binding site on the edge of $\beta$-sheet A is sensitive to this conformational change in $\beta$-sheet A as well as to similar changes associated with conversion of PAI-1 to the latent form or cleavage in the RCL by a nontarget proteinase. This sensitivity may provide a way to ensure the expression of PAI-1 activity at specific sites of action. For example, it is thought that Vn serves to localize PAI-1 to the extracellular matrix where it regulates local proteolytic activity (59) and blocks cell adhesion and migration (30, 31). In this setting it may be beneficial to permit only functionally active PAI-1 to bind to vitronectin. On a cell surface an inactive ligand can be internalized and degraded. However, this type of regulation may not be as efficient on the less dynamic extracellular matrix. Therefore, to prevent Vn from becoming saturated with inactive forms of PAI-1, a system may have been selected that is sensitive to the conformational state of PAI-1, which in turn is closely linked to its activity state.

Acknowledgments—We thank S. Muhammad for excellent technical assistance, M. Sandkvist for helpful discussions, and K. Ingham for a critical reading of the manuscript.

REFERENCES

1. Vassalli, J.-D., Sappino, A.-P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1072.
