Protein Disulfide Isomerase Catalyzes the Formation of Disulfide-Linked Complexes of Vitronectin with Thrombin−Antithrombin†

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ABSTRACT: In this study, purified preparations of platelet protein disulfide isomerase (PDI), vitronectin, α-thrombin, and antithrombin (AT) were used to demonstrate that PDI catalyzes formation of vitronectin−thrombin−AT complexes. Complex formation requires reduced glutathione (GSH) and can be prevented by *N*-ethymaleimide, and the formed complex is dissociated by reducing agents such as mercaptoethanol. No vitronectin−thrombin complex formed in the absence of AT, indicating that the thrombin−AT complex is an obligate intermediate in the reaction. Under optimal conditions, the majority of the thrombin−AT is incorporated into the complex in 60 min. Thrombospondin-1, known to form disulfide-linked complexes with thrombin−AT [Milev, Y., and Essex, D. W. (1999) *Arch. Biochem. Biophys. 361,* 120−126], competes with vitronectin for thrombin−AT in the low-Ca^{2+} environment that favors the active form of thrombospondin. The results presented here may also explain previous studies showing that vitronectin−thrombin−AT complexes form better in plasma (which contains PDI) than with purified proteins (where PDI was not used). We were able to purify a PDI from plasma that was immunologically identical to the platelet enzyme. We used the scrambled RNase assay to show that added purified PDI can function in a plasma environment. Complex formation in plasma was inhibited by inhibitors of PDI. PDI was released from the platelet surface in a soluble form at high pH (around the physiologic range), suggesting a source of the plasma PDI. In summary, these studies indicate that PDI functions to form disulfide-linked complexes of vitronectin with thrombin−AT.

The generation of thrombin and its various activities are tightly controlled (*1*). The final clearance and translocation of thrombin-containing complexes in the vascular system is believed to involve (i) a reaction of thrombin with AT,[1] (ii) formation of a ternary vitronectin−thrombin−AT complex, (iii) a conformational change in the vitronectin part of the ternary complex endowing it with heparin binding properties, and (iv) binding of the ternary complex to cell surface sites on endothelial and other cells.

Vitronectin has at least one free reactive thiol group (*2, 3*) and becomes disulfide-linked (*4, 5*) to the thrombin molecule of the thrombin−AT complex (*6*). Since thrombin contains three pairs of disulfide bonds with no unpaired cysteines (*7*), the formation of the ternary complex must occur via thiol−disulfide exchange. The secreted protein thrombospondin-1 (thrombospondin) forms an analogous disulfide-linked complex with thrombin−AT through thiol−disulfide exchange, and thrombospondin, like vitronectin, supplies a free reactive thiol group (*8*).

Several preparations of vitronectin are used in the literature. "Native" vitronectin refers to vitronectin purified in the absence of a denaturing agent (*2, 3, 9*). Vitronectin can also be treated with urea, causing a conformational alteration that allows it to bind to a heparin column (*10*). Material treated in this way is commonly termed "urea-treated" (*9*) or "conformationally altered" vitronectin (*11, 12*). The urea-treated preparation is similar to a small fraction of plasma vitronectin that is normally in a conformationally altered form; this form represents 2% of normal plasma and increases to 7% when plasma clots (*13*). Platelets have a high-molecular mass form of vitronectin which, like the urea-treated form, is capable of interacting with heparin (*14*). Vitronectin in the extracellular matrix is thought to exist predominantly in the conformationally altered form (*15*).

The majority of thrombin−AT in normal plasma is found in the disulfide-linked ternary complex with vitronectin (*6*). In vitro, however, it has been found that only a minor portion of thrombin−AT can be incorporated into the vitronectin−thrombin−AT complex if purified vitronectin, thrombin, and AT are used (*5, 6*). When fibrinogen-deficient plasma was used to assemble complexes in a plasma environment, 20% of the thrombin−AT was incorporated into vitronectin−thrombin−AT complexes after 5 min and 50% after 30 min (*6*), suggesting the involvement of another component in plasma that facilitates ternary complex assembly.

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[1] Abbreviations: ACD, acid−citrate−dextrose anticoagulant buffer; AT, antithrombin III; GSH, reduced glutathione; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate; VN, vitronectin.

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Protein disulfide isomerase (PDI), an enzyme which catalyzes thiol−disulfide exchange reactions, was recently shown to catalyze formation of the thrombospondin−thrombin−AT complex (*16*), and here we show that it also catalyzes the formation of the vitronectin−thrombin−AT complex. PDI is on the platelet surface from which it can be secreted in a microvesicle form (*17*) and is, therefore, available to catalyze disulfide bonding reactions in the vascular system. PDI contains a peptide binding site which is necessary for catalytic activity of PDI against protein substrates (*18*−*21*). PDI also has a C-terminal tetrapeptide sequence, Lys-Asp-Glu-Leu (KDEL), which functions as an endoplasmic reticulum retention signal in yeast and mammalian cells (*22*). In cells, PDI binds by this signal to a known 23−26 kDa, six- or seven-transmembrane domain receptor (*23, 24*) that facilitates its recycling from the Golgi apparatus back to the endoplasmic reticulum. Our results show that platelet PDI catalyzes the formation of a ternary vitronectin−thrombin−AT complex and that thrombospondin competes for the thrombin−AT. The work also suggests that preparations of vitronectin may contain PDI, and this may account for differing vitronectin activities reported in the literature.

EXPERIMENTAL PROCEDURES

Materials. Heparin (from porcine intestinal mucosa), bovine serum albumin, sodium dodecyl sulfate (SDS), *N*-ethylmaleimide (NEM), and cyanogen bromide-activated Sepharose were purchased from Sigma Chemical Co. (St. Louis, MO). A monoclonal mouse anti-PDI antibody (clone R 1.77) (*25*) was purchased from Affinity Bioreagents Inc. (Golden, CO). Rabbit anti-PDI antiserum was raised against human platelet PDI, and the IgG purified using a protein A column as previously described (*17*). The rabbit anti-PDI IgG was monospecific for PDI on Western blots of platelet lysate (*26*). The monoclonal antibody 1D3 which recognizes the last 10 amino acids, including the C-terminal KDEL sequence of PDI (*37*), was obtained from Stress Gen Biotechnologies Corp. (British Columbia, BC). Fibrinogen-depleted citrated plasma was obtained from Organon Teknika Corp. (Durham, NC). Purified bacitracin A was a gift from L. Kesner of the State University of New York Downstate Medical Center.

Proteins. Protein disulfide isomerase was purified from human platelets and its activity tested in the scrambled RNase PDI assay as previously described (*17*). PDI was more than 90−95% pure as determined by Coomassie Blue staining and densitometry of a 7.5% SDS−PAGE. α-Thrombin was a gift from J. Fenton, II (Division of Laboratories and Research, New York State Department of Health, Albany, NY). Iodination of thrombin was carried out by a modification of the method of Lipford et al. (*27*) using $Na^{125}I$ (Amersham, Arlington Heights, IL) and iodobeads (Pierce, Rockford, IL). Free $Na^{125}I$ was separated from the labeled protein by centrifugation by employing a 30 kDa molecular mass cutoff microconcentrator. TCA precipitation of the thrombin sample showed no free ^{125}I. The concentrate was washed six times to ensure a very low radioactivity of the final filtrate. Radiolabeled α-thrombin recovery was >95% with less than 5% loss of esterase activity as determined with S2238 (Kabi Vitrum, Stockholm, Sweden). Human antithrombin III was prepared from plasma as previously described (*28*). The biological activity of antithrombin III was >95% as determined with an activity assay (Kabi Vitrum). Thrombospondin was prepared as previously described (*16*).

Vitronectin was purified according to a modification of the procedure of Bittorf et al. (*9*). This method uses the sulfhydryl reagent *N*-ethylmaleimide (NEM) prior to urea treatment to prevent the formation of disulfide-linked multimers. In our preparation, after barium chloride absorption and precipitation of plasma with 9% (w/v) poly(ethylene glycol), NEM (5 mM) was added for 30 min (instead of 2 h) (*9*). This purified vitronectin gave only 65 and 75 kDa bands on Coomassie Blue staining of reducing gels. Unless otherwise noted, experiments were performed with this preparation which we term "urea-treated". Another preparation used for comparison was purified using the same technique without the addition of NEM. This material gave essentially the same results with the thrombin−AT experiments, but many higher-molecular mass species were observed. Because of the importance of sulfhydryl groups in PDI-catalyzed reactions, we measured the amount of free sulfhydryls in the two urea-treated preparations with the reagent 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM, Molecular Probes, Eugene, OR); NEM-treated protein had 0.4 mol of SH/mol of vitronectin, while the material not treated with NEM had approximately 1.0 mol/mol. "Native" vitronectin was purified according to the method of Preissner et al. (*3*), employing $(NH_4)_2SO_4$ fractionation, with DEAE-Sephacel, Blue Sepharose, and Sephacryl S-200 chromatography (the final anti-human albumin, anti-human C3, and anti-human α_1-antichymotrypsin columns were omitted). Fibrinogen-depleted plasma and whole blood were also used for comparison with the behavior of the urea-treated vitronectin. The protein concentration was determined spectrophotometrically by using absorption coefficients ($A^{1\%}$, 1 cm at 280 nm) of 9.0 for urea-treated vitronectin (*3*) and 6.5 for antithrombin III (*29*).

Vitronectin−Thrombin−AT Complexes. Unless otherwise indicated, mixtures of vitronectin (300 μg/mL, 4 μM), antithrombin (375 nM), [^{125}I]thrombin (250 nM), GSH (1.2 mM), and heparin (2 units/mL) were incubated in the presence or absence of PDI (32 nM) at 37 °C for the indicated time intervals. The reaction was started by the addition of labeled thrombin. All experiments were performed in 10 mM HEPES, 137 mM NaCI, 4 mM KCl, and 15 mM glucose (pH 7.4) containing 2 mM $CaCl_2$ and 0.1% BSA. The reaction was stopped by the addition of gel sample buffer containing 2% SDS and 1 mM NEM. Vitronectin−thrombin−AT complex formation in citrated fibrinogen-depleted plasma or citrated or EDTA anticoagulated whole blood was studied by addition of 5 units/mL heparin and preformed [^{125}I]thrombin−AT complex (prepared by incubating 250 nM [^{125}I]thrombin with 375 nM AT for 30 min in the presence of 2 units/mL heparin).

Electrophoretic Analysis of Proteins. The samples were analyzed using 6.5 and 7.5% homogeneous SDS−PAGE with a 3.25% stacking gel or 3.5 to 16% linear gradient SDS−PAGE with 3.25% stacking gels and Gel Bond support film as previously described (*16*). Dried gels were developed, and quantitation of incorporated radiolabeled thrombin into complexes was carried out with a Molecular Dynamics Phosphorimager SF (Sunnyvale, CA). The average back-

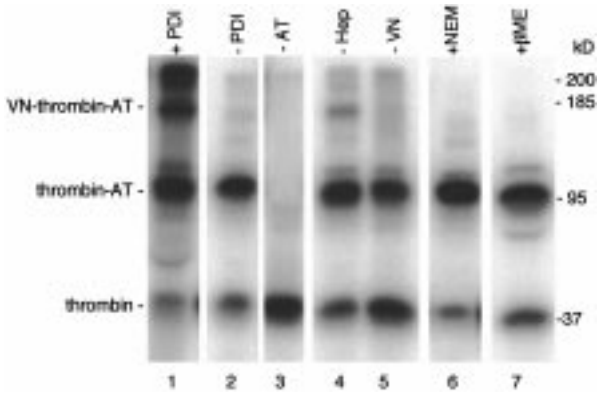

FIGURE 1: Characterization of vitronectin−thrombin−AT complex formation. The reaction was performed as described in Materials and Methods using a 60 min incubation period and analyzed on SDS−6.5% PAGE. The sample in lane 1 contained vitronectin (VN), thrombin, antithrombin (AT), PDI, and heparin. In lanes 2−5, the omitted component was PDI, AT, heparin (Hep), and vitronectin (VN), respectively. NEM (20 mM) was added before the incubation period to the sample in lane 6. β-Mercaptoethanol (βME, 2.5%) was added to the sample in the last lane just prior to electrophoresis.

ground count was subtracted from the total count of each lane. The amount of vitronectin−thrombin−AT or thrombin−AT complexes was expressed as the percentage of total radioactivity of all thrombin−AT-containing complexes.

Western Blotting, Lactate Dehydrogenase (LDH), and PDI Assays. Western blotting was performed as described previously (*30*) using 7.5% SDS−PAGE under reducing conditions. After the membrane was incubated with the rabbit anti-PDI IgG, it was developed using goat anti-rabbit IgG conjugated with peroxidase and a chemiluminescent substrate. For some Western blots, a primary monoclonal anti-PDI antibody was used with an appropriate secondary antibody. LDH release from samples was assayed on a Kodak Ektachrome Analyzer by employing a colorimetric method. Scrambled RNase was prepared from native RNase (*30*, *31*) and the PDI assay performed as described previously (*17*).

Preparation of Plasma. Blood was collected from healthy aspirin-free donors into 0.15 volume of ACD anticoagulant (85 mM sodium citrate, 65 mM citric acid, and 2% dextrose) as previously described (*30*). Platelet-poor plasma was made by centrifugation at 1000*g* for 15 min at 22 °C. The platelet-poor plasma was filtered through a 0.2 μm filter to remove any remaining platelets. Platelet-poor plasma was also made from blood collected into 4 mM EDTA. Immunodepletion of some plasma samples was performed as previously described (*16*) using 5 mg/mL rabbit anti-PDI IgG.

Anti-PDI Affinity Column. Cyanogen bromide-activated Sepharose was washed with 1 mM HCl, suspended in 0.1 M borate buffer (pH 8.3), and incubated with 5−10 mg/mL rabbit anti-PDI IgG on a rotator at 4 °C overnight. After centrifugation, the gel was suspended in the borate buffer containing 0.2 M glycine (pH 8.3). After 1 h, the gel was washed three times with 0.1 M borate buffer at pH 8.3, once with water, three times with 0.1 M acetate buffer at pH 4.2, once with water, and twice with borate buffer and suspended in phosphate-buffered saline (PBS).

RESULTS

Effect of PDI on Vitronectin−Thrombin−AT Complex Formation. Figure 1 shows formation of high-molecular mass vitronectin−thrombin−AT complexes from purified components. The presence of PDI (lane 1) greatly enhanced complex formation relative to the reaction in its absence (lane 2). With omission of AT (lane 3), no vitronectin−thrombin complex was formed, indicating that thrombin−AT is an obligate intermediate. The reaction was also highly dependent on heparin, as previously described (*5*), with little of the ternary complex being formed in its absence (lane 4). The formation of vitronectin−thrombin−AT complexes was inhibited by incubation of the reaction mixture with NEM prior to the addition of labeled thrombin. The addition of 2.5% β-mercaptoethanol, added after the reaction, completely dissociated the complex.

Time Course of Complex Formation. The time course of vitronectin−thrombin−AT complex formation is shown in Figure 2. At each time point, samples were analyzed on both 6.5% SDS−PAGE and 3.5 to 16% linear gradient SDS−PAGE. The gels show the reaction in the absence (lanes 1−6) and presence (lanes 7−12) of PDI. The gradient gel enables separation of the complexes into multimers of vitronectin complexed to thrombin−AT, with the smallest and most abundant form being the approximately 165 kDa vitronectin−thrombin−AT complex [the multimeric nature of vitronectin purified using urea and heparin affinity chromatography is well recognized (*4*, *9*)]. The kinetics of total vitronectin−thrombin−AT complex formation are shown in Figure 2B. The points include all bands larger than the thrombin−AT complex using quantitative phosphorimager analysis. Figure 2B shows that the kinetics are the same for both gel types. Maximal ternary complex formation was reached at about 60 min, with more than 50% of the thrombin−AT being incorporated into the complex.

Effect of GSH, PDI, and Vitronectin Concentration on Vitronectin−Thrombin−AT Complex Formation. The catalytic activity of PDI depends on the effect of the redox environment on either PDI or its substrate. Since reduced glutathione (GSH) is used in virtually all assays of PDI, we tested the effect of GSH concentration on complex formation using both a 30 min and a 90 min incubation period (Figure 3). The reaction showed an almost absolute dependence on GSH with maximal complex formation at about 1200 μM GSH.

To estimate whether PDI concentrations needed to catalyze vitronectin−thrombin−AT complex formation are within the range likely to be physiologic (plasma concentrations on the order of 10 nM), we tested the effect of varying the PDI concentration from 0 to 300 nM. The results are shown in Figure 3B. Activity can be detected at 5 nM PDI, with a 10 nM concentration giving close to maximal activity. Since in all these experiments there was always some thrombin−AT not incorporated into vitronectin−thrombin−AT complexes, we examined whether any of the reactants were limiting. Doubling of the concentration of vitronectin (Figure 3C) had little effect on the amount of ternary complex. Doubling the thrombin−AT concentration produced only a 10−15% increase in the amount of ternary complex (data not shown), indicating that although some of the thrombin−AT may not be active, this is not the major explanation for the plateau.

Effect of Thrombospondin-1 on Complex Formation. Thrombospondin-1 is known to form disulfide-linked complexes with thrombin−AT in a reaction analogous to the ternary complex formation of vitronectin with thrombin−

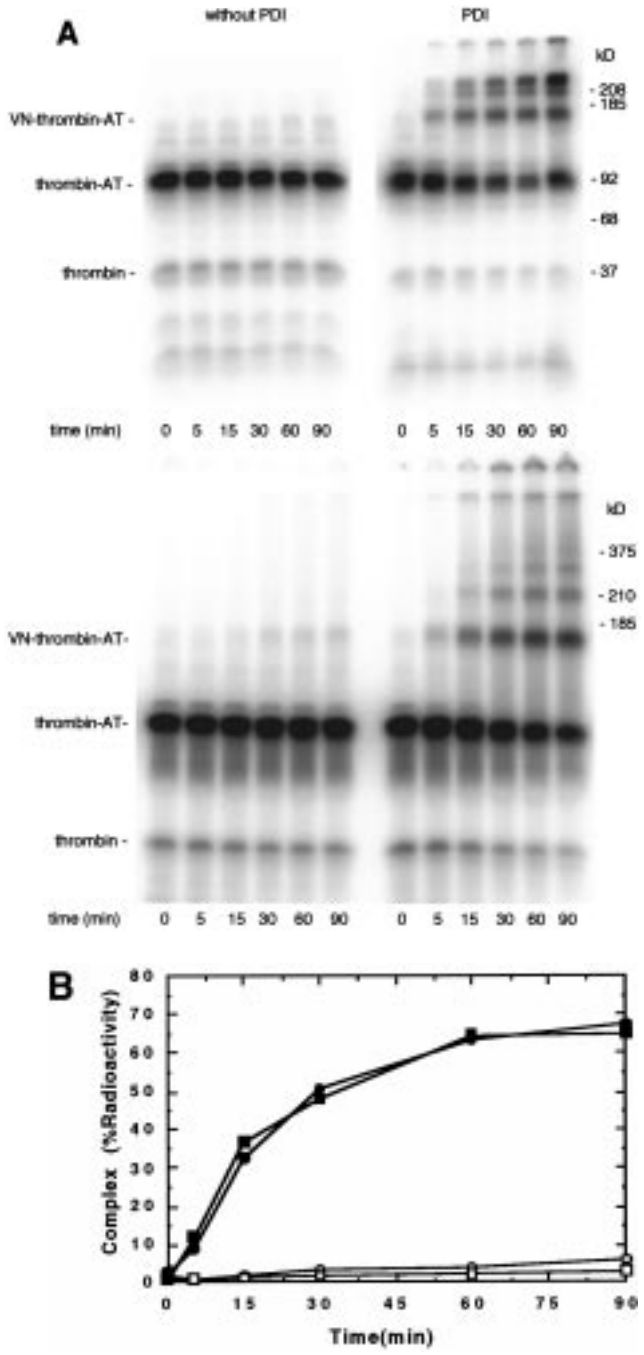

FIGURE 2: Time course of vitronectin (VN)−thrombin−AT complex formation. The reaction was performed as described in Materials and Methods with aliquots of the sample removed at various time points and the reaction stopped by the addition of NEM-containing sample buffer. Reactions were performed with or without PDI (right and left sides, respectively). In panel A, the top panels show samples analyzed using nonreducing conditions on SDS−6.5% PAGE. In the bottom panels, aliquots of the same samples were analyzed under nonreducing conditions using SDS−PAGE (3.25 to 16% gradient). In panel B, the labeled thrombin−AT complex was quantitated using laser densitometry on the Molecular Dynamics Phosphorimager SF, with the background activity subtracted out. The data for the vitronectin complex plotted in panel B are the percent radioactivity of the bands at 165 kDa and the higher bands (presumed multimeric forms) expressed as the percentage of the total radioactivity of thrombin−AT complexes. Shown are data for the VN−thrombin−AT complex with PDI, (■) 6.5% PAGE and (●) gradient gel, and without PDI, (□) 6.5% PAGE and (○) gradient gel.

AT. Thrombospondin−thrombin−AT complexes form in the supernatant solution of activated platelets (*32*, *33*) by a reaction catalyzed by PDI (*16*). Thrombospondin undergoes Ca^{2+}-dependent conformational changes; a low-Ca^{2+} environment facilitates thrombospondin−thrombin−AT complex formation, while little complex formation occurs in Ca^{2+}-containing buffers (*16*, *32*, *33*). We found that in the absence of Ca^{2+} thrombospondin effectively competes with vitronectin for thrombin−AT, even with molar concentrations of thrombospondin that are 5−6-fold lower than that of vitronectin (Figure 4, lane 3). Although there is some overlap of bands for the thrombospondin and high-molecular mass vitronectin complexes, quantitation of the gels typically showed about 50% of thrombin−AT in thrombospondin complexes and about 20% as vitronectin−thrombin−AT.

Studies on PDI in Plasma. The involvement of PDI described above explains studies in the literature showing that vitronectin−thrombin−AT complexes form better in plasma than with purified proteins [to which PDI had not been added (*6*)] and suggests that PDI is the additional component in plasma that facilitates complex formation. We were initially unable to detect PDI in plasma by Western blotting (*17*, *30*), but using a more sensitive sandwich ELISA, we have now detected PDI in plasma (Figure 5A). (Immunodepletion of PDI from plasma samples reduced the reading to the background level, indicating that the ELISA is specific for PDI. The plasma concentration of PDI was estimated to be 250−1000 ng/mL or 4−17 nM.) We partially purified PDI from plasma samples using an affinity column made with our rabbit anti-PDI antibody. In these experiments, plasma was prepared from blood collected into ACD anticoagulant, to maintain a low pH and prevent release of PDI from the platelet surface during the processing procedure (see below). Plasma PDI, thus prepared, migrated in a manner identical with that of purified platelet PDI under reducing conditions on SDS−PAGE, 4 to 15% acrylamide gradient gels (Figure 5B) and, like platelet PDI, contains the C-terminal KDEL sequence (Figure 5C). PDI was also purified from plasma (Figure 5B, lane 3), using a modification of our procedure (omitting the Triton X-100 platelet lysis step) (*17*), and found to be catalytically active in the scrambled RNase assay. (In the plasma PDI preparation shown, substantial proteolysis occurred, resulting in a 55 kDa proteolytic product.)

PDI has long been known to function in the redox environment (*34*) of the endoplasmic reticulum. It was therefore important to demonstrate activity of purified platelet PDI in a plasma environment. Using the scrambled RNase assay, we detected activity of PDI added to plasma (Figure 6).

We tested the effect of inhibitors of PDI on complex formation in fibrinogen-depleted plasma; Figure 7 shows that complex formation was inhibited by the rabbit-anti-PDI IgG (400 μg/mL, lane 2) and the PDI inhibitor bacitracin A (7 mM, lane 4) (*30*, *35*) while normal rabbit IgG (lane 3) had no effect. [The >200 kDa bands seen in the plasma system have been noted previously by others (*4*) and do not correspond to the larger bands seen with urea-treated vitronectin on gradient gels.]

Western Blot for PDI in Vitronectin Preparations. Our early attempts to reproduce the results of Figures 1 and 2 using a native vitronectin preparation (purified without urea;

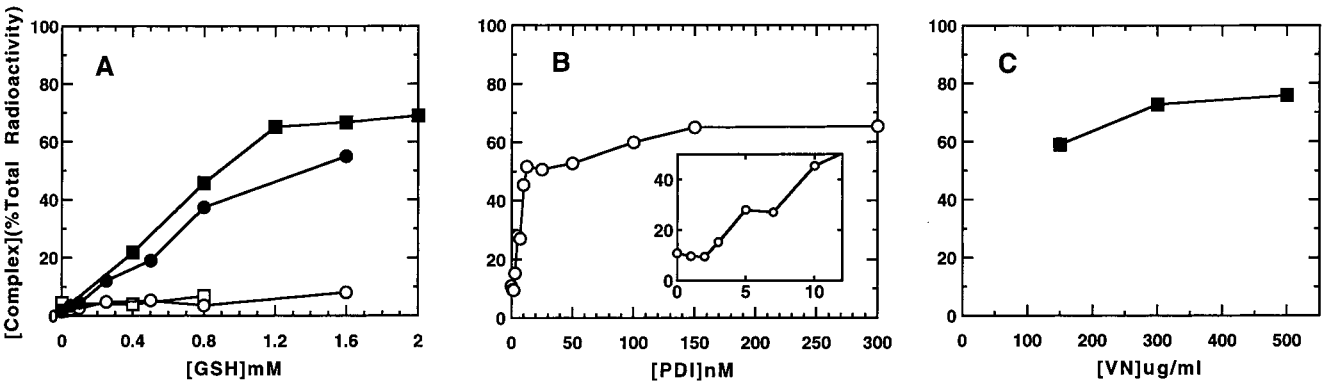

FIGURE 3: Effect of GSH, PDI, and vitronectin concentration on the formation of vitronectin−thrombin−AT complex. [^{125}I]Thrombin was added to reaction mixtures containing thrombin, AT, GSH, PDI, vitronectin, and heparin as described in Materials and Methods, and samples were analyzed on nonreducing SDS−7% PAGE. The labeled complex was quantitated as the percentage of of total thrombin−AT complexes as described in the legend of Figure 2. (A) Effect of GSH concentration. The reaction was allowed to proceed for either 30 (●) or 90 min (■). The concentration of PDI was 32 nM. Controls without addition of PDI were carried out for 30 (○) or 90 min (□). (B) Effect of PDI concentration. The reaction time was 60 min. Low concentrations of PDI are shown in the inset. The concentration of GSH was 1.2 mM. (C) Effect of vitronectin concentration. The reaction time was 60 min.

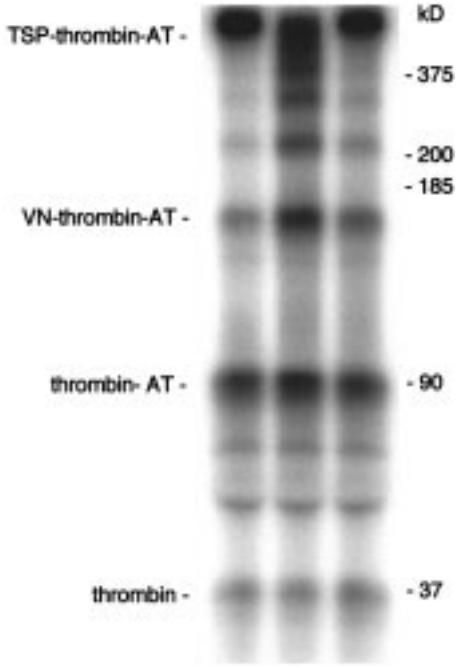

FIGURE 4: Effect of thrombospondin on the formation of VN−thrombin−AT complexes. Vitronectin (VN) complex formation was examined under standard assay conditions using 300 μg/mL vitronectin (4 μM) and 2 units/mL heparin. In these experiments, the buffer contained 4 mM EDTA for chelating Ca^{2+}. In lane 1, thrombospondin (TSP) (0.7 μM) was substituted for vitronectin [note that there is evidence for slight contamination of the purified thrombospondin with vitronectin (*42*)]. Lane 2 contained vitronectin alone. Lane 3 shows the reaction with vitronectin (4 μM) and thrombospondin (0.7 μM).

see Materials and Methods) gave qualitatively similar although somewhat variable results; vitronectin−thrombin−AT complex formation often occurred without the addition of PDI. The results above showing PDI in plasma suggested the explanation for this variability was that these preparations might contain PDI. Figure 8 shows a Western blot indicating that PDI is, in fact, present in the native vitronectin preparation but not in the vitronectin preparations purified with urea. [It is possible that the copurification of PDI with native vitronectin reflects a physiologic interaction with the peptide-binding site of PDI (*18*−*21*).] Consistent with this observation, we found that complex formation with native vitronectin was inhibited by rabbit-anti-PDI IgG (400 μg/mL) or bacitracin A (7 mM) in a manner similar to the effect of these inhibitors on PDI in plasma.

PDI and Platelet Surface Interactions. Previous studies have noted that small amounts of PDI may be released from the platelet surface in a soluble form (*17*), suggesting a possible source of plasma PDI. The potential for release of a soluble form of PDI from platelets was evaluated using washed nonactivated platelets. Figure 9 demonstrates a pH-dependent release of PDI into the supernatant solution of platelets kept in suspension for 1 h at 37 °C. The LDH in the supernatant fractions, a marker for cell lysis, was the same over the pH range that was studied, and the ratio of supernatant LDH to total platelet LDH was less than 0.5%, indicating that PDI was not released at higher pH due to disruption of the platelets. The majority of the total platelet PDI pool is on the platelet surface, and it is therefore unlikely that this PDI is released from internal platelet pools. We were not able to pellet this PDI by ultracentrifugation performed as described previously (*17*), indicating that it is in a soluble form. We were also unable to detect an effect of divalent cations or ionic strength on the interaction of PDI with the membrane (similar to what others have reported) (*36*). Since the interaction of PDI with its ER-Golgi receptor is through the C-terminal KDEL retention signal on PDI, we used the monoclonal antibody 1D3 against the C-terminal KDEL sequence of PDI (*37*) to show that platelet PDI (Figure 5C, lane 1), as well as plasma PDI, contains this sequence.

To evaluate the possibility that platelet surface PDI was a phosphatidylinositol-linked protein, we used FITC-labeled rabbit anti-PDI antibody and flow cytometry, as previously described (*17*, *30*). No deficiency of platelet PDI was found on the surface of platelets obtained from a patient with paroxysmal nocturnal hemoglobinuria, a disorder in which phosphatidylinositol-linked membrane proteins are lacking. Furthermore, phospholipase C, an enzyme which releases phosphatidylinositol-linked proteins from membranes, did not release PDI from the platelet surface as detected by Western blot. As a control, phospholipase C was shown to release labeled folate binding protein, a known phosphatidylinositol-linked protein, from placental extracts (*38*).

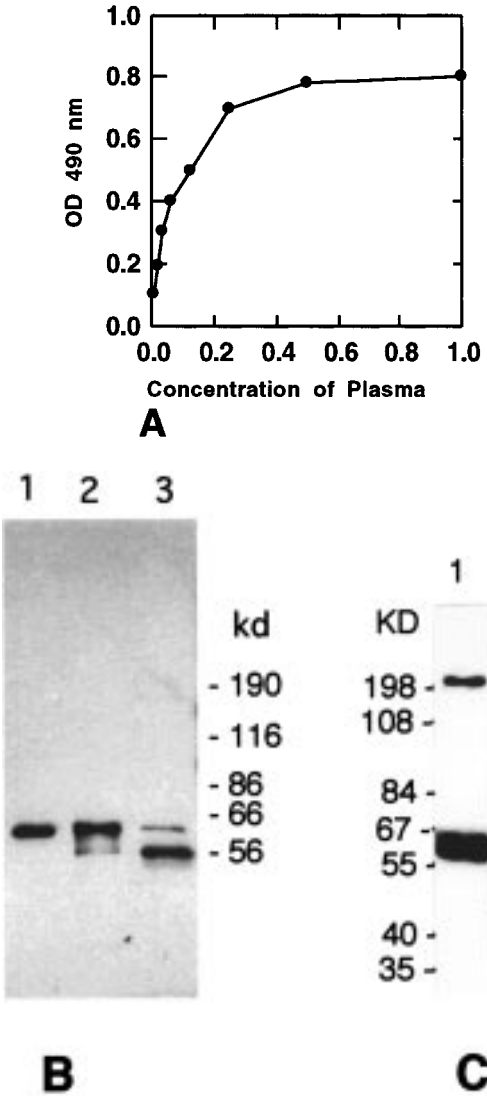

FIGURE 5: (A) ELISA assay for plasma PDI. The wells were coated with 2 μg/mL rabbit anti-PDI antibody in PBS for 18 h at 4 °C. After blocking with 1% BSA in PBS, the wells were washed with PBS/0.1% Tween. ACD anticoagulated platelet-poor plasma samples were incubated for 1 h in the wells. After washing, PDI was detected using a monoclonal anti-PDI antibody (Clone R 1.77) (*25*) followed by a secondary peroxidase-labeled anti-mouse antibody, and the chromogen was *o*-phenylenediamine. Undiluted plasma has a concentration of 1.0. Panels B and C are Western blots of platelet and plasma PDI. (B) Western blot using rabbit anti-PDI antibody after SDS−PAGE (5 to 15% gradient gel): lane 1, purified platelet PDI; lane 2, plasma PDI eluted from the rabbit anti-PDI column with a buffer at pH 8.4; and lane 3, purified plasma PDI [purified according to the method of Chen et al. (*17*)]. (C) Western blot with 1D3, a monoclonal antibody to the C-terminal KDEL tail of PDI: lane 1, purified platelet PDI; and lane 2, plasma PDI (prepared using a rabbit anti-PDI affinity column). (The larger band in lane 1 is a polymer of PDI which dissociates on urea gels.)

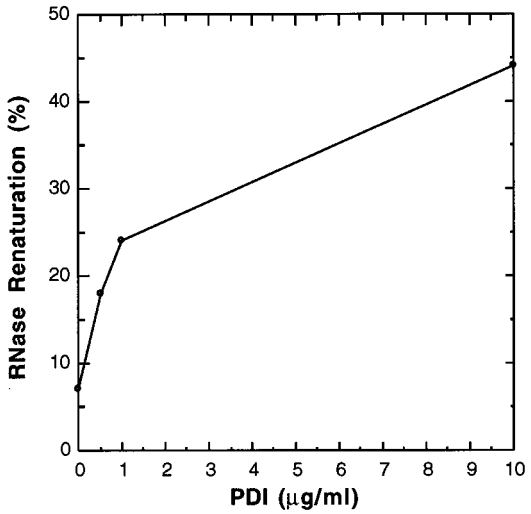

FIGURE 6: Scrambled RNase assay of PDI added to plasma. Platelet-poor plasma was prepared from blood collected in 4 mM EDTA (pH 7.4), as described in Materials and Methods. PDI in various concentrations was added to the plasma, and 10 μg/mL scrambled RNase was added. The incubation was carried out for 1 h at room temperature, and the level of renaturation of RNase was determined as previously described (*35*) except no GSH was added. The background level of renaturation of scrambled RNase in PBS (pH 7.4) with 4 mM EDTA was subtracted out. Each point represents the mean of triplicate samples.

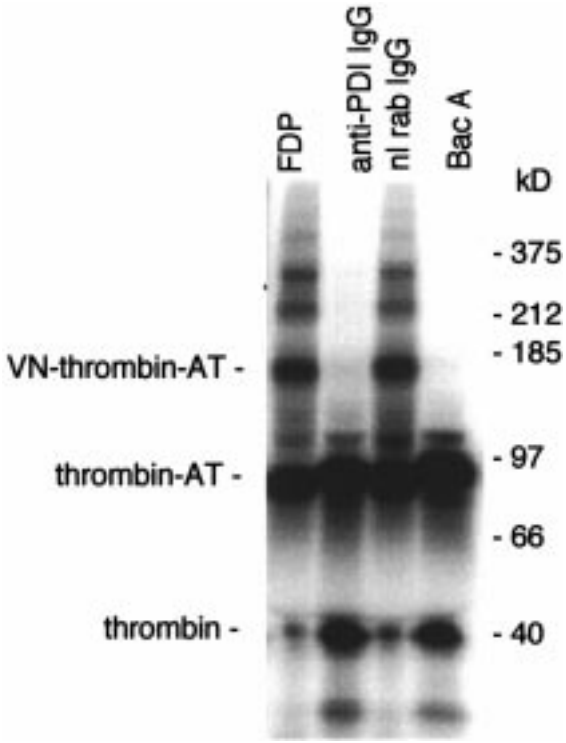

FIGURE 7: Vitronectin−thrombin−AT complex formation in plasma. The reaction was carried out as described in Materials and Methods by adding preformed [^{125}I]thrombin−AT complexes to fibrinogen-depleted plasma (FDP) for 60 min at 37 °C. Rabbit anti-PDI IgG (400 μg/mL), normal rabbit IgG (nl rab IgG, 400 μg/mL), or bacitracin A (Bac A, 7 mM) was preincubated with the indicated samples for 30 min at 37 °C before the addition of heparin (5 units/mL) and [^{125}I]thrombin−AT.

DISCUSSION

The major form of thrombin−AT in plasma is known to be the disulfide-bonded vitronectin−thrombin−AT complex. Although vitronectin is also capable of forming noncovalent complexes with thrombin−AT, in the current work we have studied the formation of the covalent complex (*6*). The significance of this work is that it identifies a catalyst, PDI, for the formation of this ternary complex. We also show that PDI, previously found in endoplasmic reticulum and more recently on the platelet surface (*17*, *30*), can also function in plasma. Our demonstration of release of soluble PDI from the platelet surface suggests a source of the plasma PDI. The involvement of PDI suggests thiol−disulfide exchange as

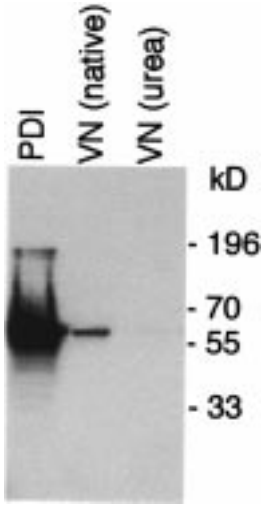

FIGURE 8: Western blot for PDI protein in vitronectin preparations: lane 1, purified PDI (100 ng); lane 2, native vitronectin [VN (native)]; and and lane 3, vitronectin purified using urea and heparin chromatography [VN (urea)]. This sample contains 20 μg of vitronectin, at no less than double the concentration of native vitronectin (lane 2) as determined by Coomassie Blue staining and densitometry.

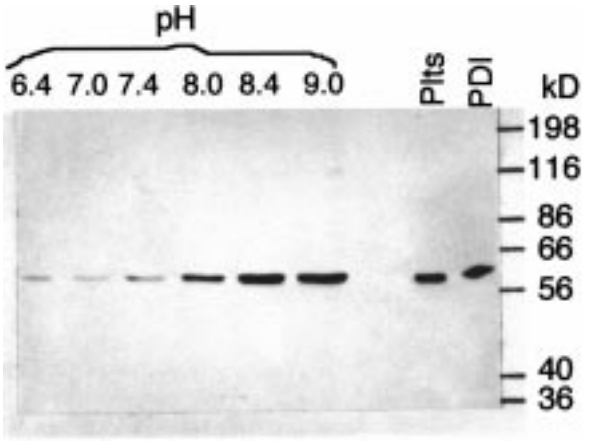

FIGURE 9: Release of PDI in the supernatant solutions of nonactivated platelets incubated at varying pHs. Western blotting was carried out using the chemiluminescent substrate kit. The first six lanes represent PDI in the supernatant solutions of platelets (1×10^9 platelets/mL) after a 1 h, 37 °C incubation at pHs ranging from 6.4 to 9.0. The buffer used, bis-tris-propane (10 mM), included PGE_1 (1 mM) to help prevent activation. A platelet lysate of 2×10^6 platelets (Plts) and a purified PDI sample (PDI) are also shown.

the mechanism, an idea supported by the observations that (1) NEM blocks the reaction and (2) β-mercaptoethanol dissociates the complex.

Using purified proteins, about 50−70% of the thrombin−AT was incorporated into vitronectin−thrombin−AT complexes, although some thrombin−AT was always unreacted. Increasing the concentration of reactants or the amount of GSH did not significantly affect the fraction of complex formed. It is possible that competing reactions with vitronectin occur, such as the formation of a vitronectin dimer, a product which would not be detected in this assay.

PDI catalyzes an analogous reaction of thrombospondin with thrombin−AT (*16*), and thrombospondin, as expected, competes with vitronectin. This thrombospondin−thrombin−AT ternary complex may account for a small amount of thrombin−AT which is complexed to an unknown plasma protein that is larger than vitronectin noted in the literature (*6*). Although it is premature to speculate about the significance of the competition between thrombospondin and vitronectin, one of the relevant variables that is important is the calcium concentration. Thrombospondin effectively competes with vitronectin for the PDI-catalyzed thiol−disulfide exchange reaction in a low-Ca^{2+} environment (Figure 4), and the addition of Ca^{2+} has an inhibitory effect on the thrombospondin reaction with thrombin−AT (*16*). Vitronectin−thrombin−AT complex formation, however, is independent of Ca^{2+} concentration (unpublished observation). Therefore, changes in Ca^{2+} concentration could regulate which end products form, with thrombospondin−thrombin−AT being favored in a low-Ca^{2+} environment and vitronectin−thrombin−AT complexes in a high-Ca^{2+} environment. The relative concentrations of these adhesive proteins are also important. In plasma, the vitronectin concentration (2−4 mM) greatly predominates over that of thrombospondin (0.1−0.2 nM), favoring vitronectin association into the ternary complex. On the other hand, in the supernatant solution of activated platelets, thrombospondin is found in higher concentrations than vitronectin.

Another factor that could affect the vitronectin−thrombin−AT reaction is the presence of heparin (Figure 1 and refs *4*−*6*). The requirement for heparin is not due to the acceleration of thrombin−AT complex formation but to an effect on vitronectin (*5*). In contrast, heparin has little effect on thrombospondin in the formation of the thrombospondin−thrombin−AT complex (*16*).

The finding of a pH-dependent release of soluble PDI from the platelet membrane is consistent with the notion that PDI is an extrinsic membrane protein (i.e., it has no transmembrane domain) and suggests that electrostatic forces are involved in its binding to the platelet membrane. This is also consistent with the pH-dependent binding of PDI to its known 26 kDa ER-Golgi receptor, where the affinity of PDI for its receptor decreases as the pH increases (*36*). Plasma PDI migrates in a manner identical with that of platelet PDI on gels and contains the C-terminal KDEL sequence. These findings taken together suggest the possibility that the platelet membrane contains the same receptor for PDI as is found in the endoplasmic reticulum and Golgi apparatus of mammalian cells.

Finally, PDI also has a role in platelet aggregation (*26*), a process which can involve both vitronectin (*39*) and thrombospondin (*40*, *41*). These results thus raise the possibility that PDI-catalyzed thiol−disulfide exchange in these proteins has a role in platelet responses.

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