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The Use of Fluorescent Probes to Characterize Conformational Changes in the Interaction between Vitronectin and Plasminogen Activator Inhibitor-1*

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Plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of tissue-type plasminogen activator and urokinase, is known to convert readily to a latent form by insertion of the reactive center loop into a central β-sheet. Interaction with vitronectin stabilizes PAI-1 and decreases the rate of conversion to the latent form, but conformational effects of vitronectin on the reactive center loop of PAI-1 have not been documented. Mutant forms of PAI-1 were designed with a cysteine substitution at either position P1′ or P9 of the reactive center loop. Labeling of the unique cysteine with a sulfhydryl-reactive fluorophore provides a probe that is sensitive to vitronectin binding. Results indicate that the scissile P1–P1′ bond of PAI-1 is more solvent exposed upon interaction with vitronectin, whereas the N-terminal portion of the reactive loop does not experience a significant change in its environment. These results were complemented by labeling vitronectin with an arginine-specific coumarin probe which compromises heparin binding but does not interfere with PAI-1 binding to the protein. Dissociation constants of approximately 100 nM are calculated for the vitronectin/PAI-1 interaction from titrations using both fluorescent probes. Furthermore, experiments in which PAI-1 failed to compete with heparin for binding to vitronectin argue for separate binding sites for the two ligands on vitronectin.

The adhesive glycoprotein, vitronectin, circulates in human plasma at concentrations of 200–400 $\mu g \cdot ml^{-1}$ and serves as a regulatory protein in humoral defense mechanisms by interacting with macromolecules in the reaction cascades of coagulation and fibrinolysis (reviewed in Refs. 1–3). The circulating form of vitronectin is a monomer of 72 kDa, and vitronectin is also found in a multimeric form in platelet releasates and in the extracellular matrix (4–6). The anti-fibrinolytic protein, plasminogen activator inhibitor-1 (PAI-1),[1] is the major inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator (7–11, reviewed in Refs. 12, 13). Like other serpins, PAI-1 has a reactive center loop that mimics the substrate of its target proteases (14, 15). The active conformation of PAI-1 is relatively unstable, so that the protein undergoes rapid conversion to a latent conformation which is characterized by the insertion of the reactive center loop into a central β-sheet within the molecule (16). Interactions between strands of the β-sheet and the reactive loop stabilize this conformation relative to the active conformation, in which the loop is thought to protrude from the surface of the molecule (7, 16).

Binding to vitronectin results in a 2–3-fold increase in the half-life of active PAI-1 (17–19). In addition to stabilizing the active conformation of PAI-1, vitronectin also alters the protease specificity of the serpin so that the vitronectin·PAI-1 complex is endowed with the additional ability to inhibit thrombin (20, 21). A vitronectin-binding site has been localized on the surface of PAI-1 using site-directed mutagenesis (22) and monoclonal antibodies (23). Binding of vitronectin is thought to restrict the movement of the central β-sheet in PAI-1 by interactions that bridge the β-sheet and adjacent secondary structural elements and thus prevent insertion of the reactive center loop (22). Based on the observation that vitronectin alters PAI-1 protease specificity, it can be hypothesized that vitronectin binding causes conformation changes in the reactive center loop as well. Fa *et al.* (24) have demonstrated that vitronectin binding causes a decrease in the anisotropy and increased rotational freedom of fluorescent reporters incorporated into the reactive center loop of PAI-1. Other details of the vitronectin-induced conformational changes in PAI-1 are uncharacterized.

Very little is known about concomitant changes that occur in vitronectin when it interacts with the serpin. Moreover, there is considerable debate in the literature regarding the PAI-1-binding site(s) in vitronectin (reviewed in Ref. 25). Reports utilizing synthetic peptides or proteolytic fragments do not agree, with some results localizing the PAI-1-binding site to the heparin-binding sequence located near the C terminus of vitronectin (26–30), others to the N-terminal somatomedin B region (31–34), and yet another to a polypeptide consisting of residues 115–121 from vitronectin (35). More recent work utilizing heterologous expression systems has focused on the somatomedin B domain of vitronectin, which contains eight cysteines thought to form a "disulfide knot" at the N terminus of

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[1] The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; tPA, tissue-type plasminogen activator; NBD, *N,N′*-dimethyl-*N*-(acetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; IANBD, iodoacetamide NBD; S338C, recombinant PAI-1 with cysteine substituted for serine 338; M347C, recombinant PAI-1 with cysteine substituted for methionine 347; NBDP1′PAI-1, M347C mutant form of PAI-1 labeled with NBD; NBDP9PAI-1, S338C mutant form of PAI-1 labeled with NBD; HOCGO, 7-hydroxycoumarinyl-3-glyoxal; HOCGO-VN, vitronectin labeled with HOCGO; PEG, polyethylene glycol; BSA, bovine serum albumin; HRP, horseradish peroxidase.

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vitronectin. Segments of the N-terminal somatomedin B domain expressed as fusion proteins with the maltose-binding protein in *Escherichia coli* were shown to bind and stabilize the active conformation of PAI-1 (33). In order to localize sequences critical for PAI-1 binding, Deng *et al.* (34) generated chimeras between segments of the vitronectin somatomedin B domain and complementary sequences in other inactive somatomedin B homology domains. These studies indicated that the essential PAI-1 binding determinant was located between residues 12 and 30 of vitronectin, and alanine scanning mutagenesis revealed that all 8 cysteines and Gly-12, Asp-22, Leu-24, Tyr-27, Tyr-28, and Asp-34 are essential to maintain PAI-1-binding activity (34).

To gain further insight into the structural changes that occur in both vitronectin and PAI-1 as they interact, and to understand more thoroughly the structural requirements for the interaction, a strategy has been employed for following the conformational changes in the molecules using fluorescent reporter groups. With site-directed mutagenesis, cysteines have been engineered into positions P1′ and P9 of the reactive center loop in PAI-1, and the mutant proteins have been labeled with the sulfhydryl-reactive probe, IANBD (36, 37). Since wild type PAI-1 contains no cysteines, these fluorescent probes can be exploited to obtain information about the local environment of the reactive center loop at these positions. This work extends the studies of Fa *et al.* (24) by evaluating probes at different sites within the reactive center loop and evaluating whether conformational changes are associated with increased or decreased solvent exposure of the probe. Also, a parallel experimental approach was taken in which an arginine-reactive coumarin derivative (38) was used for site-specific labeling of the arginine-rich heparin-binding region of vitronectin.

Fluorescence spectroscopy of the labeled protein derivatives has been used to gain insight into the interaction of intact vitronectin and PAI-1 in solution. The following questions were of interest and guided these studies. Is the conformation of the reactive center loop of PAI-1 altered upon interaction with vitronectin? Does binding of monomeric and multimeric forms of vitronectin result in similar changes in the reactive center loop of PAI-1? Can the heparin-binding domain of vitronectin be preferentially labeled with an arginine-specific coumarin derivative? Are there conformational changes in vitronectin that are induced by interaction with PAI-1? Do PAI-1 and heparin share a common binding determinant in vitronectin?

EXPERIMENTAL PROCEDURES

Materials—The fluorescent probe NBD was obtained from Molecular Probes. Heparin purified from porcine mucosa grade 1-A was obtained from Sigma. Murine monoclonal antibodies directed against human vitronectin were obtained from Quidel. Rabbit anti-PAI-1 antiserum was a generous gift of Dr. Daniel Lawrence, Holland Laboratory, American Red Cross, Rockville, MD. Polyclonal antiserum made in rabbit against human vitronectin was obtained from Rockland Laboratories. Peroxidase-linked goat anti-rabbit IgG was obtained from Vector Laboratories. All other reagents were of the highest grade commercially available and were used without further purification.

Vitronectin was purified from human plasma by a modification of the original protocol of Dahlback and Podack (39), as described by Bittorf *et al.* (40). The purity of the protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie staining (41). The molecular weight of vitronectin is 72,000, and the protein concentration was calculated using an extinction coefficient of 1.02 $ml \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm (42). Multimeric vitronectin was prepared by incubating plasma-purified vitronectin with 8 M urea overnight at room temperature, and the denaturant was removed by extensive dialysis into PBS (0.05 M phosphate, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA) (42, 43). Vitronectin was stored at 4 °C until use. Wild type recombinant PAI-1 was purified from *E. coli* strains engineered to overexpress the protein (generously provided by Dr. David Ginsburg, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor), essentially as described by Lawrence *et al.* (7). PAI-1 purified from cells expressing the protein was separated into its active and latent components using the protocol determined by Kvassman and Shore (44). Recombinant forms of PAI-1 with cysteine substituted for serine 338 (S338C) or cysteine substituted for methionine 347 (M347C) were prepared as described in Shore *et al.* (37).

Fluorescent Labeling of S338C and M347C—The protocol for labeling and quantifying the P1′ and P9 PAI-1 mutants has been described previously (37). Briefly, concentrated samples of the purified proteins were applied to a PD-10 gel filtration column (Bio-Rad) that had been equilibrated with 0.05 M sodium phosphate, pH 6.6, containing 0.15 M NaCl, 1 mM EDTA, 0.01% (v/v) Tween 80, and 150 mM IANBD. The sample was allowed to react for 8 h at 25 °C in the dark. Fractions containing PAI-1 were pooled and applied to a Sephadex G-25 superfine column (Pharmacia Biotech, Inc.) to separate the protein from free dye. Labeling stoichiometry was determined using absorbance measurements made at 280 and 497 nm in 0.1 M Tris-HCl, pH 8.5, containing 6 M guanidine and 1 mM EDTA. Extinction coefficients of 26,000 and 43,000 $M^{-1}\ cm^{-1}$ were used to calculate the concentrations of NBD label and PAI-1, respectively. A correction factor of 0.103 ($\epsilon_{280}/\epsilon_{497}$) was used to correct the 280-nm absorbance of the labeled PAI-1 for the contribution of NBD (37). Samples were stored frozen at −20 °C until use.

Fluorescent Labeling of Human Vitronectin—The arginine-reactive probe, hydroxycoumarin glyoxal (HOCGO) was prepared as described by Baburaj *et al.* (38). Vitronectin (2 μM) was mixed with 10 μM HOCGO in 50 mM HEPES, pH 7.5, containing 1 mM sodium borate. The reaction was allowed to proceed in the dark at room temperature for 16 h. Borate and unreacted probe were separated from the protein by gel filtration on a Sephadex G-25 column (Pharmacia Biotech, Inc.), equilibrated in 50 mM HEPES, pH 7.5. EDTA was added to a final concentration of 1 mM for storage of the sample at 4 °C. The labeling stoichiometry was determined spectrophotometrically at 374 nm using an extinction coefficient of 14,200 $M^{-1}\ cm^{-1}$. Protein concentration was determined using a bicinchoninic acid assay (Pierce). The same protocol was used to label vitronectin in the presence of 0.4 mM heparin.

Fluorescence Measurements—Changes in the fluorescence emission/excitation ratio of NBD at the P1′ and P9 positions of PAI-1 were measured using an SLM 8000 spectrofluorimeter with the excitation monochromator set at 480 nm and the emission wavelength set for 540 nm. Excitation and emission slits were set at 4 and 8 mm, respectively. Solutions of NBD-P1′ PAI-1 (490 nM) in 0.1 M HEPES, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, and 0.1% (w/v) PEG-8000 were titrated with small volume aliquots from a stock of purified human vitronectin (8–30 μM) to a final concentration of 1.25 μM. Reactions were performed in a total volume of 2.0 ml in acrylic cuvettes (Sarstedt) that were previously coated with a 1.0% (w/v) solution of PEG-20,000, according to the procedure of Latallo and Hall (45). A quench in fluorescence was observed that was dependent on manipulation of the sample and was possibly due to adsorption of the PAI-1 onto the surfaces of the cuvette and pipette tips. To correct for this quench, duplicate titrations were always performed. Vitronectin was added to the NBD-PAI-1 solution in one cuvette. To an identical sample in the other cuvette, equal volumes of buffer were added to obtain the F_0 values. The data were then normalized using the equation $(F - F_0)/F_0$, where F is the ratio of emission/excitation intensities at each vitronectin addition, and F_0 is the emission/excitation ratio of NBD-P1′ PAI-1 in the absence of added vitronectin. Scans were performed over a wavelength range of 500–600 nm using an excitation wavelength of 480 nm.

The interaction between HOCGO-VN and PAI-1 was analyzed by measuring changes in the fluorescence intensity of the coumarin-derived probe upon interaction with wild type PAI-1. A Perkin-Elmer LS50B luminescence spectrometer with the excitation monochromator set at 335 nm was used to measure fluorescent intensity of HOCGO-VN at 453 nm, the emission maximum of HOCGO-VN. The reaction was performed in a 1.0-ml quartz cuvette (Hellma) in 0.1 M HEPES, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, and 0.1% (w/v) PEG-8000. HOCGO-VN (290 nM) was titrated by addition of small volume aliquots of active wild type PAI-1 (26 μM stock) to a final concentration of 1.6 μM. The data were corrected for dilution and normalized using the relationship $(F - F_0)/F_0$, where F is the emission at each PAI-1 addition and F_0 is the emission of HOCGO-VN in the absence of PAI-1.

For both measurements the $(F - F_0)/F_0$ was plotted as a function of titrant concentration using Kaleidagraph software and fit to Equation 1 describing the binding isotherm to obtain the F_{max} and K_d (46):

$$\Delta F = \Delta F_{max}/2R^*(R + T + K_d - ((R + T + K_d)^2 - 4TR)^{1/2}) \quad \text{(Eq. 1)}$$

where ΔF is the change in fluorescence $(F - F_0)/F_0$, R is the total concentration of fluorescent protein, T is the total titrant concentration, K_d is the dissociation constant, and F_{max} is the fluorescence at saturating concentrations of titrant.

Iodide Quenching Studies—For iodide quenching studies, solutions of 130 nM HOCGO-VN (prepared in the presence or in the absence of 0.4 mM heparin) in 50 mM HEPES, pH 7.5, were titrated with 5 M NaI, prepared in the same buffer. Using an excitation wavelength of 335 nm, the fluorescence emission of the labeled protein was measured at 453 nm after each addition of NaI (47). Likewise, the extent of iodide quenching of the coumarin-derived probe on HOCGO-VN was measured in the absence and presence of saturating concentrations of PAI-1. A 130 nM solution of HOCGO-VN in 0.1 M HEPES, pH 7.4, containing 0.1 M NaCl, 1 mM EDTA, and 0.1% (w/v) PEG-8000 was titrated with NaI to a final concentration of 480 mM (47). A similar quenching experiment was performed after the addition of 1.1 μM PAI-1 to a vitronectin solution (130 nM). Fluorescence emission measurements were made after each addition of iodide, as described above.

Measurements of Vitronectin Binding to PAI-1 in Microtiter Dishes—Interactions between recombinant wild type PAI-1 and HOCGO-VN or unmodified vitronectin were measured using a slight modification of the competitive binding assay described by Seiffert and Loskutoff (31). Briefly, microtiter plates were coated with 50 μl of a 1 $\mu g \cdot ml^{-1}$ solution of native human vitronectin at 4 °C for 16 h. After washing the plates three times with PBS, the wells were blocked with 200 μl of 3.0% (w/v) BSA in PBS at 37 °C for 1 h. The wells were washed three times with PBS/Tween/BSA (PBS containing 0.1% BSA (w/v) and 0.1% Tween 20 (v/v)) after this and all subsequent incubation steps. Serial dilutions of vitronectin (3 $\mu g \cdot ml^{-1}$ to 0.0003 $\mu g \cdot ml^{-1}$) in PBS/Tween/BSA were added to the plates and mixed with 0.4 nM PAI-1 to give a final PAI-1 concentration of 0.2 nM in 100 μl. Polyclonal anti-PAI-1 antibodies diluted 1:10,000 in PBS/Tween/BSA, followed by HRP-linked goat anti-rabbit IgG (1:1000) were used to detect bound PAI-1. The plates were developed with a 0.2 $mg \cdot ml^{-1}$ solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 50 mM sodium citrate, pH 5.5, containing a 1:2000 dilution of 30% hydrogen peroxide. The extent of PAI-1 binding was determined by measuring the absorbance at 405 nm in a Microtek microplate reader. Several absorbance readings were performed over time after addition of substrate to ensure that data were in the linear range of the assay.

The effect of heparin on the vitronectin·PAI-1 interaction was determined by preincubating serial dilutions of vitronectin with 0, 0.4, 4.0, and 40 μM heparin before adding it to PAI-1 in the above assay. Also, a noncompetitive PAI-1 binding assay which measures PAI-1 binding to solid phase vitronectin was performed. In this experiment, PAI-1 was serially diluted and added to vitronectin-coated plates in the presence or absence of 0.5 $\mu g \cdot ml^{-1}$ heparin. PAI-1 binding was detected and quantified as described above.

Heparin Binding Immunoassay—The effect of PAI-1 on the vitronectin/heparin interaction was analyzed using an assay that measures protein binding to heparin-coated microtiter plates. For coating, a 100-μl solution of heparin (1.0 $mg \cdot ml^{-1}$ in 50 mM sodium carbonate, pH 9.6) was incubated in microtiter wells for 16 h at 4 °C. The plates were washed three times with PBS/casein/Tween (PBS containing 0.1% (w/v) casein and 0.1% (v/v) Tween 20) after this and all other incubation steps. Nonspecific binding was prevented using a blocking solution of PBS containing 0.03% casein (w/v) and 0.05% (v/v) Tween 20. After blocking, the plates were incubated with serial dilutions of vitronectin in the presence or absence of 0.6 μM PAI-1 for 2 h at 37 °C. The plates were probed for vitronectin binding using polyclonal anti-vitronectin (1:5000) followed by goat anti-rabbit IgG conjugated to HRP (1:1000). Duplicate plates were also probed for PAI-1 binding using polyclonal anti-PAI-1 (1:10,000) followed by HRP-linked goat anti-rabbit IgG. All dilutions were made in PBS/casein/Tween. The amount of bound protein was quantified by developing and measuring the absorbance at 405 nm as described above.

PAI-1 Activity Assay—Kinetic assays were designed to measure the rate at which PAI-1 converts to the latent form by measuring the concentration of active PAI-1 at any given time by its ability to inhibit urokinase. PAI-1 (0.48 μM final concentration) was incubated for varying periods at 37 °C in a buffer of 25 mM HEPES, pH 7.4, containing 50 mM NaCl, 0.01% (w/v) BSA, and 1.3 mM EDTA. A reaction was performed in parallel, with coumarin-labeled vitronectin added to the PAI-1 sample in a final concentration of 0.5 μM. Aliquots (50 μl) of PAI-1 or the PAI-1/vitronectin mixture were removed at intermediate times up to 8 h and were assayed for remaining PAI-1 activity by incubating with 0.1–0.2 μM urokinase in 200 μl of 0.1 M HEPES, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, 0.1% (w/v) PEG-8000, and 0.2% (w/v) BSA. After a 30-min incubation of PAI-1 or the PAI-1·vitronectin complex with urokinase at room temperature, residual urokinase activity was measured by hydrolysis of the chromogenic substrate, S-2444 (Kabi-Hepar, Pharmacia).

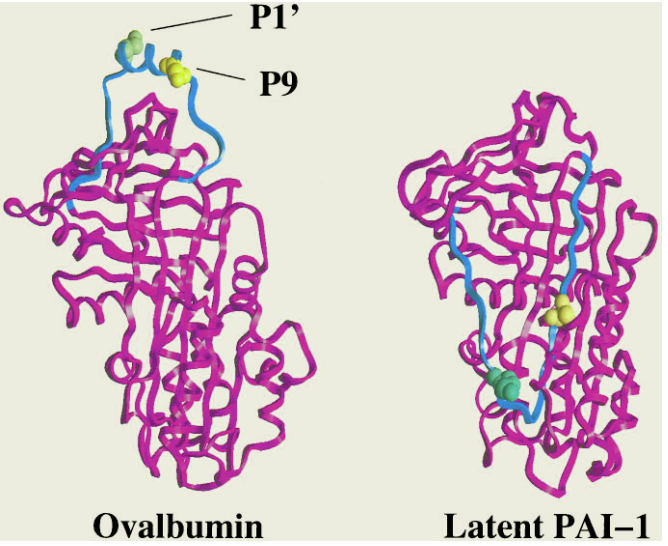

FIG. 1. **Crystal structures that represent models for latent and active serpins.** Ribbon diagrams of latent PAI-1 and ovalbumin were generated using the Insight program on a Silicon Graphics workstation. Coordinates from the x-ray crystal structures of the two proteins (20, 52) were used. β-Sheet A is visible on the surface of latent PAI-1 and has been identified as a vitronectin-binding site. The reactive center loop is shown in *blue*. In the active form of PAI-1, the reactive center loop is thought to be an exposed loop extending from the β-sheet on the surface of the protein, similar in structure to the homologous region shaded in *blue* in the ovalbumin structure. Amino acids depicted in the *green* and *yellow* space-filling models represent the P1′ and P9 positions, respectively. Note that the P1′ and P9 residues are located within the exposed reactive center loop in the model for the active structure, and these two amino acids are buried within the hydrophobic core of the protein in the latent form of PAI-1.

RESULTS

A Fluorescent Probe in the Reactive Center Loop of PAI-1 Can Be Used to Detect Vitronectin-induced Conformational Changes—Recombinant forms of PAI-1 with M347C and S338C mutations at the P1′ and P9 positions of the reactive center loop (36, 37), respectively, were expressed in *E. coli* and purified. Using the sulfhydryl-specific fluorophore, IANBD, the unique cysteines in the two proteins were fluorescently labeled at a 1:1 stoichiometry. Fig. 1 shows ribbon diagrams derived from the crystal structures of latent PAI-1 (16) and of ovalbumin (48), a serpin-like molecule thought to exhibit structural features resembling the active conformation of PAI-1. The *green* and *yellow* space-filling symbols in the diagram denote the positions of the NBD probe at the P1′ and P9 positions, respectively. The activity of both mutant forms of PAI-1 was comparable with that exhibited by the wild type protein (36, 37). The fluorescence of the labeled proteins was monitored as the NBD-PAI-1 mutants were titrated with native human vitronectin. Vitronectin binding resulted in a 10% quench in the fluorescence of the probe at the P1′ position of the reactive center loop. Fig. 2*A* shows the spectra of NBDP1′PAI-1 in the presence and absence of saturating vitronectin. When NBDP1′PAI-1 was titrated with vitronectin, a dissociation constant of 100 ± 50 nM was obtained (Fig. 2*B*). These data indicate that vitronectin induces a conformational change in the reactive center loop of PAI-1 that shifts the probe at the scissile bond into a somewhat more hydrophilic milieu. In contrast, no fluorescence changes were detected when NBDP9PAI-1 was titrated with vitronectin (Fig. 2*B*).

Native and Multimeric Vitronectin Differ in Effects on the

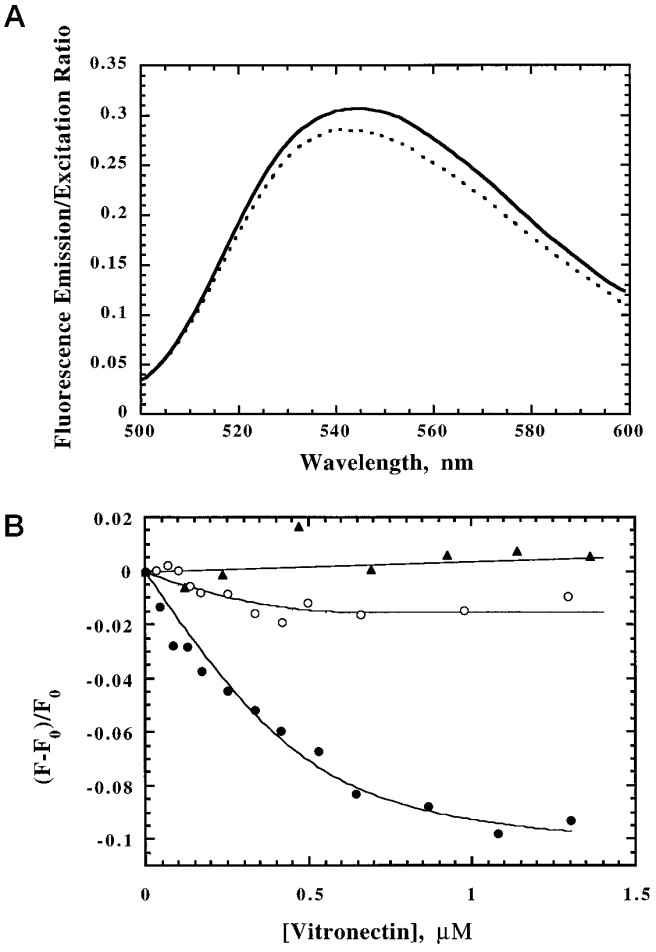

FIG. 2. **Fluorescence changes in NBDP1′PAI-1 upon interaction with vitronectin.** *A* shows the fluorescence emission spectrum (excitation wavelength = 480 nm) for a 490 nM solution of NBDP1′PAI-1 in the presence (*dashed line*) and absence (*solid line*) of native vitronectin added to a 1.3 μM final concentration. *B* shows binding isotherms for the interaction between NBDP1′PAI-1 and native vitronectin (●), NBDP1′PAI-1 and multimeric vitronectin (○), and NBDP9 PAI-1 with native vitronectin (▲). Fluorescence intensity, using an excitation wavelength of 480 nm and an emission wavelength of 540 nm, was recorded following each addition of vitronectin. The data for native vitronectin interacting with NBDP1′PAI-1 (●) were analyzed as described in "Experimental Procedures" to generate the binding isotherm shown with the *solid line*.

Scissile Peptide Bond in PAI-1—Interpretation of previous studies on the interaction between PAI-1 and vitronectin have been complicated by the fact that vitronectin, like PAI-1, exists in an alternative form. Although vitronectin purified from plasma exists as a monomer with a molecular weight of 72,000 (42), the glycoprotein is found as a high molecular weight species in the extracellular matrix and in platelet releasates (4–6). Multimeric vitronectin which is produced *in vitro* has been characterized extensively and is considered to be the product of an alternative folding pathway (6, 40, 42, 43). Potential differences between PAI-1 binding by the native and multimeric forms of vitronectin are not clear, although multimeric vitronectin may have a higher capacity for PAI-1 binding when bound to a solid phase (30). It has been demonstrated that vitronectin in the extracellular matrix stabilizes the active conformation of PAI-1 almost 10-fold (49), a much higher degree of stabilization than is observed with plasma-derived vitronectin. While many reports indicate that multimeric forms of vitronectin are capable of binding PAI-1 (17, 30), the binding of multimeric vitronectin is contrasted with native vitronectin since it does not alter PAI-1 substrate specificity (21). The data in Fig. 2*B* indicate that the multimeric form of vitronectin did not alter the fluorescence of NBDP1′PAI-1. Thus, multimeric vitronectin does not alter the conformation of the reactive center loop at the scissile bond of PAI-1 in the same manner as does native vitronectin, providing separate evidence for differential effects of the two forms of vitronectin on the reactive center loop of the inhibitor.

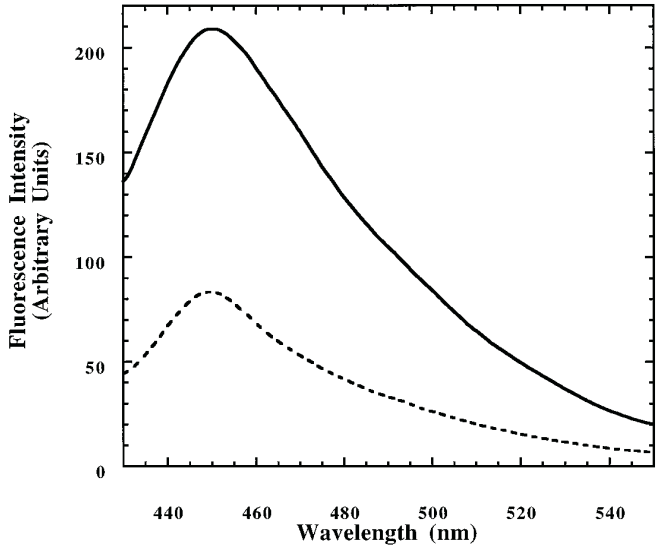

FIG. 3. **Fluorescence emission spectra of HOCGO-modified vitronectin.** Native vitronectin (2 μM) was labeled with a 5:1 molar ratio of hydroxycoumarin glyoxal to protein in the presence and absence of added heparin as described under "Experimental Procedures." The fluorescence emission spectra of the labeled products produced in the absence (*solid line*) and presence (*dashed line*) of heparin added to a 0.4 mM concentration are shown. The excitation wavelength was 335 nm.

Heparin Binding Protects Exposed Arginines from Modification with Hydroxycoumarin Glyoxal—The sequence spanning residues 341–378 near the C terminus of vitronectin has been identified as the heparin-binding determinant (28, 50) and as a putative PAI-1-binding site (26–30). The region contains 7 of the 35 arginine residues found within vitronectin and fits the heparin-binding consensus sequence described by Cardin and Weintraub (51) and Sobel *et al.* (52). The novel arginine-reactive fluorescent probe, hydroxycoumarin glyoxal (38), was exploited with the goal of preferentially labeling the arginine-rich heparin-binding domain of vitronectin with an environmentally sensitive reporter group. Native vitronectin was treated with the HOCGO probe under mild reaction conditions favoring a low labeling stoichiometry. Unreacted probe was removed from the protein by gel filtration, and the absorption and fluorescence spectra of the modified vitronectin were examined. Fig. 3 shows the fluorescence spectrum of HOCGO-VN. The labeling stoichiometry was found to be approximately 2 mol of HOCGO per mol of vitronectin.

In order to determine whether the probe was targeted to the heparin-binding domain, studies were performed to assess the effect of heparin on the labeling reaction. It was rationalized that if the probe was preferentially incorporated into the glycosaminoglycan-binding domain, heparin binding would protect this arginine-rich sequence from labeling. The *dashed line* in Fig. 3 represents the fluorescence spectrum of vitronectin labeled in the presence of saturating concentrations of heparin. Although fluorescence from the coumarin probe is detected in the vitronectin sample labeled in the presence of heparin, the fluorescence intensity is significantly diminished in comparison to the coumarin probe incorporated into vitronectin upon labeling in the absence of heparin. The decreased fluorescence intensity correlates with decreased labeling, supporting the

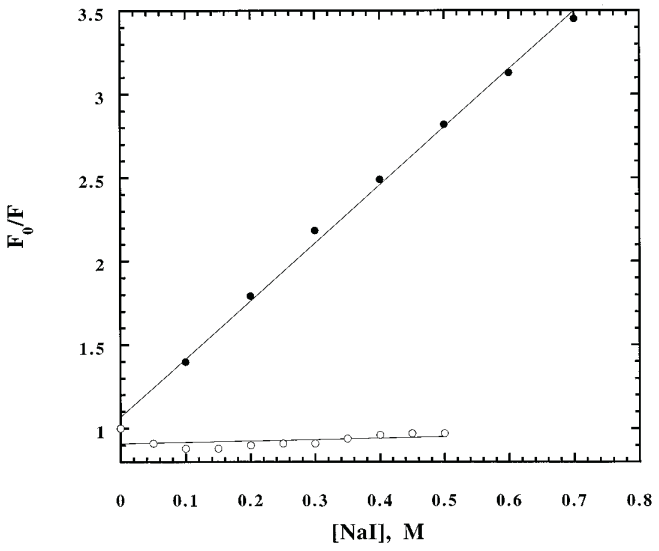

FIG. 4. **Stern-Volmer plots for iodide quenching of HOCGO-VN.** HOCGO-VN prepared in the absence (●) and presence (○) of heparin added in saturating concentrations was titrated with 5 M NaI. Data are plotted as Stern-Volmer quenching curves, with F_0/F plotted on the *y* axis against the molar concentration of iodide on the *x* axis. F_0 represents the initial fluorescence of the sample prior to addition of NaI, and *F* represents the fluorescence intensity at a given concentration of iodide. Fluorescence emission at 453 nm was measured after each addition of iodide.

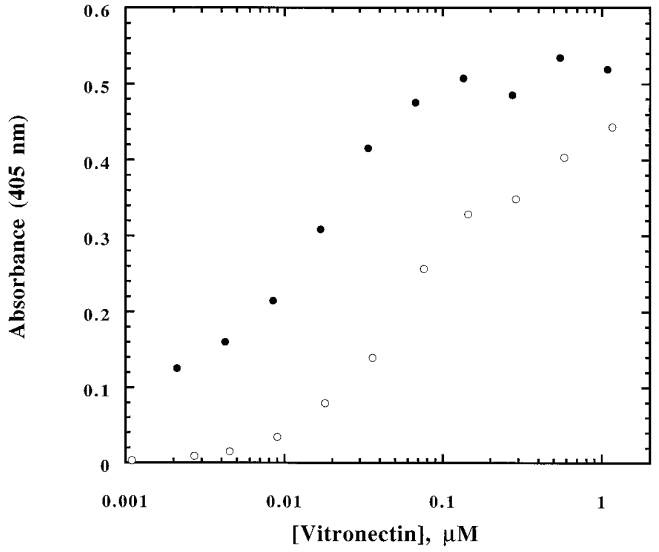

FIG. 5. **Heparin binding immunoassays comparing modified and unmodified vitronectin.** Unlabeled native vitronectin (●) or HOCGO-VN (○) was serially diluted in heparin-coated microtiter plates. Bound vitronectin was detected with anti-vitronectin antibodies as described under "Experimental Procedures." Absorbance at 405 nm (average of three measurements) is plotted *versus* the vitronectin concentration (μM) expressed on a logarithmic scale.

hypothesis that heparin competes with the coumarin label for binding to the heparin-binding site in vitronectin.

To investigate further the localization of HOCGO within vitronectin, iodide quenching studies were performed on the vitronectin samples labeled in the presence or absence of added heparin (Fig. 4). Such studies provide information about the solvent accessibility of fluorescent moieties on proteins (47). Vitronectin treated with HOCGO in the absence of heparin is susceptible to quenching by iodide; however, the product of labeling in the presence of heparin is not accessible to the quencher. Together, these data indicate that two distinct species of modified vitronectin are generated when the protein is treated with HOCGO in the absence or presence of added heparin. The results suggest that surface-exposed arginines react preferentially with coumarin glyoxal in the absence of heparin. Conversely, in the presence of heparin, these arginines are protected from labeling, and arginine(s) that are less exposed in uncomplexed vitronectin become modified. The observation that heparin protects labeling of certain arginines infers that these residues are located within the heparin-binding region.

HOCGO-VN Displays Weakened Heparin-binding Activity—It was predicted that modification of arginines within the glycosaminoglycan-binding domain should have a deleterious effect on heparin binding. To determine whether incorporation of the probe had disrupted heparin-binding determinants, interaction of heparin with HOCGO-VN was compared with that of the unlabeled protein. To achieve the same level of heparin binding, 10-fold higher concentrations of HOCGO-VN were required compared with unmodified vitronectin (Fig. 5). These data indicate that modification of arginine residue(s) disrupts critical heparin-binding elements. Along with observations that heparin protects exposed arginines from modification, these data suggest that the fluorescent probes in HOCGO-VN are incorporated into the heparin-binding domain of vitronectin. Studies are currently underway to identify the specific arginines labeled with coumarin glyoxal.

HOCGO-modified Vitronectin Binds and Stabilizes PAI-1—HOCGO-VN was tested for its ability to bind PAI-1. If heparin and PAI-1 share the same binding determinants, a modification that weakens heparin binding would also be expected to alter PAI-1 binding. HOCGO-VN was tested for the ability to bind PAI-1 using a competitive binding assay in which PAI-1 was incubated with vitronectin-coated microtiter plates in the presence of increasing concentrations of competing vitronectin in solution (31). The fluorescently labeled vitronectin competed effectively with immobilized, unmodified vitronectin for binding of PAI-1 (Fig. 6).

A direct consequence of vitronectin binding to PAI-1 is that the half-life of the active conformation of the serpin is increased 2–3-fold (17–19). In order to test whether the arginine-modified vitronectin could stabilize the serpin, PAI-1 was incubated with HOCGO-VN, and the ability of PAI-1 to inhibit urokinase was measured over time. As shown in Fig. 7, the activity of PAI-1 in the absence of vitronectin decreases 50% within 1 h, indicative of conversion of the serpin to its inactive, latent form. When PAI-1 is incubated with HOCGO-VN, activity of the serpin is stabilized considerably, with the half-life increasing to 6 h. This behavior reflects the PAI-1 stabilizing property which has been well characterized with unmodified vitronectin (17–19). Thus, while the fluorophore on HOCGO-VN weakens heparin binding, it does not prevent PAI-1 binding or PAI-1 stabilization. From these results, it can be concluded that the heparin-binding element on vitronectin that is disrupted by the arginine-reactive fluor is not essential for binding or stabilizing PAI-1.

PAI-1 Induces Conformation Changes in Vitronectin That Lead to a Quench in the Fluorescence of the Coumarin Probe—The arginine-reactive probe incorporated into vitronectin was exploited for the purpose of detecting PAI-1-induced conformational changes in vitronectin. Fig. 8 demonstrates that the emission of the fluor is quenched approximately 15% when HOCGO-VN is complexed with PAI-1. When a 260 nM solution of HOCGO-VN is titrated with PAI-1, a binding isotherm is obtained which gives a K_d of 112 ± 15 nM, a value that is in excellent agreement with the dissociation constant for the vitronectin·NBDP1′PAI-1 reaction. The quench in fluorescence suggests that when PAI-1 binds vitronectin, a conformational

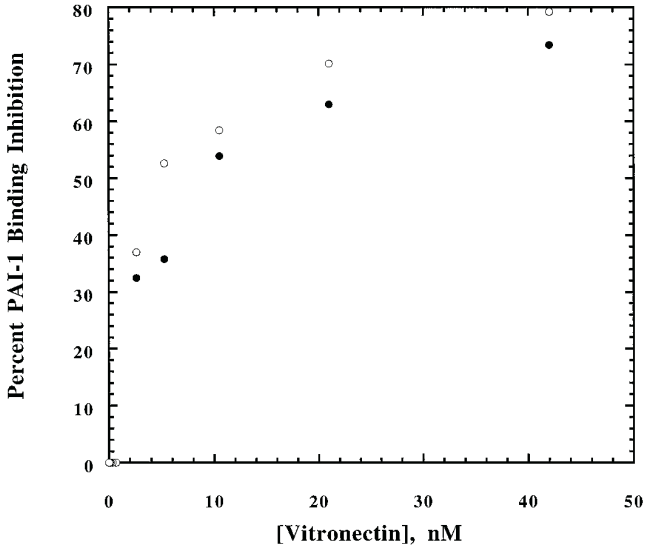

FIG. 6. **PAI-1 binding activity of HOCGO-VN.** HOCGO-VN (○) was assayed for the ability to compete with immobilized unmodified vitronectin for binding PAI-1. The binding activity of modified vitronectin is compared with the PAI-1 binding of unmodified vitronectin (●) in the same assay. The absorbance of samples in wells containing PAI-1 and no competing vitronectin provided the value for 100% binding. Percent binding inhibition was determined by subtracting the absorbance at each concentration of competing vitronectin from this value and then dividing by the absorbance in the absence of competing vitronectin.

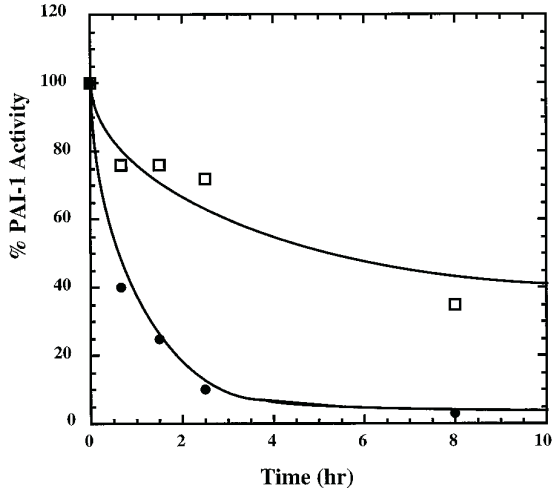

FIG. 7. **Kinetics of PAI-1 stabilization by HOCGO-VN.** PAI-1 was incubated at 37 °C in the presence (□) or absence (●) of HOCGO-VN for periods up to 8 h to measure stabilizing effects of the vitronectin sample on PAI-1 activity. At intermediate time intervals, remaining active PAI-1 was determined by its ability to inhibit urokinase in a colorimetric assay, as described in "Experimental Procedures."

change occurs such that the fluorescent probe is shifted somewhat to a more hydrophilic environment. In contrast, the addition of saturating concentrations of heparin did not induce any changes in the fluorescence of the probe (data not shown), a result which is not surprising since heparin binding to HOCGO-VN is compromised.

PAI-1 Binding Does Not Protect the Coumarin Label on Vitronectin from NaI Quenching—Alterations in the fluorescence of the coumarin probe upon PAI-1 binding could result from direct interaction of PAI-1 in the vicinity of the coumarin probe on vitronectin. Alternatively, conformational changes induced by PAI-1 binding to sites elsewhere in vitronectin could result in changes in the fluorescence properties of the probe. To determine which scenario was responsible for the changes in

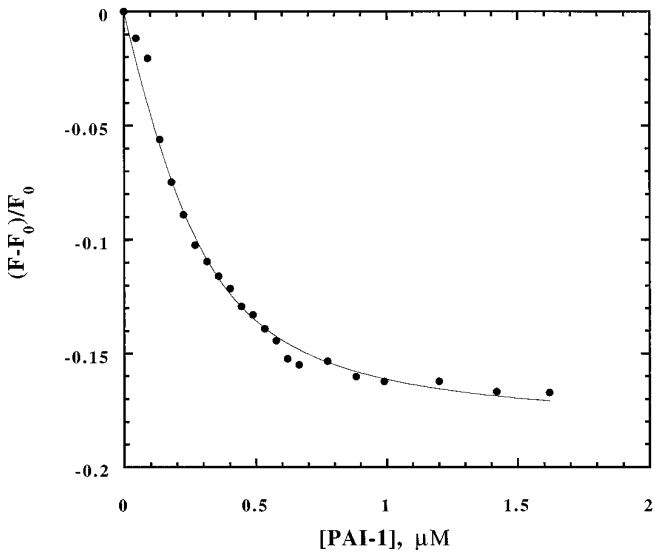

FIG. 8. **Fluorescence changes in HOCGO-VN observed upon interaction with PAI-1.** Using an excitation wavelength of 335 nm and emission wavelength of 453 nm, the fluorescence of a 260 nM sample of HOCGO-VN was measured upon addition of varying amounts of PAI-1 up to saturating concentrations. The data (●) were fit to a binding equation to obtain the binding isotherm shown in the *solid line*.

the coumarin probe when PAI-1 binds, HOCGO-VN was titrated with NaI in the absence and presence of saturating concentrations of PAI-1 (Fig. 9). If PAI-1 binding were to occur at or near the site(s) where the protein has been labeled, formation of complexes with PAI-1 would be expected to reduce the accessibility to diffusible quencher. However, although PAI-1 binds and is stabilized by HOCGO-VN, the serpin does not confer protection from iodide quenching to the fluor (Fig. 9). From these results, it is concluded that PAI-1 does not bind HOCGO-VN in a way that masks the modified arginines or greatly alters their degree of solvent exposure.[2] Furthermore, these data indicate that PAI-1 does not bind to the heparin-binding domain, as that would alter the quenching behavior of the coumarin probe. PAI-1-induced conformational changes in HOCGO-VN are manifested only in slight changes in the hydrophilic milieu of the probe. Although the measurement of a 15% quench in fluorescence upon formation of a complex between HOCGO-VN and PAI-1 (Fig. 8) indicates that there is an increased exposure of the coumarin probe in the complex, its accessibility to small diffusible quenchers is not greatly affected.

Heparin and PAI-1 Do Not Compete for Binding Sites on Vitronectin—A series of assays was performed to determine the effect of heparin on the PAI-1·vitronectin interaction. If PAI-1 and heparin share the same binding site on vitronectin, heparin should inhibit PAI-1 from binding to vitronectin. In an experiment in which vitronectin was adsorbed to a microtiter plate and various concentrations of PAI-1 were added in the presence or absence of heparin, it was found that heparin did not inhibit and, in fact, slightly enhanced the interaction between PAI-1 and vitronectin (Fig. 10*A*). In a competitive assay,

[2] In order to test for potential disruption of the PAI-1·vitronectin complex at the higher ionic strengths achieved in the quenching experiment, salt effects on the interaction were determined by comparing the binding of PAI-1 to immobilized vitronectin at 0.15 and 2 M NaCl. The protocol for the non-competitive PAI-1 binding assay is described under "Experimental Procedures." From this analysis, there were no significant differences in the binding of PAI-1 to vitronectin at the two ionic strengths tested. This is consistent with the observed lack of salt effects on the PAI-1/vitronectin interaction reported by Sigudattor and Wiman (66).

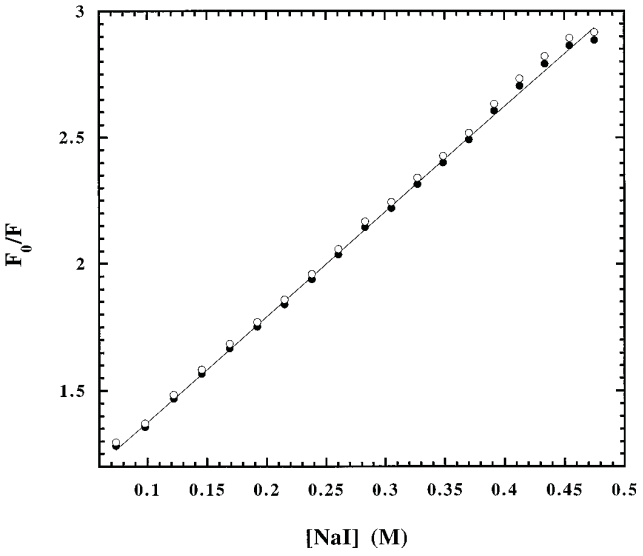

FIG. 9. **Stern-Volmer plot for iodide quenching of the HOCGO-VN·PAI-1 complex.** The fluorescence of the extrinsic probe on HOCGO-VN (○) or the HOCGO-VN·PAI-1 complex (●) was titrated with NaI as described under "Experimental Procedures." The concentration of HOCGO-VN was fixed at 130 nM in both experiments, and the HOCGO-VN complex was formed upon addition of PAI-1 to a final concentration that equaled 1.1 μM PAI-1. The data were plotted as F_0/F, where F_0 is the fluorescence in the absence of iodide and F is the fluorescence at a given concentration of iodide.

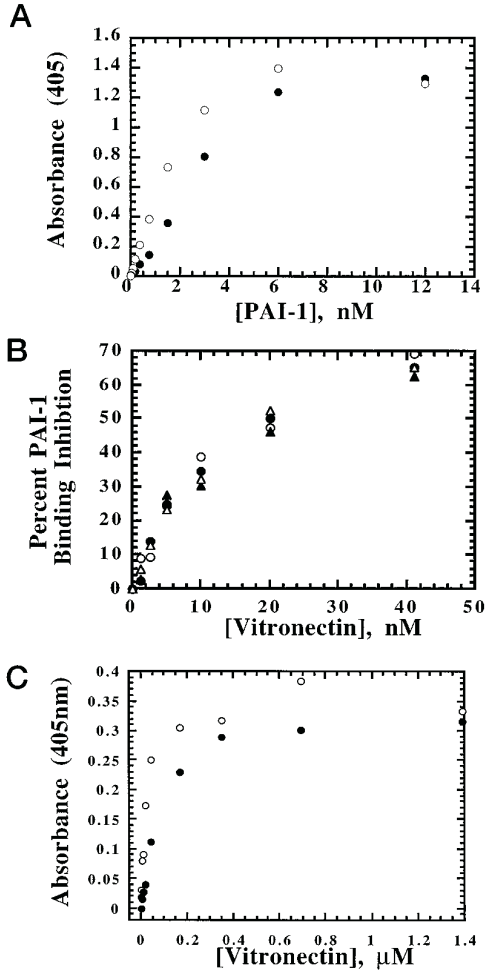

FIG. 10. **Assays to distinguish independent *versus* competitive binding of heparin and PAI-1 to vitronectin.** *A*, serial dilutions of PAI-1 were incubated with surface-immobilized vitronectin in the absence (●) or presence (○) of heparin, and bound PAI-1 was detected immunochemically as described under "Experimental Procedures." Assays were performed using conditions in which the absorbance shows a linear relationship with the amount of PAI-1 bound to vitronectin. *B,* the effect of heparin on a competitive PAI-1-binding assay was determined as described under "Experimental Procedures." The percent inhibition of PAI-1 binding observed at various concentrations of vitronectin is shown. The assays were performed in the presence of 0 (●), 0.4 (○), 4.0 (▲), and 40 μM heparin (△). *C*, the effect of PAI-1 on the interaction between vitronectin and heparin was determined by incubating various concentrations of vitronectin in microtiter plates coated with 1 mg·ml^{-1} heparin. Serial dilutions of vitronectin in buffer alone (●) or in a solution containing 0.6 μM PAI-1 (○) were tested in the assay for effective binding to the heparin-coated plates.

vitronectin was immobilized and PAI-1 was incubated in the presence of increasing concentrations of competing vitronectin or vitronectin-heparin complexes. Again, heparin did not prevent PAI-1 binding to either the surface- immobilized vitronectin or to the competing vitronectin in solution (Fig. 10*B*). In a final experiment, the effect of PAI-1 on the heparin-binding activity of vitronectin was assessed. Increasing concentrations of vitronectin or pre-formed vitronectin·PAI-1 complexes were added directly to heparin-coated microtiter plates and assayed for binding. The amount of vitronectin bound to heparin was increased slightly when vitronectin was allowed to form complexes with PAI-1 prior to being added to the heparin-coated plate (Fig. 10*C*). Although a quantitative analysis of these experiments is complicated by the fact that heparin binds both PAI-1 and vitronectin, the results from this series of experiments clearly indicate that PAI-1 and heparin do not compete for binding to vitronectin.

DISCUSSION

Vitronectin has been found to associate with PAI-1 *in vivo* and has been shown to stabilize its active conformation (17–19). The stabilization of PAI-1 that occurs as a result of binding to vitronectin represents a regulatory factor in the cascade of reactions that control fibrinolysis. In fact, vitronectin directs PAI-1 activity through three separate mechanisms as follows: 1) vitronectin stabilizes the active conformation of PAI-1 (17–19); 2) it alters the protease specificity of the serpin (20, 21); and 3) it may help maintain a distribution between PAI-1 in plasma and the extracellular matrix, where vitronectin is found in native and multimeric forms, respectively (53).

Elucidation of the conformational changes and requirements associated with the interaction of PAI-1 and vitronectin has been complicated by the propensity of both molecules to exist in alternative conformations. Moreover, the methods traditionally used to study this interaction have involved harsh treatments of the proteins, which are likely to have altered their conformations, or proteolytic fragments, which have different binding characteristics compared with the native molecules. The objective of this study was to develop a method for analyzing structural aspects of the PAI-1·vitronectin interaction under conditions that maintain the proteins in their native conformation. Fluorescent probes targeted to specific regions of the proteins were exploited to monitor conformational changes that occur as PAI-1 and vitronectin interact. These results are discussed in response to the questions posed in the introduction.

Conformational Changes in the Reactive Center Loop of PAI-1 Are Associated with Vitronectin Binding—To determine the effects of vitronectin binding on the reactive center loop, the fluorescent probe, NBD, was reacted with the unique sulfhydryl group in each of two mutant forms of PAI-1, M347C and S338C. The two sites of labeling provide environmentally sensitive probes at the P1′ and P9 positions of the reactive center loop, respectively. The usefulness of these probes to detect conformational changes in the reactive center loop of PAI-1 was

demonstrated previously by Shore *et al.* (37). In those studies, the fluorescence of NBDP9PAI-1 was found to be enhanced upon conversion to latency or upon complex formation with tPA, consistent with burial of the probe within the hydrophobic core of the protein. In the present studies, vitronectin binding induced a 10% decrease in probe fluorescence at the P1′ position, but a corresponding fluorescence change at the P9 position was not observed. The fluorescence change at the P1′ site is as expected for movement of the probe at the scissile bond to a slightly more hydrophilic milieu.

The results demonstrate that a conformational change at the scissile bond in the reactive center loop is associated with vitronectin binding. In serpins, protease specificity is determined by the primary sequence and conformation of the reactive center loop. Lawrence *et al.* (54) have shown that the substrate specificity of PAI-1 can be altered by substitutions in the reactive center loop based on the sequences of other serpins (20, 54). Other studies have indicated that the P1 residue of the scissile bond in PAI-1 is critical for both substrate specificity and activity against tPA (55–57). A methionine to arginine substitution at this position renders PAI-1 inactive against tPA but active against thrombin (57). It has also been reported that vitronectin restores the tPA inhibitory activity of this mutant form of PAI-1 (57). This conclusion is consistent with the observation in this work that vitronectin induces changes in the conformation of PAI-1 within the reactive center loop. The data from the present studies are the first to indicate that vitronectin induces local changes in the vicinity of the scissile bond, rather than changes that affect the entire reactive center loop. This study and that of Fa *et al.* (24) are significant in demonstrating that subtle changes in the conformation of the loop affect the specificity toward target proteinases.

The affinity of the interaction between vitronectin and PAI-1 is high. Reports of the K_d vary from 0.1 to 190 nM (18, 21, 22, 58). Furthermore, some differences in the binding affinity between native and multimeric vitronectin have been suggested (21, 30). The wide range in estimates of affinity of vitronectin for PAI-1 is surprising and may be attributed in part to the conformational lability of both proteins. The result is that different forms of both proteins, including active and latent forms of PAI-1, as well as monomeric and multimeric forms of vitronectin, have not always been distinguished in binding assays. Furthermore, previous estimates of the K_d have relied on kinetic and Scatchard analyses of PAI-1 binding to immobilized vitronectin or vitronectin binding to immobilized PAI-1 (18, 22, 58). It is difficult to accurately determine K_d values with these solid phase methods, because one must assume a concentration for the immobilized protein and one must also assume that binding sites are accessible. In fact, vitronectin has been shown to lose β-sheet structure upon adsorption to plastic (59). Moreover, Deng *et al.* (25) have observed that the vitronectin-binding site in PAI-1 is disrupted when the serpin is immobilized. Estimates of the K_d for the interaction based on ligand binding directly to immobilized proteins, therefore, may not accurately reflect the K_d of the interaction in solution.

The K_d values determined for the interaction of native vitronectin and PAI-1 in this study, using fluorescently labeled forms of either protein, were estimated to be equal to or lower than 100 nM. These represent the first determinations of the affinity of the interaction between the two proteins in solution. This approach obviates difficulties with the solid phase methods, providing a more reliable estimate of the energetics of interaction between vitronectin and PAI-1. It should be noted that these experiments were performed at protein concentrations (200–400 nM) that exceed the estimates for the K_d. Attempts were made to reduce the concentration of receptor protein; however, the low fluorescent yield from the labels and loss of protein due to adsorption precluded further analyses. Other methods that are more sensitive to binding interactions in the required concentration range (1–50 nM) are being pursued in this laboratory.

Binding of Native or Multimeric Vitronectin to PAI-1 Does Not Lead to Identical Changes in the Conformation of the Reactive Center Loop—PAI-1 originally purified from denatured plasma was found to co-purify with vitronectin in a complex of 450 kDa (17). That both multimeric and native vitronectin bind PAI-1 is clearly established (17, 30, 35); however, it is unclear whether both forms of vitronectin have the same effect on PAI-1 conformation. Indeed, the different forms of vitronectin have diverse effects on PAI-1 activity. Stabilization of PAI-1 by the extracellular matrix, where vitronectin is found in its multimeric conformation, is significantly higher than the stabilization of PAI-1 by purified plasma vitronectin. Unlike native vitronectin, multimeric vitronectin does not alter PAI-1 substrate specificity (21). Also, it has been reported that native, but not multimeric, vitronectin can enhance the PAI-1-induced cellular clearance of thrombin by low density lipoprotein receptors (60).

To assess whether multimeric PAI-1 causes conformational changes in the reactive center loop of PAI-1, the fluorescence of NBDP1′PAI-1 was measured upon addition of multimeric vitronectin. This form of vitronectin did not alter the fluorescence of the probe, suggesting that the conformation at the scissile bond is not affected by PAI-1 binding to multimeric vitronectin. These data imply that the contrasting effects of native and multimeric vitronectin on the substrate specificity of PAI-1 result, at least in part, from differential effects of the two molecules on the conformation at the scissile bond.

An Arginine-specific Probe, Hydroxycoumarin Glyoxal, Is Targeted to the Heparin-binding Region of Vitronectin—The conformation of the heparin-binding sequence in vitronectin has been the subject of a great deal of discussion in the literature. Observations that vitronectin in denatured plasma binds heparin more efficiently than native vitronectin (61, 62) resulted in the hypothesis that the heparin-binding domain is buried in the native molecule (62–64). Conformational changes induced by treatments with chaotropic agents, ligands, heat, or acid were thought to expose the heparin-binding domain (6, 40, 42, 43, 64, 65). A recent study by Zhuang *et al.*[3] provided evidence that the heparin-binding domain of vitronectin is not buried in the native state of the molecule and that apparent differences in affinity between native and multimeric vitronectin are due to differences in binding valency and are not the result of increased exposure of the heparin-binding domain.

Based on this observation, it was hypothesized that an arginine-selective fluorescent probe might be preferentially targeted to the arginine-rich heparin-binding domain of vitronectin. The product could then serve as an agent for monitoring PAI-1-induced changes in the heparin-binding region of vitronectin. When treated with hydroxycoumarin glyoxal, vitronectin was labeled at a stoichiometry of approximately 2 mol of probe per mol of protein. Further analysis revealed that HOCGO-VN bound heparin more weakly than unlabeled vitronectin. Including heparin during the labeling reaction resulted in protection of a set of arginines from labeling. Together, these data provide evidence that the heparin-binding domain of vitronectin serves as a target for the arginine-reactive probe. Peptide mapping of the labeled protein will be required to identify the exact location of the probe(s) among the seven arginine residues within the heparin-binding domain.

[3] P. Zhuang, T. Chen, and C. B. Peterson, submitted for publication.

PAI-1-induced Conformational Changes in Vitronectin Can Be Detected Using the HOCGO-labeled Protein—Coumarin-modified vitronectin provided a tool for analyzing conformational changes in vitronectin associated with PAI-1 binding. HOCGO-VN competed effectively with immobilized vitronectin for PAI-1 binding. The HOCGO-VN behaved similarly to unlabeled native protein with respect to PAI-1 binding and stabilization, indicating that the probes did not disrupt elements essential for either activity. Fluorescence studies showed that PAI-1 binding was associated with a 15% quench in HOCGO-VN fluorescence. When PAI-1 was bound to vitronectin, the Stern-Volmer plots for iodide quenching were identical to those of HOCGO-VN in the absence of PAI-1. The evidence that PAI-1 does not affect the susceptibility of the probe to iodide quenching indicates that alterations in coumarin fluorescence upon PAI-1 binding are not the result of direct PAI-1 interactions with the probe. Rather, the binding of PAI-1 results in an altered conformation at the heparin-binding site on vitronectin. The PAI-1-induced conformational change within the heparin-binding region of vitronectin is also detected by enhanced binding of PAI-1·vitronectin complexes over vitronectin alone to surface-immobilized heparin.

Heparin and PAI-1 Do Not Share a Binding Determinant in Vitronectin—Three lines of evidence from this work dispute the concept that PAI-1 and heparin share a binding determinant in vitronectin. Modification of arginines in vitronectin with hydroxycoumarin glyoxal weakens heparin binding but does not affect PAI-1 binding or stabilization. Fluorescent probes, thought to be incorporated into the heparin-binding domain of vitronectin, are not protected from iodide quenching when HOCGO-VN is bound to PAI-1. Heparin and PAI-1 do not compete for binding to vitronectin. If heparin and PAI-1 did share a binding determinant in vitronectin, modifications of residues in the sequence would be expected to weaken vitronectin binding to both macromolecules. HOCGO-VN would then be expected to have a weakened affinity for PAI-1. Only affinity for heparin is affected by the modification, while interactions with PAI-1 are unperturbed.

Second, it could be argued that PAI-1 and heparin share a binding determinant but that specific residues critical for interaction within that determinant are different for the serpin and the glycosaminoglycan. The iodide quenching data indicate that the heparin-binding residues that undergo modification are not located near the PAI-1-binding sites. Otherwise, the modified residues would be expected to have decreased solvent exposure in the presence of PAI-1.

Finally, data from several competition experiments further dispute the possibility that heparin and PAI-1 share a binding determinant. PAI-1 bound to vitronectin-coated microtiter plates somewhat more effectively in the presence of heparin. Likewise, vitronectin complexed with PAI-1 bound heparin-coated plates somewhat more effectively than vitronectin alone. These observations contradict a previous report by Kost *et al.* (26) in which high concentrations of heparin prevented PAI-1 binding to immobilized vitronectin. However, a different report by the same group failed to demonstrate competition between heparin and PAI-1 for binding a CNBr fragment of vitronectin (30).

The results from competition experiments described in this document provide evidence that PAI-1 and heparin do not share a common binding site on vitronectin. Otherwise, at the saturating concentrations used, heparin and PAI-1 would be expected to compete for vitronectin binding. The observation that each of the two molecules increases the binding affinity of vitronectin for the other could have several interpretations. 1) Because both vitronectin and PAI-1 bind heparin, the glycosaminoglycan could serve as a template for their interaction, providing a common surface on which they both interact. This function has been attributed to heparin in the interaction between thrombin and antithrombin (67–70). 2) Since heparin bound to immobilized vitronectin could provide additional binding sites for PAI-1, more PAI-1 may bind in the well. 3) As both heparin and PAI-1 have been demonstrated to alter the conformation of vitronectin, the phenomenon could be an example of allostery, where binding of one of the macromolecules results in conformational changes that increase the affinity of vitronectin for the other.

Concluding Remarks—The goal of these studies was to investigate conformational changes in vitronectin and PAI-1 using fluorescently labeled proteins. The probes incorporated into the proteins have provided useful information about the conformational changes that occur in the molecules as a result of their interaction. The data obtained with NBD PAI-1 demonstrate that binding native but not multimeric vitronectin alters the conformation of the reactive center loop of PAI-1. Changes in the fluorescence of probes incorporated into the heparin-binding domain of vitronectin indicate that the heparin-binding region is not an important binding determinant for PAI-1. Rather, this region experiences a conformational change when vitronectin and PAI-1 interact. Furthermore, the fluorescently labeled proteins serve as valuable tools for continued investigation of mechanistic aspects of the PAI-1·vitronectin interaction.

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