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A Mechanism for Assembly of Complexes of Vitronectin and Plasminogen Activator Inhibitor-1 from Sedimentation Velocity Analysis*

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Plasminogen activator inhibitor-1 (PAI-1) and vitronectin are biological cofactors involved in pathological conditions such as injury, inflammation, and cancer, during which local levels of PAI-1 are increased and the active serpin forms complexes with vitronectin. These complexes become deposited into surrounding tissue matrices, where they regulate cell adhesion and pericellular proteolysis. The mechanism for their co-localization has not been elucidated. We hypothesize that PAI-1-vitronectin complexes form in a stepwise and concentration-dependent fashion via 1:1 and 2:1 intermediates, with the 2:1 complex serving a key role in assembly of higher order complexes. To test this hypothesis, sedimentation velocity experiments in the analytical ultracentrifuge were performed to identify different PAI-1-vitronectin complexes. Analysis of sedimentation data invoked a novel multisignal method to discern the stoichiometry of the two proteins in the higher-order complexes formed (Balbo, A., Minor, K. H., Velikovsky, C. A., Mariuzza, R. A., Peterson, C. B., and Schuck, P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 81–86). Our results demonstrate that PAI-1 and vitronectin assemble into higher order forms via a pathway that is triggered upon saturation of the two PAI-1-binding sites of vitronectin to form a 2:1 complex. This 2:1 PAI-1-vitronectin complex, with a sedimentation coefficient of 6.5 S, is the key intermediate for the assembly of higher order complexes.

Plasminogen activator inhibitor type 1 (PAI-1) and vitronectin are biological cofactors that play a central role in extracellular matrix remodeling during pathological events such as tissue injury and cancer progression (1–3). The serpin PAI-1 is the primary inhibitor of tissue- and urokinase-type plasminogen activators and thus is the main regulator of plasmin-mediated vascular and pericellular proteolysis (4–6). The circulating form of PAI-1 is found primarily in a complex with vitronectin, with the inhibitor bound at a high affinity site located within the amino-terminal SMB domain of vitronectin (7–9). Vitronectin, which is found both in blood and surrounding tissues, also contains binding sites for cell surface receptors and ECM components, both of which serve in the attachment of cells to their surrounding matrices (10–17).

In many instances of tissue injury, cell invasion, or tumor development, PAI-1 and vitronectin are co-localized to the areas of vascular damage, necrosis, angiogenesis, and inflammation (2, 18–23). In contrast to PAI-1, which is locally produced and secreted by cells into the pericellular environment, the main source of vitronectin for tissue deposition is probably its recruitment from circulation. Incorporation of vitronectin into tissue ECM is linked to “activation” of the protein into an altered, adhesive form, which is both structurally and functionally distinct from its native, plasma counterpart (3). Adhesive functions adopted by this altered form of vitronectin work in parallel with the inhibitory functions of PAI-1, in a fashion that allows for co-regulation of cell adhesion and tissue degradation. The exact mechanism by which plasma vitronectin adopts the altered form, is relocalized, and becomes associated with PAI-1 in tissues under pathological scenarios is currently undefined.

Interaction of PAI-1 and vitronectin affects the structural and functional properties of both proteins. The active structure of free PAI-1 is relatively unstable, and it readily converts into a latent, inactive form by spontaneous insertion of its reactive center loop into the central β-sheet core of the protein (24). However, vitronectin bound to PAI-1 stabilizes the serpin in an active, inhibitory conformation (7) by binding across the base of the central β-sheet of PAI-1 and constraining the movement necessary to accommodate insertion of the reactive center loop (25). In addition to this stabilization, association with vitronectin endows PAI-1 with inhibitory properties toward a wider repertoire of serine proteases, including thrombin and activated protein C, thus expanding the antiproteolytic potential of PAI-1 (26–28).

Binding of PAI-1 also affects the adhesive properties of vitronectin that arise from its association with cell surfaces and the ECM. Cell surface receptors (e.g. integrins and urokinase-type plasminogen activator receptor) bind vitronectin at sites that lie proximal to the high affinity PAI-1 site in the SMB
domain of vitronectin (8, 9, 29–31). Under some conditions, PAI-1 disrupts cell receptor binding to vitronectin via a presumed steric effect (32, 33). However, under other conditions, PAI-1 actually promotes the association of native vitronectin with purified integrins αvβ3 and GPIIIbIIa (34) or cultured cells (34).2 In addition to such variable effects on interactions with receptors, PAI-1 binding also enhances interactions of vitronectin with heparin, osteonectin, and purified ECM from human placenta (34–36). A similar effect of PAI-1 is apparent in cell culture, in which the PAI-1-vitronectin complexes preferentially are relocated to the ECM of cultured fibroblasts in a manner that requires glycosaminoglycans.2 These findings suggest that the changes that accompany vitronectin binding may define a mechanism for the recruitment of PAI-1 and vitronectin to tissues in pathological situations.

Sedimentation equilibrium analysis first showed that PAI-1 interacts with vitronectin to form a complex with a 2:1 (PAI-1/vitronectin) stoichiometry that associates into a higher order form comprising four PAI-1 molecules and two vitronectin molecules (37). Formation of the 4:2 complex was postulated to proceed via transient 1:1 and 2:1 species, although these intermediates were not significantly populated in the equilibrium studies (37). This study provided the first evidence that PAI-1 binds simultaneously to two sites on vitronectin and prompted our working hypothesis, that formation of these 2:1 complexes is the step that controls the pathway for assembly of higher-order PAI-1-vitronectin complexes that adopt altered adhesive properties. In the present study, hydrodynamic approaches were expanded to investigate the mechanism of assembly with defined intermediates to produce multivalent, higher order complexes containing PAI-1 and vitronectin.

MATERIALS AND METHODS

Purification and Labeling of Vitronectin—Monomeric vitronectin was purified from human plasma as described previously (38, 39). For multiwavelength studies, vitronectin was labeled with fluorescein isothiocyanate using a FluoroReporter® protein labeling kit (Molecular Probes, Inc., Eugene, OR) that labels primary amines. The molar ratio of dye to vitronectin in the labeling reaction typically resulted in conjugation of ~2 fluorescein molecules per vitronectin monomer; labeling ratios varied from 1.5 to 1.8 mol of fluorescein per mol of vitronectin. The labeled vitronectin monomer was fully functional, with no adverse effects of labeling on PAI-1 binding.

Mutagenesis, Expression, and Purification of Recombinant PAI-1—The stable I4–I8 mutant PAI-1 (40) and latent PAI-1 were obtained from Molecular Innovations, Inc. The W175F variant (41) and PAI-1R (42) were produced as described (in Refs. 41 and 42, respectively). The M347C (P1 containing two amino acid replacements, T333R and A335R (42)) recombinant human PAI-1 gene was cloned into the pET-24d vector (Novagen) as described previously (43). The W175F variant (41) or 0.83 ml mg−1 cm−1 for wild-type PAI-1 (45) or 0.53 ml mg−1 cm−1 for W175F PAI-1 (41). Concentrations of labeled PAI-1-fluorescein were determined by the Bradford dye-binding assay (Bio-Rad) using wild-type PAI-1 as the standard (46).

Kinetic Assay to Quantify Active PAI-1—Equimolar amounts of PAI-1 and vitronectin (2 μM) were mixed in phosphate-buffered saline (10 mM dibasic sodium phosphate, 2 mM monobasic potassium phosphate, pH 7.4, containing 0.14 mM NaCl and 3 mM KCl) and incubated at room temperature. At various time points from 15 min to 35 h, an aliquot of the vitronectin/PAI-1 mixture was added to 2 μM tPA and incubated at 37 °C for 10 min to allow inactivation of tPA by active PAI-1. The vitronectin/PAI-1/tPA mixture was added to 0.1 mM Spectrozyme® tPA (American Diagnostica Inc., Stamford, CT), and the absorbance at 405 nm was read after 5 min.

Sedimentation Velocity Experiments—A Beckman Optima XL-A analytical ultracentrifuge equipped with both absorbance and interference optical detection systems were used to follow the formation of higher-order complexes between vitronectin and PAI-1. Epon double sector cells were filled with 400 μl of samples containing either vitronectin or PAI-1 alone or mixtures of the two at varying molar ratios. All samples were first dialyzed extensively against phosphate-buffered saline. For all experiments, the dialysate was loaded into the reference sector. Samples were centrifuged at a rotor speed of 50,000 rpm, and data were collected in fringes using interference optics and/or by absorbance at 494 nm, the extinction maximum for fluorescein.

Hydrodynamic Modeling of the Sedimentation Velocity Data—Sedimentation velocity analyses were performed with the program SEDFIT (available on the World Wide Web from www.AnalyticalUltracentrifugation.com) as described in detail in Ref. 47 that carry out size distribution analyses based on Lamm equation modeling (48–50). Briefly, the analysis of velocity profiles was performed by direct boundary modeling by distributions of Lamm equation solutions c(s) with the following,

\[
a(r,t) = \int c(s) \chi(s,F,r,t) ds + b(r) + \beta(t)
\]

(Eq. 1)

where a(r,t) denotes the measured absorbance or interference profiles at position r at time t, b(r) and \(\beta(t)\) are the characteristic systematic offsets (50), and \(\chi(s,F,r,t)\) are solutions of the Lamm equation,

\[
\frac{\partial \chi}{\partial t} = \frac{1}{\rho} \frac{\partial}{\partial r} \left[ D \frac{\partial \chi}{\partial r} - s \omega^2 \rho^2 \chi \right]
\]

(Eq. 2)

(\(\omega\) with \(\rho\) the denoting the rotor angular velocity, D the diffusion coefficient, and \(s\) the sedimentation coefficient) at unit loading concentration (51). D and s are related via the frictional ratio \(F = (\beta(s)/\beta)\) and the hydrodynamic scale relationship,

\[
D(s) = \frac{c^2}{18\pi^3} kT s^{-2/3} (1 - \nu)/\nu^{3/2}
\]

(Eq. 3)

(\(\nu\) and \(\eta\) the solvent viscosity and density, respectively, and \(\nu\) the partial specific volume of the macromolecules, which were calculated using the software SEDNTERP (50)). The nonlinear regression with Equation 1 was combined with the determination of the best fit weight average frictional ratio, the best fit meniscus position, and the algebraic calculation of systematic time-invariant and radial-invariant noise components and maximum entropy regularization at a confidence level of 0.70 (47, 51). Because these systematic signals are arbitrary offsets introduced from the detection system, calculated systematic offsets can be subtracted from the raw data without introduction of bias if the degrees of freedom in the analysis are not reduced. Therefore, the final calculated best fit offsets are subtracted from the raw data for presentation purposes. In all calculations, both meniscus and frictional ratio parameters were optimized by carrying out nonlinear regression to obtain best fits. s values were converted to standard conditions in water at 20 °C with the program SEDNTERP (50).

Multisignal Analysis of Sedimentation Data for Fluorescently Labeled PAI-1/Vitronectin Mixtures—Sedimentation velocity data acquired simultaneously at different signals (reflective index and absorbance) were globally modeled with a superposition of component sedimentation coefficients \(c_i(s)\) according to the following,

\[
a_i(r,t) = \sum_{k=1}^{K} c_i(s) \chi_i(s,F,r,t) ds
\]

\(\lambda = 1 \ldots \Lambda, K \leq \lambda\)

(Eq. 4)
where \( c_{s_k} \) denotes the extinction coefficient (or generalized signal increment) of component \( k \) at signal \( \lambda \). This multisinusoidal generalization of Equation 1 for heterogeneous protein interactions is described in detail elsewhere (50) and was implemented in the software SEDPHAT (available on the World Wide Web at www.AnalyticalUltracentrifugation.com). Briefly, the extinction coefficients were predetermined in separate experiments with each protein component alone. As a result, the global modeling of data acquired at different wavelengths (or signals) permits a spectral distinction of the different sedimenting protein components and allows for separate deconvolution of the sedimentation coefficient distributions \( c_{s_k}(s) \) of species containing protein component \( k \). Although this may lead also to slightly enhanced deconvolution of the hydrodynamic separation, it has properties very similar to those of the \( c(s) \) with regard to the influence of reaction kinetics on the obtained sedimentation coefficient distributions (48). Reactions that are fast on the time scale of sedimentation can be diagnosed by concentration-dependent peak positions in \( c_{s_k}(s) \), which reflect the characteristic \( s \) values of the reacting system, but with different \( c_{s_k}(s) \) still reflecting the correct stoichiometry (52). Like \( c(s) \) (48, 50, 53), reactions that are slow on the time scale of sedimentation, such as those studied in the present communication, result in peak positions of \( c_{s_k}(s) \) that correspond to different sedimenting species.

**RESULTS**

**Characterization of PAI-1 and Vitronectin**—The design of this study is to use hydrodynamic methods to critically evaluate the formation of PAI-1-vitronectin complexes. The SV method, which provides first principle hydrodynamic information concerning the size, shape, and stoichiometries of macromolecules in heterogeneous populations of interacting species, was adopted to characterize all complexes that form upon PAI-1 binding to vitronectin.

As a first step in the analysis, experiments were conducted to characterize the hydrodynamic properties of the individual proteins. Fig. 1 shows the sedimentation profiles for PAI-1 and vitronectin (Fig. 1A and B, respectively) and the sedimentation coefficient distributions \( c(s) \) for both PAI-1 and vitronectin obtained from these analyses (Fig. 1C). For both samples, a major species is apparent, with peak \( s \) values centered at 3.4 and 4.2 \( S \) for PAI-1 and vitronectin, respectively (Table I). This observation was made essentially independent of protein concentration. The small peak at a higher \( s \) value of 6.2 \( S \) corresponds to a small amount of vitronectin dimer that appears to a variable extent in purified vitronectin samples (7). The quality of fit was high, with root mean square deviations in both observation was made essentially independent of protein concentration. The small peak at a higher \( s \) value of 6.2 \( S \) corresponds to a small amount of vitronectin dimer that appears to a variable extent in purified vitronectin samples (7). The concentration range from 8 to 1.6 \( \mu \)M significantly alters the sedimentation rates of the different species formed stably during the time frame of individual SV experiments (48, 50, 53). Representative SV data on the PAI-1/vitronectin mixture are shown in Fig. 2A. From the SV data acquired on an equimolar 8 \( \mu \)M mixture of vitronectin and PAI-1, three major peaks at \( s \) values of 6.5, 8.6, and 19.9 \( S \), respectively, were resolved in addition to several higher order complexes ranging from 12 to 25 \( S \) (Fig. 2B). Consistent with previous studies (34, 35, 37), the hydrodynamic profiles show that PAI-1 binds to vitronectin and results in the formation of a variety of complexes, including multiple higher order oligomers.

To test the reversibility in assembly of these higher order complexes, the equimolar 8 \( \mu \)M PAI-1/vitronectin mixture was diluted to varying concentrations that were analyzed by SV (Fig. 2, C and D). The results demonstrate that dilution over a concentration range from 8 to 1.6 \( \mu \)M significantly alters the \( c(s) \) distribution profiles of the PAI-1/vitronectin mixtures. As summarized in Table I, the 1–5, 5.1–7.4, and 7.5–25 \( S \) intervals of sedimentation coefficients are hydrodynamically consistent with free vitronectin and/or PAI-1, intermediate 1:1 and 2:1 PAI-1-vitronectin complexes, and higher order complexes, respectively. To quantify changes observed in Fig. 2, B–D, peaks within these three different ranges of \( s \) values were integrated to obtain total amounts of the corresponding species (Table II).

Only the distribution of PAI-1/vitronectin complexes, characterized by sedimentation coefficients above 5 \( S \), is shown in Fig. 2. For each of the protein concentrations tested, the pro-

**FIG. 1. Sedimentation velocity analysis of PAI-1 and vitronectin using SEDFIT.** PAI-1 (3.5 \( \mu \)M) and vitronectin (3.5 \( \mu \)M) were analyzed individually by centrifugation at 50,000 rpm at 22 °C in a Beckman-Coulter XL1 analytical ultracentrifuge, and sedimentation was monitored by interference detection. SV data were analyzed using a continuous \( c(s) \) distribution model implemented in the program SEDFIT. Partial specific volumes calculated on the basis of amino acid and carbohydrate content for recombinant PAI-1 and human vitronectin were 0.74 and 0.705 \( ml/g \) (Table I). The frictional ratio for each sample was a fitted parameter in the analysis. Representative sedimentation data, with global fits to each data set, are shown for PAI-1 and vitronectin in A and B, respectively, and resulting residuals for each analysis are given below each SV data profile. Scans were collected at approximate 11-min intervals. All data were included in the calculations of \( c(s) \) distributions. \( c(s) \) distribution profiles for the two proteins are shown in C (black line, PAI-1; blue line, vitronectin).
portion of signal corresponding to s-values between 1 and 5 S, representing free PAI-1 and/or vitronectin, consistently was ~30% of the total signal (not shown), consistent with our prior sedimentation equilibrium studies on 1:1 mixtures of the two proteins (37). The 5.1–7.4 and 7.5–25 S integration ranges, corresponding to intermediate and higher order complexes, respectively, are indicated by colored bars at the top of Fig. 2B. What is most significant about the findings is the apparent reversibility of formation of higher order complexes with s values between 7.5 and 25 S, with a concomitant emergence of intermediate complexes in the 5.1–7.4 S range. As demonstrated in Fig. 2D, with an equimolar concentration of PAI-1 and vitronectin at 1.6 μM, only 6% of the total reaction mixture is present in the higher order complexes with s values above 7.5 S. This contrasts with the mixtures containing the 8 and 3.2 μM concentrations, which showed complexes in this range equal to 6 S at lower protein concentrations tested. As discussed below, this sedimentation coefficient of ~6 S represents a mixture of 1:1 and 2:1 PAI-1-vitronectin complexes that are not resolved in this experiment. Because of the apparent interdependence of species with intermediate (5.1–7.4 S) and high (7.5–25 S) sedimentation coefficients, we focused on characterizing the composition of the intermediate species in further experiments.

**Formation of Higher Order Complexes Occurs in a Concentration-dependent Fashion**—To test the concentration dependence of the assembly of oligomeric complexes, SV analysis was performed on mixtures where one of the proteins was held constant while the other was varied. For one set of experiments (Fig. 3A), vitronectin was maintained at a constant concentration of 1.6 μM. This particular vitronectin concentration leads to the assembly of a predominant species in the intermediate range with a sedimentation coefficient near 6 S (Fig. 2). Furthermore, this concentration is physiologically relevant and reflects the levels of vitronectin found in normal circulation (56–58). As shown in Fig. 3A, increases in the molar concentration of PAI-1 relative to vitronectin alter the c(s) distributions, with several higher order complexes that form at high concentrations of PAI-1.

A clearer picture of this dose dependence is seen in Fig. 3B, which focuses more closely on species distributed between 4.5 and 15 S. A substoichiometric molar ratio of PAI-1 to vitronectin (1:4) results in the assembly of complexes primarily distributed between ~5 and 7 S. When PAI-1 is mixed with vitronectin at equimolar levels, a significant increase in complexes in this range is evident. Moreover, the partial resolution of the broad peak between 5 and 7 S into a peak at ~5.5 S and a shoulder at 6.5 S is noteworthy. These species appear to represent PAI-1-vitronectin complexes with molar stoichiometries of 1:1 and 2:1, respectively (Table I; see below). Also, some higher order species with s values greater than 7 S emerge in this mixture. A further increase in PAI-1 concentration to a molar ratio of 4:1 results in further redistribution of sedimenting species that is quite dramatic. First, the broad peak be-

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**Table I**

| Species       | Molecular Weight* | Partial Specific Volume (mL·g⁻¹) | Maximum Sedimentation Coefficient (sₚₑᵥₚₚ) | Observed Sedimentation Coefficient (sₚₑᵥₚₚ) | Standard Sedimentation Coefficient (sₚₑᵥₚₚ) | Frictional Ratio (f/F) |
|---------------|------------------|---------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|----------------------|
| Vitronectin   | 62,000           | 0.705°                          | 6.4 S                                       | 4.1 - 4.7 S                                 | 4.2 ± 0.2 S                                 | 1.5                  |
| PAI-1         | 43,000           | 0.740°                          | 4.3 S                                       | 3.2 - 3.7 S                                 | 3.4 ± 0.2 S                                 | 1.2                  |
| 1 PAI-1:1 VN  | 105,000          | 0.73°                           | 8.1 S                                       | 5.0 - 5.6 S                                 | 5.5 ± 0.2 S                                 | 1.5                  |
| 2 PAI-1:1 VN  | 148,000          | 0.73°                           | 10.2 S                                      | 6.0 - 6.7 S                                 | 6.3 ± 0.2 S                                 | 1.6                  |
| 4 PAI-1:2 VN  | 296,000          | 0.73°                           | 16.3 S                                      | 8.0 - 10.0 S                                | 8.5 ± 0.5 S                                 | 1.8                  |

* The molecular weight of PAI-1 was calculated from its amino acid sequence. The molecular weight of vitronectin originally determined by equilibrium analytical ultracentrifugation (35) was recalculated as 62,000 using a corrected v that includes contributions of carbohydrate side chains (53).
* Partial specific volumes were calculated using SEDNTERP from the known sequences of PAI-1 and vitronectin. The partial specific volume of vitronectin also included the contribution from an average of two biantennary and one triantennary carbohydrate side chain per molecule, with an average sequence as reported (77).
* A partial specific volume typical for protein samples was used in calculations for complexes of PAI-1 and vitronectin.
* Maximum sedimentation coefficients for a spherical, nonhydrated molecule calculated from the molecular weights using SEDFIT.
* Range in sedimentation coefficients observed under experimental conditions.
* Sedimentation coefficients converted to standard conditions of 20 °C in water ± S.D. (n = 4). sₚₑᵥₚₚ values were calculated using the SEDNTERP and SEDPHAT programs.
Sedimentation Velocity Analysis on PAI-1-Vitronectin Complexes

Equimolar amounts of PAI-1 and vitronectin were mixed for 1 h and were analyzed by SV at 50,000 rpm at 25 °C. Representative data collected using interference optics are shown in A. These data were fit using the continuous c(s) distribution model in SEDFIT, and the fit to the data for an 8 μM PAI-1/vitronectin mixture is given by the solid lines in A, with residuals for the fit shown below the data. Scans collected at different time intervals are shown in different colors for clarity, and only a subset (every other scan) of the interference data is shown. Scans were collected at approximate 1-min intervals. Resulting c(s) distributions for a dilution series originating from the 8 μM PAI-1/vitronectin mixture are shown in B (8 μM mixture), C (3.2 μM mixture), and D (1.6 μM mixture). A range of s values from 5 to 25 S is plotted in B-D, corresponding only to intermediate (5.1–7.4 S) and higher order (>7.5 S) PAI-1-vitronectin complexes. These ranges in s values, corresponding to the integration ranges in Table I, are denoted, respectively, by the red and blue bars above B. Species with s values of <5 S, corresponding to free vitronectin and/or PAI-1 and quantified in Table I, are not shown.

comes more resolved with a predominant peak at ~6.5 S and a corresponding decrease in the ~5.5 S complex. Thus, the 2:1 PAI-1-vitronectin complex is favored at higher PAI-1 concentrations. Second, at this point, there is an accumulation of significant levels of higher order species with s values greater than 7 S. Multiple higher order complexes are present, including those at s values of 8.7, 12.9, 16.4, 20.7 S, and greater. Thus, it appears that appreciable formation of these higher order complexes is only seen when the intermediate 6.5 S complex is populated.

A converse experiment also was performed in which the PAI-1 concentration was fixed and vitronectin was varied. As shown in Fig. 3C, accumulation of PAI-1-vitronectin complexes depends on the relative abundance of the two proteins. The results of this experiment are similar to those in Fig. 3A, in which higher protein concentrations lead to the population of a 6.5 S complex and a concomitant generation of higher order complexes. Although the formation of higher order complexes at the highest protein concentrations is apparent, the abundance of these complexes relative to total protein is less than observed at the highest protein concentrations measured in Fig. 3A. This is an expected result, since the highest protein concentrations in Fig. 3C correspond to excess vitronectin (i.e. experimental conditions that favor primarily the occupation of the high affinity PAI-1-binding site rather than the second, lower affinity site on vitronectin). As a result, there is less accumulation of the 6.5 S (2:1) intermediate and pursuant higher order species compared with the highest protein concentrations in Fig. 3A in which PAI-1 is in excess and both binding sites on vitronectin are occupied. The analyses of varying protein ratios in Fig. 3 support a mechanism for the accumulation of higher order species (>7.5 S) via formation of the 6.5 S species as an intermediate.

Multisignal Sedimentation Velocity Analysis Confirms the Binding of Two PAI-1 Molecules to Vitronectin—What is this critical complex that sediments with an s value of 6.5 S? Our hypothesis is that it is a 2:1 complex with two molecules of PAI-1 bound to distinct sites on a single molecule of vitronectin (Table I). We suggest that these two sites on vitronectin differ in affinity, with the high affinity site (Kd ~ 1–10 nM (29, 59)) resident in the SMB domain and a second lower affinity site (Kd ~ 25–50 nM (34)) located outside the SMB region at a yet unidentified locus. Because of the differences in affinity, these binding sites should be occupied in a concentration-dependent fashion, and our SV results support this notion. Furthermore, we hypothesize that it is the saturation of both binding sites that forms the crucial 2:1 complex that then serves as a physiological “trigger” to induce the formation of higher order PAI-1-vitronectin complexes. To test this hypothesis, a definitive identification of the composition and stoichiometry of this 6.5 S complex is needed.

For this purpose, we have used a new multiwavelength approach that allows simultaneous analysis of each protein in the complex (52). We have performed a global analysis of multisignal SV data for unraveling the populations and stoichiometries of heterogeneous protein complexes formed between PAI-1 and vitronectin. We have focused our attention primarily on the species identified at ~6.5 S. Multiwavelength analysis has been used fruitfully with this system of interacting proteins in our prior sedimentation equilibrium measurements (37), taking advantage of different absorbance properties for proteins selectively labeled with chromophores. In a similar strategy, the SV analysis was carried out using labeled PAI-1 and/or labeled vitronectin by collecting absorption and interference optical data from the same cell.

The analysis was implemented in the program SEDPHAT, for which features are described in detail elsewhere (52). From a global multisignal analysis, the new method permits a direct calculation of the separate c(s) distributions of the different protein components, termed c(s), based on the predetermined contributions of each protein to all of the observed signals (52). The analysis shown in Fig. 4 was performed on data acquired using fluorescein-labeled vitronectin and unlabeled PAI-1 (Fig. 4, A and B, interference and absorbance data, respectively). Both sets of data were globally fit by the multisignal/wave-length analysis approach using experimentally determined values representing both chromophoric and interference-coupled extinction coefficients. Analyses resulted in well fit data for the experiments, with both local and global errors in the acceptable range, as shown in the residuals shown below the data. Similar results were obtained from the analysis using the opposite labeling strategy with fluorescein-labeled PAI-1 and unlabeled vitronectin (data not shown).

3 C. S. Schar and C. B. Peterson, unpublished data.
Sedimentation Velocity Analysis on PAI-1-Vitronectin Complexes

**Table II**

Assembly of vitronectin and PAI-1 into intermediate and higher order complexes

| Protein samples            | Protein concentrations | Unbound proteins (fraction in s range from 1 to 5 S) | Intermediate complexes (fraction in s range from 5.1 to 7.4 S) | Higher order complexes (fraction in s range from 7.5 to 25 S) |
|---------------------------|------------------------|--------------------------------|--------------------------------------------------|--------------------------------------------------|
| Vitronecin + wild-type PAI-1 | 8 μM vironectin 8 μM PAI-1 | %                              | %                                         | %                                        |
| Vitronecin + wild-type PAI-1 | 3.2 μM vitronectin 3.2 μM PAI-1 | %                              | %                                         | %                                        |
| Vitronecin + wild-type PAI-1 | 1.6 μM vitronectin 1.6 μM PAI-1 | %                              | %                                         | %                                        |
| Vitronecin + Y175F PAI-1   | 3.3 μM vitronectin 3.3 μM PAI-1 | %                              | %                                         | %                                        |
| Vitronecin + PAI-1 R       | 3.3 μM vitronectin 3.3 μP MA-1 | %                              | %                                         | %                                        |

*Sedimentation data were evaluated as c(s) profiles, and data were integrated using SEDFIT to determine the relative amounts of various PAI-1-vitronectin complexes formed in the three mixtures. Values were normalized by dividing the integrated values by the total loading signal in fringes and multiplying by 100 to obtain a percentage of the various species formed. Errors are ± 5%.

**Fig. 3.** SV analysis to evaluate the concentration dependence of formation of higher order PAI-1-vitronectin complexes. PAI-1 and vitronectin were mixed under conditions where the concentration of one protein was held constant while the other was varied. The proteins were mixed for 1 h and were analyzed by SV at 50,000 rpm at 20 °C with detection by interference optics. Data were analyzed using SEDFIT. A and B show c(s) distributions for mixtures of 1.6 μM vitronectin with 0.4 μM PAI-1 (black line), 1.6 μM PAI-1 (blue line), or 5.6 μM PAI-1 (red line). B gives an expanded view of the c(s) distribution between s values of 4.5–15 S. An asterisk denotes the critical 6.5 S complex. C shows the converse titration in which PAI-1 was held constant at 3.5 μM, and the vitronectin concentration was varied between 0.87 μM (black line), 1.7 μM (blue line), and 7 μM (red line).

The analysis of the mixture using fluorescein-labeled vitronectin and unlabeled PAI-1 shows both protein components in co-sedimenting peaks, as expected, since they form complexes (Fig. 4C). The relative amounts of signal indicate that the complex with a sedimentation coefficient of ~6.5 S contains a molar excess of PAI-1, and integration of the peak areas in this c(s) distribution shows that this 6.5 S complex contains a molar ratio of 2:1 PAI-1/vitronectin. The calculation of this molar ratio relies on the relative heights of the boundaries of the sedimenting components detected using the two signals from interference and absorbance optics. Thus, the multiwavelength approach explicitly identifies the 2:1 intermediate that is required for assembly to higher order species. Also, the higher order species that exhibits a sedimentation coeffi-

**Fig. 4.** Global multisignal analysis demonstrates the formation of a 2:1 PAI-1-vitronectin complex at 6.5 S. Solutions of either fluorescein-labeled PAI-1 (8 μM) or fluorescein-labeled vitronectin (2 μM), mixed for 1 h with the corresponding nonlabeled protein, were analyzed by sedimentation velocity at 50,000 rpm at 20 °C. For each mixture, both interference and absorbance at 494 nm were collected. Interference and absorbance scans were taken every 4 min. Resulting profiles were analyzed using a novel multisignal analysis feature implemented in the program SEDPHAT. Prior experiments were carried out to determine the chromophoric and interference-coupled extinction coefficients, which were included in the analysis of mixtures. SV data, with fits to each data set, are shown for the mixture of fluorescein-labeled vitronectin with unlabeled PAI-1 in A (interference data) and B (absorbance data at 494 nm). Vertical green lines in panels A and B represent the boundaries on data included in the global fits. Resulting residuals for each analysis are given below each SV data set. A representative multisignal c(s) analysis is shown in C, with the distribution of PAI-1 and vitronectin represented by red and blue lines, respectively.
cient of ~8 S (Fig. 4C) contains the same molar stoichiometry of vitronectin and PAI-1, identifying this species as the 4:2 complex (Table I). The same approach applied to other higher order complexes was less definitive (mainly due to their limited abundance and small contribution in the visible absorbance signal) but clearly indicated the presence of molar excesses of PAI-1 in several cases (data not shown).

A detailed interpretation of the peak shapes from this multiwavelength analysis is not possible currently, due to probable effects of the signal/noise ratio on the degree of regularization in the calculated curves and the potential influence of slight mismatches in the meniscus position of the different data sets. It should be noted that in theory the component c(s) distributions of interacting species cannot necessarily be expected to be in perfect register, due to the effects of finite reaction kinetics and the dispersion of composition in the reaction boundary predicted by Gilbert-Jenkins theory (60), which can apply to c(s) distributions. Despite these effects, the integral over the peaks of the reaction boundary reports the stoichiometry of the complexes. Thus, the multisignal analysis provides conclusive evidence that PAI-1 and vitronectin interact to form a 2:1 complex that sediments at ~6.5 S, supporting our hypothesis that formation of a 2:1 PAI-1-vitronectin complex serves as a building block in the formation of higher order oligomers.

Oligomeric Forms of Vitronectin Persist at Long Incubation Times—PAI-1 is a unique member of the serpin family that spontaneously converts over time to a latent, noninhibitory conformation (61, 62). This conformational change in PAI-1 also renders the protein incapable of binding to vitronectin (63). To evaluate the effects of this transition to latent PAI-1 on the distributions of higher order complexes, a time course was evaluated over the distributions of higher order complexes, a time course was evaluated over ~24 h. In these experiments, equimolar mixtures of PAI-1 and vitronectin were incubated at room temperature for different time intervals and then subjected to SV analysis. As shown in Fig. 5A, c(s) distributions for mixtures incubated at 1 and 6 h are similar, with a nearly equivalent population of the species at ~6.5 S. However, there is a redistribution of higher order species with s values greater than 7.5 S, most apparent for the species with a sedimentation coefficient ~8 S (Table I). It is probable that this redistribution reflects an accumulation of higher order forms that assemble on a longer time scale than the 2:1 intermediate at ~6.5 S.

As incubation times are increased to 12 and 24 h, there is further redistribution, with a time-dependent loss of higher order species. These changes are attributed to dissociation of the complexes as PAI-1 is converted to the latent form that does not bind to vitronectin (37). Consistently, the time-dependent loss of higher order species correlates well with the rate at which PAI-1 converts to a latent form under these conditions (Fig. 5B). Also consistent is the loss over time of the ~6.5 S (2:1) intermediate, coincident with the accumulation of significant amounts of free vitronectin and latent PAI-1. In agreement with previous work (34, 35), some oligomeric forms of vitronectin persist even at the longest times that correlate with complete loss of active PAI-1. Indeed, almost 44% of the total loading signal for vitronectin was still present as oligomers after 24 h.

Assembly of PAI-1-Vitronectin Complexes Is Independent of the Anti-protease Activity of the Inhibitor—Is the spontaneous conversion of PAI-1 to a latent conformation required for accumulation of vitronectin oligomers that persist at long times, as shown in Fig. 5? Must PAI-1 be active as a protease inhibitor to be effective in promoting the formation of higher order com-

\[ P \text{A} \text{I-1} + \text{V} \rightarrow \text{P} \text{A} \text{I-1-V} \]

complexes? To address such questions, mutant forms of PAI-1 were used that exhibited either slower rates of conversion to the latent form (40, 41) or impaired anti-protease activity (42). Several forms of PAI-1 have been engineered by introduction of strategic mutations that disfavor conversion to a latent structure and thus allow PAI-1 to retain activity for a long period of time.

Analysis of a stable form of PAI-1, the W175F mutant (41), gave a common c(s) distribution pattern of PAI-1-vitronectin complexes induced by W175F versus wild-type PAI-1, with the distribution of species in the 1–5, 5.1–7.4, and >7.5 S ranges summarized in Table II. It is clear from the results on this “stable” mutant that the inherent rate of conversion to a latent form is not a critical feature that determines the ability of PAI-1 to form higher order complexes with vitronectin. Consistent with previous studies (34, 63), mixtures of vitronectin with latent, inactive PAI-1 did not result in formation of any complexes (data not shown).

Another PAI-1 mutant tested in these experiments was the noninhibitory mutant PAI-1R (42). This double arginine mutant (T333R and A335R) is impaired in its ability to act as an inhibitor of either tissue- or urokinase-type plasminogen activator due to the introduction of charges into strategic positions in the reactive center loop that impede the insertion of the reactive center loop into the central β-sheet of the protein upon cleavage by plasminogen activators (42). However, this mutant PAI-1 retains the ability to bind vitronectin. The structural changes result in PAI-1R acting as a substrate, not an inhibitor, for plasminogen activators; as such, this mutant serves as a valuable tool for studying a variety of nonproteo-
lytic roles of PAI-1 that are important during processes like cell migration and tissue remodeling (32, 33, 64, 65). As summarized in Table II, PAI-1,14 induces a similar formation of higher order species as wild-type PAI-1. These findings indicate that the ability of PAI-1 to promote the formation of higher order complexes with vitronectin is independent of its ability to inhibit plasminogen activators.

DISCUSSION

PAI-1 is an acute phase reactant that increases in concentration rapidly and locally in response to tissue damage, inflammation, and cancer (66). Large increases in PAI-1 concentration occur in cancerous (66, 67) and in wounded tissues (66, 68, 69). Indeed, plasma concentrations of PAI-1 in cancer patients have been shown to approach micromolar concentrations (70), and high levels of PAI-1 are associated clinically with poor outcome and survival (66, 67, 70). Our hypothesis is that PAI-1-vitronectin complexes form by sequential occupation of two PAI-1-binding sites on vitronectin, with occupation of the high affinity site (Kd ~1–10 nM) under normal conditions at which PAI-1 circulates in approximately nanomolar concentrations. Occupation of the lower affinity site (Kd ~25–50 nM) should occur in pathological situations with elevated PAI-1 concentrations that approach the dissociation constant for the second site, a value ~10-fold weaker than that exhibited by the primary binding site housed within the SMB domain. Furthermore, we suggest that the 2:1 PAI-1-vitronectin complex, which is only formed at higher PAI-1 concentrations, is the key intermediate that regulates formation of the higher order adhesive forms. We propose that the formation of these complexes in the pericellular environment, with their concomitant targeting to the ECM, accounts for the co-localization of PAI-1 and vitronectin that is observed in damaged and diseased tissues (2, 18–23).

The objective of this work was to evaluate this stepwise assembly of PAI-1-vitronectin complexes in a concentration-dependent fashion via the 2:1 intermediate using SV analysis. By selectively labeling each of the two reactant proteins, the optical capabilities of the analytical ultracentrifuge can be exploited so that discrete assignments of the composition and stoichiometry of complexes is feasible. Moreover, a newly developed analytical method, multisignal analysis of SV data by global analysis of c(s) distributions in the program SEDPHAT (52), makes the quantitative aspects of this work more reliable. Experiments were designed to analyze the distribution of oligomeric species that evolve when monomeric forms of PAI-1 and vitronectin are mixed under a variety of conditions and protein concentrations. The novel method using SEPHAT was instrumental in determining the molar ratios of PAI-1 and vitronectin to conclusively demonstrate formation of the key 2:1 (PAI-1/vitronectin) regulatory intermediate under conditions in which higher order complexes are produced.

A scheme based on the SV analysis summarizes a proposed mechanism for assembly of PAI-1-vitronectin complexes (Fig. 6). Evaluation of the dose-dependent effects of PAI-1 show that in the first assembly step, PAI-1 binds with vitronectin to form a complex at 5.5 S, which consists of a single molecule of PAI-1 bound to a high affinity binding site on vitronectin (Table I, Fig. 6). As PAI-1 levels are increased, occupation of the second, relatively lower affinity binding site on vitronectin leads to the formation of the 6.5 S (2:1) species. The crucial 2:1 complex promotes oligomerization, serving as a building block for assembly of higher order complexes. Once this 6.5 S species accumulates, higher order complexes are produced, with a broad distribution in s values ranging from 7.5 to 25 S. In agreement with an earlier study using sedimentation equilibrium analysis (37), the new results demonstrate the formation of a dominant oligomeric species with a 4:2 PAI-1/vitronectin molar stoichiometry that exhibits sedimentation coefficients of ~8–10 S (Table I).

What are the structural requirements for assembly of these higher order forms? Experiments with mutant forms of PAI-1 indicate the inherent rate of conversion of PAI-1 to a latent conformation has a relatively minimal effect on whether or not the higher order complexes are formed. However, there are some differences when comparing our other work on SV analyses of the 14-1B PAI-1 mutant2 with the observations here on the W175F “stable” PAI-1 mutant. The well characterized 14-1B mutant of PAI-1 is another “stable” PAI-1 mutant that converts more slowly to the latent form. It contains four mutations, three of which are found within the central β-sheet core (40). The SV analyses showed that fewer higher order complexes with s values greater than 15 S are formed with the 14-1B isoform than with W175F PAI-1, given comparable conditions. Other recent observations substantiate differences be-

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**FIG. 6. Model for formation of higher order PAI-1-vitronectin complexes via the 2:1 intermediate.** In this model, PAI-1 binds to vitronectin in a concentration-dependent fashion to form the intermediate 1:1 and 2:1 PAI-1-vitronectin complexes. A conformational change indicated by the species marked with an asterisk is proposed to occur uniquely in the 2:1 complex. This conformational change producing “altered” vitronectin promotes oligomerization, leading to the assembly of the 4:2 PAI-1-vitronectin complex and additional higher order complexes. Free PAI-1 also converts at a slow rate to a latent form, which cannot associate with vitronectin. This structural conversion over time leads to accumulation of latent PAI-1, along with higher order vitronectin oligomers.
between two "stable" forms of PAI-1, including a report that 14-B-PAI-1 shows reduced activity against TPRA and thrombin under certain experimental conditions (71). Also, structural work using fluorescence resonance energy transfer indicates that 14-B and wild-type PAI-1 differ in the orientation of the reactive center loop, with the loop of wild-type PAI-1 being inserted (72). Consistently, the frictional ratio from the SV work on 14-1B-PAI-1 is 1.4, indicative of a larger hydrodynamic form. In our model in Fig. 6, PAI-1R, also demonstrates that an active serpin inhibitor is not independent of the rate at which the serpin converts to a latent form. These findings should provide insight into the observed dependence of processes like tumor growth and angiogenesis on local levels of PAI-1 (78, 79).

Behavior of another mutant form of PAI-1 in this study, PAI-1R, also demonstrates that an active serpin inhibitor is not required to promote the accumulation of higher order oligomers. In fact, the noninhibitory PAI-1R protein merely acts as a substrate for proteases, and cleaved PAI-1R product does not undergo insertion of the cleaved reactive center loop into the central β-sheet (42). From the analyses on these mutant forms of PAI-1, it appears that the conformational change to an "altered" or "open" form of PAI-1, it appears that the conformational change to an "open" form of PAI-1 leads to the higher order complexes, irrespective of whether the β-sheet reorientation in PAI-1 ensue. However, as suggested in our study using fluorescence resonance energy transfer indicates that 14-B and wild-type PAI-1 differ in the orientation of the reactive center loop, with the loop of wild-type PAI-1 being inserted (72). Consistently, the frictional ratio from the SV work on 14-1B-PAI-1 is 1.4, indicative of a larger hydrodynamic form. In our model in Fig. 6, PAI-1R, also demonstrates that an active serpin inhibitor is not independent of the rate at which the serpin converts to a latent form. These findings should provide insight into the observed dependence of processes like tumor growth and angiogenesis on local levels of PAI-1 (78, 79).

Behavior of another mutant form of PAI-1 in this study, PAI-1R, also demonstrates that an active serpin inhibitor is not required to promote the accumulation of higher order oligomers. In fact, the noninhibitory PAI-1R protein merely acts as a substrate for proteases, and cleaved PAI-1R product does not undergo insertion of the cleaved reactive center loop into the central β-sheet (42). From the analyses on these mutant forms of PAI-1, it appears that the binding of PAI-1 to vitronectin leads to the higher order complexes, irrespective of whether the β-sheet reorientation in PAI-1 ensue. However, as suggested in our model in Fig. 6, binding in itself is presumably not sufficient to produce these higher order complexes. Instead, we propose that a conformational change occurs in vitronectin within the 2:1 complex, leading to the higher order complexes containing the "altered" form of vitronectin that adopts an adhesive role.

What support is there for this proposed conformational change in vitronectin that favors its association to the higher order forms? Evidence for a structurally altered form of vitronectin comes from studies using monoclonal antibodies to demonstrate conformational differences in the native and altered ("activated") forms of vitronectin that become tissue-associated in pathological settings (3, 73, 74). Also relevant is work by Seiffert and Smith (75, 76) that suggests that ligand-binding sites housed in the SMB domain are cryptic in the native structure of vitronectin but become more exposed upon interaction of ligands with the heparin-binding site near the C terminus of the protein. Consistent with this idea, we have shown that the higher order PAI-1-vitronectin complexes that exhibited altered binding to receptors also localize to the ECM via interactions with glycosaminoglycans.2 Furthermore, our prior work on denaturation and renaturation of vitronectin demonstrated that the conformational change to an "altered" or "activated" form in vitronectin is irreversible, since native, monomeric protein was never recovered from the altered structure of vitronectin generated from such experiments (39, 77).

In conclusion, we have utilized SV analysis to elaborate on the mechanism that regulates the assembly of PAI-1-vitronectin complexes. Here, we show that PAI-1, via stepwise formation of intermediates in a concentration-dependent manner, forms a 2:1 complex that regulates the formation of higher order PAI-1-vitronectin complexes. This process of assembly of higher order complexes that are relocalized to the ECM provides a molecular mechanism for depositing PAI-1 and vitronectin into the pericellular space of tissues, where functional properties of both proteins become locally manifested. These findings should provide insight into the observed dependence of processes like tumor growth and angiogenesis on local levels of PAI-1 (78, 79).
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