Latency and Substrate Binding Globally Reduce Solvent Accessibility of Plasminogen Activator Inhibitor Type 1 (PAI-1)

AN ADAPTATION OF PAI-1 CONFORMER CRYSTAL STRUCTURES BY HYDROGEN-DEUTERIUM EXCHANGE*

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Plasminogen activator inhibitor type 1 (PAI-1) plays key regulatory roles in fibrinolysis, cell migration, and tissue remodeling. A regulatory protein without known catalytic activity, PAI-1 modulates plasminogen activators through protein-protein interactions. Although global conformational alterations that occur in PAI-1 determine its regulatory activity, comprehensive assessments of concurrent dynamic, structural, and functional alterations of this critical regulatory protein have not yet been clearly defined. X-ray crystallographic studies have described four distinct PAI-1 conformational states: active, latent, reactive center loop peptide-annealed (RCL-PA), and cleaved mutant. In this study, backbone amide hydrogen-deuterium exchange detected by mass spectrometry was used to characterize dynamic and structural alterations of human PAI-1 (hPAI-1) in relation to its function. hPAI-1 conformers were defined by surface mapping the solvent-accessible sites for strategic secondary structural components of the protein. We observed a global protection from solvent for a majority of peptides in the latent conformer relative to the active conformer. Significant differences were observed in the RCL, helix A, helix D, and sheet 1C, and these regions were markedly more dynamic or solvent-exposed in the active conformation. The RCL-PA form adopts an intermediate conformational state between the active and the latent conformers. Our results demonstrate that the most dynamic regions of PAI-1 (the RCL, helices D and A, and sheet 5A) are flexible in the transition toward latency. They also show that the dynamic surface structures of the active, latent, and peptide-annealed conformers of PAI-1 are underestimated by theoretical solvent accessibility calculations derived from crystallographic data.

Plasminogen activator inhibitor type 1 (PAI-1) is a key regulator of fibrinolysis, cell migration, and tissue remodeling.

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1 The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; ESI, electrospray ionization; h, α-helix; hPAI-1, human PAI-1; (1–3). As a member of the serine protease inhibitor family, PAI-1 serves as a major inhibitor of the proteases tissue plasminogen activator and urokinase plasminogen activator (uPA) (4, 5). Recent clinical studies suggest important links between PAI-1 and accelerated development of atherosclerosis and a prothrombotic state, with attendant enhanced risks for development of myocardial infarction, stroke, and thromboembolic events (6–9). Animal model studies further demonstrate that PAI-1 plays a critical role in ventricular remodeling following an acute myocardial infarction, a process important in the development of heart failure (10). The many links between PAI-1, cardiovascular disease, and thrombosis have thus made this regulatory protein a recent focus of intense research interest. Despite this, the structural and dynamic conformational alterations that underlie the regulatory functions of PAI-1 are incompletely understood.

In vivo, synthesized PAI-1 is secreted and bound to an adhesive, stabilizing glycoprotein, vitronectin, in a noncovalent complex (11). PAI-1 bound to vitronectin remains folded in an “active” conformation, as defined by its ability to bind and inhibit plasminogen activators. When unbound to vitronectin, PAI-1 undergoes a slow spontaneous conversion (t1/2 = 2 h, 37 °C) into an inactive “latent” conformation that no longer is capable of binding to and inhibiting plasminogen activators (5, 12, 13). The active form of PAI-1 can be partially regenerated from the latent form by undergoing refolding transitions under kinetically controlled denaturation conditions (14).

Because of its size of 45 kDa, decoding the structural changes involved in the conformational alterations of PAI-1 has been relegated mostly to x-ray crystallography. Three-dimensional studies by x-ray crystallography have revealed the structure of the latent form (15), a cleaved form (16), an engineered active form (17), and a reactive center loop RCL-PA form to mimic substrate binding (19). These structures reveal that the RCL is solvent-exposed, protruding from one end of the elongated molecule in the active form, and markedly unexposed in the latent form. Accompanying this conformational change is the insertion of the RCL during latency as β-sheet 4A into a well packed scaffold of 9 α-helices and 3 β-sheets (15–17).

Obtaining a crystal structure of the native active state and the substrate-bound complex of PAI-1 has been problematic because of their functional instability (12, 18). A conformer of PAI-1 with substrate binding properties has been mimicked by annealing PAI-1 to an N-acetylated octapeptide comprised of

1H → 2H exchange, hydrogen → deuterium exchange of amide protons; N-Ac-, N-terminal-acetylated; PDB, protein data base; RCL, reactive center loop; RCL-PA, reactive center loop peptide annealed; RSA, relative solvent accessibility; s, β-sheet; uPA, urokinase-type plasminogen activator; serpin, serine protease inhibitor.
the N-terminal residues from the RCL (N-Ac-TVASSSTA, P14–P7, T333–A340), resulting in loss of PAI-1 activity (i.e., inhibition of PAI-1 binding to and inhibition of plasminogen activators) (19). In addition, a smaller pentapeptide fragment (TVASS, P14–P10, residue T333–S337) was also used to generate a substrate mimic bound to PAI-1 (19). Results from studies of these substrate mimics show that PAI-1 functions as a protease inhibitor via a “bait” peptide bond (P1–P1', R346–M347) on its RCL that imitates the binding site of the plasminogen activator substrate (20–22). An ester bond is formed between the active site serine residue of the protease and the carboxyl group of the methionine residue (P') of the RCL. During this process of substrate inhibition, conformational changes are induced that include partial insertion of the N-terminal side of the RCL into the scaffold of β-sheet A (23, 24), followed by hydrolysis of the ester bond and release of PAI-1 in what is termed the substrate or cleaved form (21, 25, 26).

Existing high resolution crystal structures of PAI-1 have thus provided a solid reference point for characterization of average/static structures of distinct PAI-1 conformers. Fluorescence spectroscopy (20), surface plasmon resonance (19), antibody-specific mapping (28, 29), and site-directed mutagenesis (30–32) have provided some additional evidence of sites on the body specific mapping (28, 29), and site-directed mutagenesis thus provided a solid reference point for characterization of what is termed the substrate or cleaved form (21, 25, 26).

Plains, NJ), and acetonitrile (HPLC grade), HEPES, urea, and guanidinium chloride (99 atom % D2O; 99% active) were purchased from Sigma. The RCL peptide, N-Ac-TVASSSTA, was synthesized and purified, and its sequence was confirmed at the Molecular Biotechnology Core of the Lerner Research Institute, Cleveland Clinic Foundation.

**Generation and Functional Confirmation of PAI-1 Conformational States—Activity assays in control studies demonstrated that the active form of PAI-1 (purchased as >95% active) stored in a solution containing 50 mM ammonium acetate, pH 6.0, and 50 mM EDTA had a halflife of 12 h after thawing to 20 °C and diluting into working buffer. The latent form of PAI-1 was obtained by incubating the native form of PAI-1 (purchased as 20% active) for 24 h at room temperature. The RCL-PA conformation (substrate mimic) of PAI-1 was obtained by incubating active PAI-1 for 24 h at room temperature with a 10-fold excess of N-Ac-TVASSSTA in 0.1 mM HEPES buffer at pH 7.4. This peptide mimics the critical stretch of amino acids of the RCL that serves as bait and therefore deteres latency formation by sterically hindering the insertion of the RCL. Immediately prior to use, the functional states of the active and the conformationally altered forms of PAI-1 were confirmed by a chromogenic PAI-1 activity assay following 15 min of incubation with uPA.

PAI-1 activities were defined as a function of inhibition in the initial rate of uPA activity relative to residual uPA activity observed in the absence of added PAI-1, as described previously (10).

PAI-1 Activity Assay—PAI-1 (72 nM) in each of its three conformational states was individually incubated for 15 min at 25 °C in reaction mixtures containing uPA (24 μg/ml, 2000 IU urokinase), 100 mM sodium phosphate buffer, pH 7.4, 10 mM NaCl, 100 μM diethylenetriaminepentaacetic acid, and 0.02 mM CaCl2. Replicates of incubated mixtures were then transferred individually to single wells of a 96-well plate containing the uPA-specific chromogenic substrate (Spectrozyme UK, American Diagnostica, Inc.) and calcium chloride (final concentration of 5 and 400 μM, respectively). PAI-1 activity was monitored spectrophotometrically by measuring the initial rate of residual uPA activity by following real time changes in absorbance at 405 nm and 25 °C for 15 min, during which time interval absorption changes were approximately linear under the conditions employed. Initial velocities of reactions were calculated from the slopes of best fit curves using instrument software. PAI-1 activity is expressed as the percent inhibition of uPA activity relative to reactions run in the absence of PAI-1. 1% PAI-1 activity was defined as uninhibited uPA activity (as determined by sham incubations in the absence of PAI-1). 100% PAI-1 activity was defined by the assay system devoid of uPA. Assays points were routinely performed in triplicate with results reported as the mean ± S.D. % inhibition of uPA activity. Data shown are results (mean ± S.D.) from experiments performed at least three independent times.

** Backbone Amide 1H → 2H Exchange (Pulse Labeling) **—Pulse labeling by 1H → 2H exchange was done on 5-μl aliquots of 2.0 mg/ml solutions of active, latent, and RCL-PA PAI-1 conformers. Samples were equilibrated at room temperature for 10 min prior to exchange. Each PAI-1
sample was pulse-labeled by the addition of a 20-fold excess volume of deuterated solution comprised of 50 mM D$_2$OAc, pH 6.5, in D$_2$O for 10 s. Deuterium labeling of PAI-1 samples was quenched by rapid reduction of the pH and temperature by addition of 10 μl of quenching solution (10% trifluoroacetic acid, 8 M guanidine hydrochloride, pH 2.2, 0 °C) and immersion in ice/water/methanol bath (−10 °C). Control studies demonstrated that addition of guanidine hydrochloride to the quenching solution under the conditions employed (guanidine hydrochloride, 0.4 M final) did not measurably impact upon measured peptide $^1$H → $^2$H exchange, yet facilitated more efficient pepsin digestion. The final pH of the solutions after quenching was pH 2.5, optimal for minimizing back exchange (34).

**Pepsin Digestion of Labeled PAI-1 Samples**—Immediately following pulse labeling, PAI-1 was rapidly digested under conditions that minimize back exchange in 40 μl of pre-washed and pre-chilled agarose-immobilized pepsin for 5 min. Following digestion, samples were immediately filtered in 0.22-μm ultrafilter centrifugal filter devices for 1 min in a biofuge set at −2 °C. Filtered samples were immediately injected on-line and analyzed by electrospray ionization mass spectrometry. The entire process from pulse labeling to mass spectrometric analysis occurred within 15 min.

**HPLC with On-line Electrospray Ionization Tandem Mass Spectrometry Analysis of Modified PAI-1 Residues**—A Phenomenex reverse phase C$_{18}$ (1 mm × 15 cm) column on a micro-HPLC system equipped with a binary pump (Micro-Tech Scientific, Vista, CA) interfaced to a Finnigan (San Jose, CA) liquid chromatography ion trap DECAT mass spectrometer was used for separation and mass spectrometric analysis of peptic peptides. The injector, analytical column, tubings, and solvents were all immersed in ice/water bath during analysis to minimize back exchange. The column was equilibrated at a flow rate of 50 μl/min with 2% solvent B (solvent A, 0.05% trifluoroacetic acid in H$_2$O; solvent B, 0.05% trifluoroacetic acid in acetonitrile) prior to injecting a 20-μl aliquot of the sample with a pre-chilled syringe. Peptides were eluted following a 1-min desalting step using 40% solvent B over 6 min, followed by a linear gradient over the next 4 min to 60% solvent B.

To identify and sequence all peptic peptides, mass spectra were initially collected for an unlabeled pepsin-digested PAI-1 sample with sequences searched against the data base Sequest (La Jolla, CA). Corresponding mass spectra with well resolved isotopic peaks of deuterium-labeled peptides were then obtained in duplicate using full scan mode of analysis for the following mass ranges: 300–1000, 1000–1600, and 1400–2000 m/z. This process was also applied to a functional maximally labeled PAI-1 sample, denoted as 100% exchanged (achieved by incubating PAI-1 in D$_2$O buffer overnight at 37 °C), as well as a nonlabeled sample of PAI-1, denoted as 0% exchanged. Data were processed using the Finnigan Xcalibur software, and deuterium content of labeled peptides was calculated from the difference between labeled and unlabeled mass-to-charge ratio (m/z) values of the peptide peptide centroids, as described below.

Quantitation of $^1$H → $^2$H Exchange into PAI-1 Peptides—The extent of $^1$H → $^2$H exchange into PAI-1 peptides in its three conformational states was quantified as the deuterium content ($D$), as shown in Equation 1. Minimal back-exchange of the labeled amide protons to unlabeled protons occurs during digestion as well as during the chromatographic separation and mass spectrometric analysis. To correct for this phenomenon, a normalization factor was implemented in the calculations for deuterium content using a functional maximally labeled peptide (34). The deuterium content is therefore an established corrected measure of the number of deuteriums incorporated by $^1$H → $^2$H exchange into proteins (34).

\[
D = \frac{\langle m \rangle - \langle m_{0%} \rangle}{\langle m_{100%} \rangle - \langle m_{0%} \rangle} \times N
\]  
(Eq. 1)

where $\langle m_{0%} \rangle$, $\langle m \rangle$, and $\langle m_{100%} \rangle$ represent the average molecular masses of the unlabeled peptide (0% exchange), the partially deuterated (exchange by conformation), and the functional maximally deuterated peptide (100% exchange), respectively, and $N$ represents the number of amino acid residues in the peptide.

**Molecular Graphics and Sequence Analysis**—Accelrys Viewerite50 (Norwalk, CT) was used to color code and display the three-dimensional crystal structures from the respective protein data base (PDB) files. Solvent accessibility values were color-coded based on the analogous color scheme for anisotropic temperature (B) factors. Typically, these B factors give a measure of the mobility or uncertainty of the position of a given atom (35). High solvent accessibility values are colored in warmer (red) colors and lower values in colder (blue) colors. Differences in excess of 50% between the active and the RCL-PA PAI-1 conformers relative to the latent conformer were illustrated on their respective crystal structures using this color scheme.

Experimentally determined $^1$H → $^2$H exchange deuteron contents of each peptide were compared with theoretical relative solvent accessibility (RSA) values. These RSA values were calculated by entering the PDB $x$, $y$, and $z$ coordinates and atomic radii into GETAREA 1.1 (Sealy Center for Structural Biology, Galveston, TX). To differentiate between theoretical RSA values and $^1$H → $^2$H exchange derived deuteron
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Conformational States of PAI-1—Prior to deuterium labeling studies, confirmatory functional assessments were performed on active, latent, and RCL-PA PAI-1 conformers. Fig. 1 shows a plot of % PAI-1 activity measured for each PAI-1 conformer. Consistent with expectations, the active conformer of PAI-1 retained 95.2 ± 11.1% activity (i.e. inhibited uPA activity 95.2%), the latent conformer possessed only 9.8 ± 8.3% PAI-1 activity, and the RCL-PA conformer had minimal (1.1 ± 8.9%) residual PAI-1 activity (i.e. failed to significantly inhibit uPA activity).

Mass Spectrometric Characterization and Sequence Coverage of PAI-1—An important requirement of the use of $^{1}$H → $^{2}$H amide exchange as a structural probe of proteins is maximal sequence coverage of the protein. To achieve this, PAI-1 was digested with pepsin, which rapidly cleaves diverse sites of proteins at low pH and at low temperature. By collision-induced dissociation-ESI-mass spectrometry, product ions of the peptic peptides were analyzed for sequence determination. An illustrative collision-induced dissociation spectrum of a PAI-1 peptic peptide (L46–Q59, correlation factor 4.95) is shown in Fig. 2. Greater than 90% of hPAI-1 sequence was covered by ESI tandem mass spectrometry for all conformers studied. The peptides underlined (Fig. 3) were all well resolved when scanned and studied by $^{1}$H → $^{2}$H exchange/mass spectrometry for each of the active, latent, and RCL-PA conformers of PAI-1.

Assessment of Solvent Accessibility of Distinct PAI-1 Conformers by $^{1}$H → $^{2}$H Exchange—Real-time assessments of distinct conformational states of PAI-1 in solution were performed using backbone amide $^{1}$H → $^{2}$H exchange. This pulse-labeling process involved incubating the different conformational states of PAI-1 in D$_{2}$O for 10 s and analyzing the labeled pepsin-digested peptides of the protein by full scan mass spectrometry. A 10-s pulse exchange in deuterium was chosen as a practical way of evaluating solvent accessibility over a snapshot of the dynamic events that occur faster than ~0.1 s$^{-1}$ across the polypeptide backbone (35). Peptides that were characterized by $^{1}$H → $^{2}$H exchange for each PAI-1 conformer (active, latent, and RCL-PA) are listed in Table I. Included in the table are the average masses (centroids of isotopic envelope) of the peptides, the secondary structural components they represent in known crystal structures, and their deuterium factors (D).

Illustrative mass spectra for peptic peptides examined by $^{1}$H → $^{2}$H exchange for each PAI-1 conformer are shown in Fig. 4. The peptides shown, W139–L151 (Fig. 4, top panel) and I237–L250 (Fig. 4, bottom panel), represent components of helices F and G, respectively. Spectrum A in each panel shows the mass isotopomer distribution of the unlabeled peptides (0% exchange), with centroid masses at $m/z$ 1514.7 and 1500.8, respectively. Spectrum E in each panel shows the mass isotopomer distribution of functionally maximally exchanged peptide obtained from PAI-1 that was extensively incubated in D$_{2}$O, a value that is used to calculate a deuterium factor (Equation 1), which approximates a normalized deuterium content of a peptide.

Most interestingly, upon pulse labeling with deuterium, distinct and characteristic shifts in the mass isotopomer distribution for a given peptide in PAI-1 are noted depending upon the specific conformer (active, latent, and RCL-PA, Fig. 4, spectra B–D, respectively). The observed shift in the mass distribution of the isotopic envelope to higher $m/z$ for each peptide relative to the unlabeled peptide (Fig. 4, spectrum A) occurs as a consequence of deuterium exchange of labile hydrogens on amino acids within the polypeptide chain. Shifts to higher $m/z$ (i.e. more deuterium-incorporated) correspond to enhanced solvent accessibility induced by conformational alterations and dynamic motions. Peptide W139–L151 (Fig. 4, top panel) was selected for illustrative purposes because it shows characteristic relative rankings of solvent accessibility observed in a majority of peptides: increased in active conformer (Fig. 4, spectrum B) relative to latent conformer (Fig. 4, spectrum C), with RCL-PA conformer demonstrating intermediate solvent accessibility (Fig. 4, spectra D). Peptide I237–L250 Fig. 4 (bottom panel) is illustrative of a peptide demonstrating a distinct pattern of deuterium incorporation among the PAI-1 conformers, with enhanced deuterium incorporation in the latent form relative to the active form, implying that the latent conformer is more solvent-accessible or dynamic in this region of the protein (Fig. 4, spectra C).

By using the above outlined approach, alterations in solvent accessibility, a parameter sensitive to alterations both in protein conformation and dynamics, were evaluated throughout the length of the polypeptide backbone of each PAI-1 conformer. Fig. 5 graphically illustrates the calculated deuterium factors for all monitored PAI-1 peptides in each of the functional conformational states examined. In general, the peptides
Peptic digests of PAI-1 conformers were studied by \(^1\)H \(\rightarrow^2\)H exchange and analyzed by ESI mass spectrometry in the full scan mode. Prior to scanning the peptides, peptide sequences were confirmed by ESI tandem mass spectrometry and peptide mass mapping using the SEQUEST data base search program. Reported peptides had correlation factors above 1.5 and were fully resolved in the full scan mass spectra for all three conformers. The deuterium contents were calculated as described under “Materials and Methods.”

Recent studies identifying PAI-1 as a predictor for cardiovascular risks have heightened research interest in this regulatory protein of the plasminogen-plasmin cascade. The dynamic nature of PAI-1, including its ability to change spontaneously its conformation among functionally distinct forms, is a critical determinant of its physiological function, i.e. binding and cleaving plasminogen activators. Our current understanding of alterations in structure among the PAI-1 conformers is largely based upon crystal structures for the distinct PAI-1 forms. To augment these and provide insights into alterations in PAI-1 conformation and protein dynamics, complementary studies have been reported using a variety of methods, including surface plasmon resonance spectroscopy, epitope mapping with monoclonal antibodies, alterations in protein fluorescence, and structure/function studies coupled with site-directed mutagenesis (19, 20, 27–32). In general, these complementary methods either focus on specific domains or amino acids within the protein or lack spatial resolution for defining detailed structural and dynamic alterations throughout the polypeptide of the folded protein. Here, through application of a relatively new approach, pulse labeling by \(^1\)H \(\rightarrow^2\)H exchange coupled with mass spectrometry, we have obtained complementary structure-function information for each PAI-1 conformer through determination of solvent accessibility indices (D fac-
 tors) throughout the polypeptide chain. This comprehensive approach relies upon labile backbone amide protons as individualized probes throughout the PAI-1, each serving as sensors of alterations in protein conformation and dynamics. The present results thus complement existing studies by providing global assessments of PAI-1 solvent accessibility throughout the protein polypeptide backbone within functionally distinct conformers, a measure that is sensitive to both structural and dynamic alterations.

To highlight global alterations in solvent accessibility we have merged the D factors obtained experimentally with x-ray crystallographic data. By comparing and contrasting the experimentally determined solvent accessibility values within the discrete crystal structures of the conformers of PAI-1, the secondary structural components of the protein with marked differences in solvent accessibility have been determined. In Fig. 6A, differences in solvent accessibility between the active conformer relative to the latent conformer are superimposed upon the crystallographic structures of the active conformer. In our color scheme red identifies regions that are more solvent-ac-
The present results indicate that peptides G16–F34, F64–E93, V99–F113, S310–Q336, and K323–Q336, corresponding to helices A and D, β-sheet 5A, and the reactive center loop, are the most solvent-accessible and thus may be considered as critical participants in the structure/function alterations of PAI-1. Consistent with these observations, site-directed mutagenesis studies on comparative sites of PAI-1, i.e. T33 to a charged residue, F100A, Q324H, K325A, amino acids located in helices A and E, β-sheet 5A and the reactive center loop, respectively, have been shown to be critical in modulating the stability and activity of the states of PAI-1 (13, 37).

A marked disparity in surface exposure in the RCL between the active and latent conformation was observed (Fig. 6), consistent with crystallographic studies identifying the buried nature of the RCL within the latent form (15). The data are also consistent with the report (31) that mutations of residues S41 in helix B and K323 in sheet 5A accelerate PAI-1 latency. Conversely, we observed F114–D125 (hE and s1A) and I237–L250 (hG) to be more solvent-accessible in the latent conformation relative to the active conformation. This finding suggests that these peptides may serve as dynamic participants in the active to latent transition.

In the absence of a crystal structure of a PAI-1-protease complex, an alternative complex of a stable mutant of PAI-1 (A335Q) has been studied. This form was bound to two molecules of an N-acetylated fragment peptide of the N terminus of the RCL (N-Ac-TVASS, P14–P10) (19). When PAI-1 is annealed to this pentapeptide, the complete insertion of the RCL is delayed, presumably due to steric hindrance in the pocket between sheet 3A and sheet 5A (typically referred to as the shutter region) (31). In an effort to understand the conformational state of this substrate mimic, termed RCL-PA form of PAI-1, we incubated the active form of PAI-1 with an excess of the N-capped RCL peptide, N-Ac-TVASSSTA, and then in parallel, we measured PAI-1 activity and performed 1H exchange/mass spectrometry to probe its conformational properties. This PAI-1 model lost its inhibitory activity, validating the ability of the RCL-derived peptides to transform active PAI-1 into an inactive substrate form for its target proteases. Fig. 6C illustrates in red peptides that were experimentally determined to be more solvent-accessible in the RCL-PA conformation relative to the latent conformation, and illustrates in blue peptides that are relatively less solvent-accessible, superimposed upon the RCL-PA crystal structure. Alternatively, the solvent accessibility of peptides in the RCL-PA conformer relative to the active conformer is shown in Fig. 6D. These results illustrate an overall intermediate solvent accessibility to the RCL-PA conformer relative to active and latent forms. These results are consistent with the proposed model of a relatively rigid PAI-1-proteinase complex (acyl enzyme complex) where there exists only partial insertion of the RCL (P14–P7, T333–A340) (39) and not (P14–P1, T333–R344) as initially assumed (40). Closer analysis of the RCL-PA conformation reveals that the following peptides situated in the “gate region,” A203–E212 (s3C) and A345–E351 (which includes the beginning of the distal hinge or s1C) are markedly less dynamic. This may result from steric constraint generated at the gate region when the RCL peptide binds. The diminished solvent accessibility of parts of the gate region likely arise from obstruction by the partial insertion of the RCL, suggesting gate opening as a rate-limiting step for substrate binding.

Amino acid residues Q55 (hC), F109, M110, L116 (hE), and Q123 (s1A) have been associated by random and site-directed mutagenesis studies to vitronectin/PAI-1 binding (13, 38). Crystal structural analysis of these vitronectin/PAI-1-binding site residues showed that in the latent form they were mostly surface-exposed. Our 1H → 2H exchange studies support these observations as peptide V99–F113 (hE) and F114–D125 (hE, s1A) of the latent conformation remains relatively solvent-exposed. However, in contrast to expectations based upon crystallographic studies that predict increased solvent accessibility in Q55, we observed reduced solvent accessibility in the corre-
sponding region, L46–Q59 (end of hB and hC), of the latent conformer (Fig. 5). These results may imply that vitronectin preserves PAI-1 in the active form by sterically locking it into a comparable local conformation at this region.

Another informative analysis was to compare and contrast experimentally determined $^1$H → $^2$H-derived deuterium factors with calculated theoretical RSA values derived from x-ray crystallographic Cartesian coordinates and atomic radii. Fig. 7 illustrates the differences in experimentally determined versus predicted solvent accessibilities superimposed upon the crystal structure lattices of the active (Fig. 7A), latent (Fig. 7B), and RCL-PA (Fig. 7C) conformers of PAI-1. Structures were color-coded based on the differences between $^1$H → $^2$H exchange-derived deuterium factors versus the theoretical RSA factors. Regions in red and orange illustrate sites that are more solvent-accessible than predicted by the crystallographic data, suggesting an enhanced dynamic state within each of the PAI-1 conformers. Regions in gray depict no significant differences noted between theoretical (crystallographic) and experimentally determined ($^1$H → $^2$H exchange) solvent accessibility, whereas light and dark blue regions illustrate those that were less solvent-exposed based on our observed $^1$H → $^2$H exchange.
results. A remarkable finding is that there is enhanced solvent accessibility in the vast majority of peptides in the experimentally derived model relative to the theoretical model based upon crystallographic predictions. This result undoubtedly derives from the dynamic motion or “breathing” of a protein in solution relative to that within a crystalline lattice.

In conclusion, here we have defined the dynamic surface structures of the active, latent, and substrate conformational states of PAI-1 by a robust probe of protein surface accessibility. The majority of the solvent-accessible peptides in the active conformation became markedly less solvent-accessible in the latent conformation, whereas the RCL-PA form had intermediate solvent exposures between the active and latent state. Substrate characteristics were established in the RCL-PA form by the marked solvent inaccessibility of peptides located in the gate region, validating restriction from latency formation. A peptide in the vitronectin-binding site, F114–D125, was more solvent-accessible in the latent conformation compared with the active conformation, confirming previous reports from vitronectin stabilizing studies of PAI-1 (13, 15).

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