Accelerated Conversion of Human Plasminogen Activator Inhibitor-1 to Its Latent Form by Antibody Binding*  

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The serpin plasminogen activator inhibitor-1 (PAI-1) slowly converts to an inactive latent form by inserting a major part of its reactive center loop (RCL) into its β-sheet A. A murine monoclonal antibody (MA-33B8), raised against the human plasminogen activator (tPA)-PAI-1 complex, rapidly inactivates PAI-1. Results presented here indicate that MA-33B8 induces acceleration of the active-to-latent conversion. The antibody-induced inactivation of PAI-1 labeled with the fluorescent probe N,N,N'-dimethyl-N-(acetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene diamine (NBD) at P9 in the RCL caused a fluorescence enhancement and shift identical to those accompanying the spontaneous conversion of the P9-NBD PAI-1 to the latent form. Like latent PAI-1, antibody-inactivated PAI-1 was protected from cleavage by elastase. The rate constants for MA-33B8 binding, measured by NBD fluorescence or inactivation, were similar (1.3–1.8 × 10^4 M⁻¹ s⁻¹), resulting in a 4000-fold faster inactivation at 4.2 μM antibody binding sites. The apparent antibody binding rate constant, at least 1000 times slower than one limited by diffusion, indicates that exposure of its epitope depends on an unfavorable equilibrium of PAI-1. Our observations are consistent with this idea and suggest that the equilibrium involves partial insertion of the RCL into sheet A: latent, RCL-cleaved, and tPA-complexed PAI-1, which are inactive loop-inserted forms, bound much faster than active PAI-1 to MA-33B8, whereas two loop-extracted forms of PAI-1, modified to prevent loop insertion, did not bind or bound much more weakly to the antibody.

Plasminogen activator inhibitor-1 (PAI-1) is a serine proteinase inhibitor of the serpin family, a class of inhibitory and noninhibitory proteins sharing a common tertiary structure. Its most prominent features are the major, 5-stranded β-sheet A, which can accommodate an additional strand in position 4, and a flexible reactive center loop (RCL) of about 20 residues denoted P15-P5 (1, 2). Inhibitory serpins function as suicide substrate inhibitors and acylate their target proteinases (3, 4). The acyl-enzyme complex is stabilized by insertion of the cleaved RCL as strand 4 of β-sheet A, accompanied by a translocation of the acylated enzyme from its initial binding site to its "locking" site. If RCL insertion is hindered by mutating the P14 threonine to an arginine (5), or by blocking position 4 of β-sheet A with a peptide analog of the P14-P7 part of the RCL (6), PAI-1 becomes a substrate that is completely hydrolyzed at the scissile bond by its target proteinases.

PAI-1 is the only serpin known to fold spontaneously to an inactive latent form (7), and the t½ for this conversion is 9.5 h at 25 °C and pH 7.4 (6, 8). In this process, the P15-P3 portion of the RCL, which is normally exposed at the apex of the molecule, is inserted as strand 4 in β-sheet A, forcing the remainder of the loop, including the reactive center, to adopt an extended conformation alongside the protein scaffold. The term "latent" was introduced because PAI-1 traditionally has been isolated in the inactive form, and its activity was partly restored by transient treatment with chaotropic agents (9). In the absence of such treatment, however, formation of latent PAI-1 is a virtually irreversible process (6). Latent PAI-1 has greater thermal stability than native PAI-1 (10), and its reactive center is unreactive with proteinase. Another marker for the conversion is that in the latent form, the RCL is protected from cleavage by elastase (11).

Two types of murine monoclonal antibodies against the human tPA:PAI-1 complex have been documented with respect to their effect on the structure and function of PAI-1: one type inactivates PAI-1 so it neither forms a covalent complex with its target enzymes nor is hydrolyzed at the scissile bond; the second type inactivates PAI-1 by making it a substrate for its target proteinases (12). The present work focuses on one particular antibody of the first type, denoted MA-33B8, which has been observed to inactivate PAI-1 rapidly (13).

Studies using a phage-displayed library of PAI-1 peptides or site-directed mutagenesis have failed to identify the epitope for MA-33B8 and the mechanism by which it inactivates PAI-1 remains obscure. Binding of the antibody can shield the reactive center bond in PAI-1 from proteinase by one of two mechanisms. We must assume either that antibody binding interferes sterically with binding of the proteinase or that antibody binding induces a conformational change that translocates the reactive center into a shielded position, which would be the case if the active-to-latent conversion was triggered by the antibody.

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† The abbreviations used are: PAI, plasminogen activator inhibitor; NBD, N,N,N'-dimethyl-N-(acetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene diamine; Bicine, N,N-bis(2-hydroxyethyl)glycine; PAGE, polyacrylamide gel electrophoresis; RCL, reactive center loop; tPA, human tissue-type plasminogen activator; Ab, antibody.
We have previously shown that the S338C mutant PAI-1, with the cysteine at P9 in the RCL, and labeled with NBD at the mercapto group (P9-NBD PAI-1), responds to the spontaneous conversion to the latent form by a marked increase and a distinct blue shift of the NBD fluorescence and that these changes are caused by loop insertion (8). The fluorescence enhancement is consistent with a translocation of the NBD probe from a solvent exposed environment of the extracted RCL of active PAI-1 to the more shielded position in the latent form between apolar groups in the tip of the helix F loop and β-sheet A, respectively. Similar fluorescence changes were observed after reacting P9-NBD PAI-1 with tPA, urokinase-type plasminogen activator, and trypsin, as well as with elastase, all of which cleave the RCL and trigger insertion of the RCL into β-sheet A, whereas no changes resulted from complexing P9-NBD PAI-1 with trypsin and tPA after these had been inactivated by converting their catalytic triad serine residues to dehydroalanine and alanine, respectively (8, 14). In the present study, we demonstrate that the antibody-accelerated inactivation of P9-NBD PAI-1 occurs concomitant with changes in the NBD fluorescence that were within experimental error identical to those characterizing the spontaneous inactivation of the labeled mutant. We also show that the differences between active and latent PAI-1 in susceptibility to hydrolysis by elastase are reproduced by the antibody-induced inactivation of PAI-1.

Using either the fluorescence increase of P9-NBD PAI-1 or the inactivation of wild type PAI-1 as a signal, we measured second order rate constants for MA-33B8 binding of the order of 10^4 m^-1 s^-1. The second order rate constants determined for the antibody antigen interaction usually fall within the range 10^6-10^7 M^-1 s^-1 (15, 16), approaching the limit set by molecular diffusion. The much lower rate constant for binding MA-33B8 to PAI-1 suggests an extra step in the binding of MA-33B8 to PAI-1, involving an unfavorable conformational change in PAI-1. To determine whether this conformational change involves loop insertion, various loop-inserted forms of PAI-1, as well as two loop-exposed variants with a documented reduced rate of loop insertion, were tested for their ability to compete with P9-NBD PAI-1 for binding to MA-33B8. These measurements revealed a striking difference in reactivity, consistent with antibody binding to a site on PAI-1 that is exposed only when the RCL is inserted into β-sheet A. Studies involving the complexes of P9-NBD PAI-1 with heparin and vitronectin indicate that binding of these ligands does not interfere with binding of MA-33B8.

MATERIALS AND METHODS

General Conditions, Reagents, and Procedures—If not otherwise indicated, spectral and kinetic measurements were performed at pH 7.4 and 25 °C in a reaction buffer containing 0.1 M HEPES, 0.1 M NaCl, 1 mM EDTA, 0.1% polyethylene glycol 8000. Fluorescence measurements were performed using an SLM 8000 spectrophotometer. The excitation wavelength used for studying NBD fluorescence was 480 nm. Sample cuvettes were coated with polyethylene glycol 20,000 to reduce protein adsorption. Peptide analysis by SDS-PAGE was performed under nonreducing conditions according to Laemmli (17). Pancreatic elastase and analytical grade buffer chemicals were from Sigma. Commercial USP reagents were from American Hospital Pharmacy Biotech. The octapeptide NAc-TVASSSTA was provided by the University of Michigan Protein Facility (Ann Arbor, MI).

PAI-1 Variants and tPA—Human wild type PAI-1 was expressed in Escherichia coli using the expression vector pET24d (9). The T333R (P14 Arg) and S338C (P9 Cys) PAI-1 single mutants, as well as a PAI-1 carrying both mutations, were obtained by site-directed mutagenesis as described elsewhere (9). PAI-1 was purified and separated into the antibody binding and latent forms according to a previous report from this laboratory (18). P9-NBD PAI-1 protein concentrations were measured at 280 nm, using an extinction coefficient of 0.93 ml mg^-1 cm^-1 and a M_r of 43,000. The mercapto group of the P9 Cys PAI-1 mutant was labeled with NBD (Molecular Probes) as described previously (8). To quantitate latent PAI-1, formed in storage or handling, unlabeled and P9-NBD-labeled PAI-1 preparations were analyzed by SDS-PAGE after reaction with excess two-chain tPA.

Unlabeled and P9-NBD PAI-1 were complexed with the octapeptide NAc-TVASSSTA as described elsewhere (6), and excess peptide was removed by gel filtration on a PD-10 column. In the preparation with native PAI-1, latent PAI-1, formed in the process, was removed by chromatography on phenyl-Sepharose (18). Analysis of the product by SDS-PAGE, before and after addition of tPA, indicated that it was free of active PAI-1 and contained less than 1% (latent) PAI-1 that was not hydrolyzed by tPA. The PAI-1-octapeptide complex is very stable and does not release the octapeptide even during prolonged dialysis (6) or after cleavage of the RCL by plasminogen activators, as demonstrated below. PAI-1 hydrolyzed at the P3-P4 peptide bond in the RCL was prepared with elastase, using 50 μM PAI-1 and 50 μm enzyme. Elastase was activated by affinity chromatography on immobilized heparin (9), which binds PAI-1 but not elastase. The tPA/PAI-1 complex was generated by incubation of PAI-1 (5 μM) with two-chain tPA (8 μM) for 30 min in the reaction buffer at room temperature. Excess tPA was inactivated by treatment with p-amidino-phenylmethanesulfonyl fluoride (0.1 μM).

Human recombinant tPA (Activase®) was a gift of Dr. B. Keyt (Genentech, South San Francisco, CA). The predominantly single-chain enzyme was converted to the two-chain form by treatment with immobilized plasminin (18).

MA-33B8—The monoclonal antibody was raised against the tPA-PAI-1 complex as described previously (12). The IgG fraction was purified from ascitic fluid by affinity chromatography on protein A-Sepharose (20). The antibody protein concentration was determined at 280 nm, using an extinction coefficient of 1.3 mg^-1 cm^-1 and a M_r of 150,000. The antibody binding site concentrations is given as normality (n), assuming two independent and noninteracting binding sites on each molecule.

Effect of MA-33B8 on the Rate of PAI-1 Inactivation—Unlabeled wild type and P9-NBD PAI-1 (0.3–0.5 μM) were incubated separately with MA-33B8 (0.04–4.2 μM) in reaction buffer at room temperature. Aliquots, a sample from each incubation was withdrawn and added to excess tPA. Residual active PAI-1 was determined based on the depression of the activity of tPA toward the chromogenic substrate SpectrozymePA and plotted against time.

P9-NBD PAI-1 Fluorescence Spectra—The latent form of P9-NBD PAI-1 was obtained by incubation of active P9-NBD PAI-1 (2.5 μM) in a sealed and foil-wrapped Eppendorf tube at 25 °C for 5 days at pH 8.2 in 0.1 M Bicine, 0.1 M NaCl, 0.1% sodium azide (6, 8). The content of the tube was assayed for residual inhibitory activity and analyzed by SDS-PAGE after incubation with tPA at pH 7.4. Antibody-induced inactivation of the labeled mutant was achieved by incubating P9-NBD PAI-1 (0.1 μM) with excess MA-33B8 (0.4 μM) and was complete after 20 min. Active P9-NBD PAI-1 and the spontaneously inactivated and MA-33B8-inactivated P9-NBD PAI-1 were adjusted to 0.1 μM in the reaction buffer, and the 500–600 nm fluorescence emission spectrum of each species was recorded. The effect of MA-33B8 (0.4 μM) on the NBD fluorescence of spontaneously latent P9-NBD PAI-1 (0.1 μM), as well as on that of the octapeptide-complexed and P14 Arg variants of P9-NBD PAI-1 (0.1 μM), was studied by recording the relative emission spectrum before and after adding antibody. The P9-NBD PAI-1 used in this study was obtained from the same stock.

Kinetics of MA-33B8 Binding Measured by P9-NBD PAI-1 Fluorescence—The rate of the fluorescence increase induced by treating P9-NBD PAI-1 with MA-33B8 was measured at 529 nm as a function of antibody concentration up to 3 μM, keeping P9-NBD PAI-1 at 0.03–0.3 μM to maintain first order conditions. The corresponding rate constants were evaluated by nonlinear least squares fitting of a single exponential to the fluorescence progress curves. The apparent second order rate constant for antibody binding was determined by plotting the rate constants against the concentration of MA-33B8.

Competitive Binding of P9-NBD PAI-1 and Unlabeled MA-33B8 Forms to MA-33B8—P9-NBD PAI-1 and MA-33B8 were reacted at essentially equimolar concentrations (0.18 and 0.17 μM, respectively), and the time
The susceptibility of various PAI-1 forms to hydrolysis by elastase was tested by incubating mixtures of inhibitor (1 mM) and enzyme (0.1 mM) for 20 min at room temperature. The reactions were quenched with SDS (1%) and analyzed by SDS-PAGE. The species generated in the absence and presence of antibody, respectively, the emission spectra of P9 NBD PAI-1 before and after complete spontaneous or MA-33B8-induced inactivation were recorded. The data in Fig. 2 show that antibody-induced inactivation of P9 NBD PAI-1 causes changes to the NBD emission spectrum within experimental precision identical to the changes observed following spontaneous inactivation of P9 NBD PAI-1 (i.e. a 13-nm blue shift of the emission maximum and a 6.7-fold fluorescence enhancement). The NBD fluorescence of latent P9 NBD PAI-1 was not affected by addition of MA-33B8. The spontaneously and antibody-induced inactive forms of P9 NBD PAI-1 were compared also with respect to hydrolysis by elastase. Data in Fig. 3B show that when subjected to catalytic amounts of elastase and treated with SDS, active P9 NBD PAI-1 releases a ~3-kDa peptide, consistent with hydrolysis of the P3-P4 RCL peptide bond, as previously reported for hydrolysis of wild type PAI-1 by elastase (11). Following inactivation by antibody, neither native PAI-1 nor P9 NBD PAI-1 was hydrolyzed by elastase (Fig. 3C).

KinetiCs of MA-33B8-Induced P9 NBD PAI-1 Fluorescence Increase—The time course of the NBD fluorescence increase triggered by reacting 0.1 μM P9 NBD PAI-1 with 0.08 μM MA-33B8 is shown in Fig. 4. The first order rate constant for a set of such curves increased linearly with antibody concentration up to 3 μM, the highest concentration tested (Fig. 4, inset). The concentration dependence gave an apparent second order rate constant for antibody-induced fluorescence enhancement of 1.3 × 10^6 M^-1 s^-1, similar to the value of 1.4 × 10^6 M^-1 s^-1 presented above and calculated based on the rate of inactivation of P9 NBD PAI-1 induced by the antibody.

Relative Rates of Binding of P9 NBD PAI-1 and Wild Type PAI-1 Variants to MA-33B8—Data presented above indicate that MA-33B8 binding to P9 NBD PAI-1 promotes insertion of the RCL into β-sheet A. To determine whether there is a reciprocal enhancement of antibody binding when the RCL is reinserted into sheet A, unlabeled active PAI-1 and RCL-inserted variants of the inhibitor were compared for their ability to compete kinetically with P9 NBD PAI-1 for binding to MA-33B8. When PAI-1, P9 NBD PAI-1, and MA-33B8 were mixed in equimolar proportions, the amplitude of the NBD fluorescence progress curve was decreased to about half of that registered in the

**RESULTS**

Effect of MA-33B8 on the Rate of Inactivation of Wild Type and P9-NBD PAI-1—The effects of MA-33B8 on wild type and P9-NBD PAI-1 were compared by incubating the respective PAI-1 form with excess MA-33B8 and measuring residual inhibitory activity as a function of time. Fig. 1 demonstrates that with 4.2 μM MA-33B8, the antibody-induced inactivation of wild type and P9-NBD PAI-1 occurred at comparable rates, corresponding to inactivation rate enhancement factors of 4000 and 8000, respectively. The larger factor for P9-NBD PAI-1 derives from its 2-fold lower rate of spontaneous inactivation. Based on the rates of inactivation of wild type and P9-NBD PAI-1 induced by excess MA-33B8 at three different concentrations, the apparent second order rate constant for antibody binding to the respective PAI-1 form was 1.8 ± 0.2 and 1.4 ± 0.2 × 10^4 M^-1 s^-1.

Comparative Characterization of Latent and Antibody-inactivated PAI-1—To compare the inactive forms of P9 NBD PAI-1 generated in the absence and presence of antibody, respectively, the emission spectra of P9 NBD PAI-1 before and after complete spontaneous or MA-33B8-induced inactivation were recorded. The data in Fig. 2 show that antibody-induced inactivation of P9 NBD PAI-1 causes changes to the NBD emission spectrum within experimental precision identical to the changes observed following spontaneous inactivation of P9 NBD PAI-1 (i.e. a 13-nm blue shift of the emission maximum and a 6.7-fold fluorescence enhancement). The NBD fluorescence of latent P9 NBD PAI-1 was not affected by addition of MA-33B8.

The spontaneously and antibody-induced inactive forms of P9 NBD PAI-1 were compared also with respect to hydrolysis by elastase. Data in Fig. 3B show that when subjected to catalytic amounts of elastase and treated with SDS, active P9 NBD PAI-1 releases a ~3-kDa peptide, consistent with hydrolysis of the P3-P4 RCL peptide bond, as previously reported for hydrolysis of wild type PAI-1 by elastase (11). Following inactivation by antibody, neither native PAI-1 nor P9 NBD PAI-1 was hydrolyzed by elastase (Fig. 3C).
absence of the wild type inhibitor (Fig. 5). This demonstrates that P9-NBD and wild type PAI-1 compete equally for binding to MA-33B8. The loop-inserted wild type PAI-1 forms (i.e. latent, elastase-cleaved, and tPA-bound), on the other hand, decreased the amplitude of the NBD fluorescence progress curve in a stoichiometric fashion. This is most convincingly shown by the set of curves displayed in Fig. 6, which exhibit a linear decrease in the MA-33B8-induced fluorescence enhancement of P9-NBD PAI-1 with the concentration of latent PAI-1. It is also demonstrated by the proportionate depression of the reaction of MA-33B8 with P9-NBD PAI-1 by the other loop-inserted PAI-1 forms shown in Fig. 5. The stoichiometric effect indicates that binding of the loop-inserted PAI-1 forms to MA-33B8 is faster than binding of active PAI-1 by a factor considerably larger than 10, that is, that the corresponding second order rate constants are close to or exceed $10^6$ M$^{-1}$ s$^{-1}$.

**Rates of Loop Insertion in Peptide-complexed and P14 Arg**

**FIG. 3.** The reactivity of wild type and P9-NBD PAI-1 with tPA and elastase. A demonstrates the reactivity of active wild type and P9-NBD PAI-1 toward excess tPA. Lanes 1 and 3 were obtained with wild type and P9-NBD PAI-1, respectively, and lanes 2 and 4 were obtained with the same species after treatment with tPA. The bands at $a$ and $b$ are latent and reactive center bond hydrolyzed PAI-1, respectively. The tPA-PAI complex is at $c$, and the surplus tPA is at $d$. $B$ demonstrates the susceptibility of active PAI-1, active P9-NBD PAI-1, and latent PAI-1 (all at 1 M) to hydrolysis by elastase (0.1 M). Lanes 5–7 contain the elastase-treated PAI-1, P9-NBD PAI-1, and latent PAI-1, respectively. $C$ demonstrates the susceptibility of wild type and P9-NBD PAI-1 (both at 1 M), preactivated by MA-33B8 (3 M, band e), to hydrolysis by elastase. Lanes 9 and 10 contain antibody-inactivated and elastase-treated PAI-1 and P9-NBD PAI-1, respectively. Lane 8 is antibody and wild type PAI-1 before treatment with elastase.

**FIG. 4.** The time course of the antibody-induced fluorescence enhancement of P9-NBD PAI-1. P9-NBD PAI-1 (0.2 μM) was reacted with MA-33B8 (0.8 μM), and the fluorescence was monitored at 529 nm (excitation at 480). The regression curve is a first order growth function with $k_{obs} = 0.011$ s$^{-1}$. The inset shows the MA-33B8 concentration dependence of $k_{obs}$. The slope of the regression line corresponds to a second order rate constant of $1.3 \pm 0.5 \times 10^5$ M$^{-1}$ s$^{-1}$, and its intercept with the y axis is to an apparent dissociation rate constant indistinguishable from zero.

**FIG. 5.** Kinetic competition for MA-33B8 between P9-NBD PAI-1 and unlabeled PAI-1 forms. All fluorescence progress curves were obtained at essentially equimolar concentrations of P9-NBD PAI-1 and MA-33B8 binding sites (0.2 and 0.19 μM in curves 1 and 2; 0.1 and 0.08 μM in all other curves). The two superimposed curves 1 and 2 were obtained in the absence and presence of P14-Arg PAI-1 (2 μM), and the two superimposed curves 3 and 4 were obtained in the absence and presence of monomeric vitronectin (1 μM). Curve 5 was recorded in the presence of NAc-TVASSSTA-PAI-1 (1 μM), and curves 6–8 were recorded in the presence of active wild type PAI-1, the tPA-PAI-1 complex, and elastase-cleaved PAI-1, respectively (all at 0.1 μM).

**FIG. 6.** Stoichiometric suppression of MA-33B8-induced inactivation of P9-NBD PAI-1 by latent PAI-1. A, the fluorescence enhancements recorded after reacting 0.18 μM P9-NBD PAI-1 with 0.17 μM MA-33B8 in the absence (topmost trace) and presence of 0.06, 0.12, 0.15, and 1.2 μM latent PAI-1 (traces in descending order). $B$, the amplitudes of the curves in $A$ plotted against the concentration of latent PAI-1. The regression line indicates complete suppression of the fluorescence enhancement at a concentration of latent PAI-1 equal to that of antibody binding sites.

**P9-NBD PAI-1**—To assess directly the constraints against loop insertion in PAI-1 caused by mutating the threonine at P14 in the RCL to an arginine or by binding the NAc-TVASSSTA octapeptide to position 4 in sheet A, these modifications were introduced into P9-NBD PAI-1. This enabled loop insertion to be monitored by means of the NBD fluorescence. As anticipated based on the effects of the same modifications on wild type PAI-1, the modified P9-NBD PAI-1 forms were substrates for tPA and hydrolyzed completely at the scissile bond. Following hydrolysis, insertion of the RCL was extremely slow with P14 Arg P9-NBD PAI-1 ($k_{obs} = 2.3 \text{ h}^{-1}$) and too slow to be measured with the peptide-complexed P9-NBD form. In contrast, the RCL of inhibitory P9-NBD PAI-1 inserts with a rate constant of 3.4 s$^{-1}$ in the reaction with saturating tPA (8).

**Relative Rates of Binding P9-NBD PAI-1 and Loop Con- strained PAI-1 Variants to MA-33B8**—The comparatively slow binding of MA-33B8 to active PAI-1 could be caused by an unfavorable equilibrium in PAI-1 that exposes the epitope in a conformation of the inhibitor associated with insertion of the RCL into β-sheet A. To investigate this possibility, the competitive binding measurements were extended to include P14 Arg PAI-1 and PAI-1 in complex with NAc-TVASSSTA, which, by inference from the data presented above on the corresponding variants of P9-NBD PAI-1, are prevented from inserting the RCL into β-sheet A at a significant rate. With the P14 Arg...
MA-33B8 binding to P9 observed when peptide-complexed PAI-1 (10-fold molar excess over P9) could be observed, even at 2 μM P14 Arg PAI-1, representing a 10-fold molar excess over P9-NBD PAI-1 and MA-33B8 binding sites (Fig. 5, curves 1 and 2). A small effect on the rate of the fluorescence change, but not on its amplitude, was observed when peptide-complexed PAI-1 (1 μM) was added to a mixture of P9-NBD (0.1 μM) and MA-33B8 (0.1 μM) (Fig. 5, curve 5). Based on this rate depression and the assumption that binding of peptide-complexed PAI-1 to MA-33B8 equilibrates fast compared with the duration of the experiment, the $K_d$ for binding the peptide-complexed PAI-1 to MA-33B8 was estimated to be 1–2 μM. The absence of an effect of P14 Arg PAI-1 on MA-33B8 binding to P9-NBD PAI-1 indicates that the antibody binds this species with a $K_d$ that exceeds 10 μM.

Direct Assessment of the Affinities of MA-33B8 for Latent and Peptide-complexed PAI-1—The contrasting high and low affinities of MA-33B8 for latent and peptide-blocked PAI-1, respectively, were demonstrated directly by attempts to separate each PAI-1 form from a slight excess of antibody binding sites on immobilized protein A. Protein A binds the immunoglobulin via the Fc region. Latent PAI-1 alone was not retained on the column. Latent PAI-1 and antibody, however, were retained completely on the column and were eluted together at pH 3. Under the same conditions, most of the peptide-complexed PAI-1 was separated from the antibody in the equilibration buffer and eluted in the column void. The elution peak, however, was extended at its trailing end, in keeping with the concentration of antibody used and the $K_d$ reported above. These results, as well as electrophoresis gels demonstrating the contents of each elution peak, are documented in Fig. 7.

Effects of Heparin and Vitronectin on Binding MA-33B8 to PAI-1—PAI-1 has high affinity for heparin and vitronectin (21, 22). Experiments were performed to determine whether binding of these macromolecular ligands interferes with binding of MA-33B8. Heparin and vitronectin had no effect per se on the NBD fluorescence of P9-NBD PAI-1, consistent with the suggested binding sites remote from the exposed loop. Neither ligand reduced the rate or amplitude of the fluorescence enhancement observed after addition of MA-33B8, indicating that the binding sites for heparin and vitronectin on PAI-1 (22, 23) do not overlap with that for MA-33B8 and that the two ligands do not noticeably interfere with the associated step of loop insertion.

Effect of Native Monomeric Vitronectin on PAI-1 Stability—Vitronectin binding has been reported to reduce the rate by which PAI-1 becomes latent (24). The absence of an effect of vitronectin on the rate of change of the P9-NBD fluorescence induced by MA-33B8 therefore had potential implications for the mechanisms by which vitronectin stabilizes and MA-33B8 destabilizes PAI-1. However, the monomeric vitronectin (M, 70,000) used in this study was shown to have a 1.3-fold stabilizing effect on native PAI-1, less than the factor of 2 reported for multimeric vitronectin (~400 kDa) (24). The effect of monomeric vitronectin on P9-NBD PAI-1 was even further reduced.

**DISCUSSION**

We have presented results that strongly suggest that the murine monoclonal antibody MA-33B8, directed against the human tPA-PAI-1 complex, rapidly converts inhibitory PAI-1 to its latent form. Antibody-induced inactivation of the P9 Ser → Cys mutant PAI-1, labeled with the fluorescent probe NBD at the mercapto group, resulted in a 6.7-fold enhancement and 13-nm blue shift of the NBD fluorescence, within experimental precision identical to the changes documented for the spontaneous conversion of the labeled mutant to the latent form. Like latent PAI-1, antibody-inactivated PAI-1 was unreactive with target proteinases, as well as with elastase (11). The rate of the fluorescence enhancement of P9-NBD PAI-1 was proportional to antibody concentration up to 3 μg, the highest concentration tested, with an apparent bimolecular rate constant of $1.3 \times 10^4$ M$^{-1}$ s$^{-1}$. Similar rate constants (1.4 and $1.8 \times 10^4$ M$^{-1}$ s$^{-1}$) were calculated based on the rate of inactivation of P9-NBD PAI-1 and wild type PAI-1 by MA-33B8, which at 4.2 μg antibody resulted in rates of inactivation 4000 and 8000 times faster than the spontaneous inactivation of the respective PAI-1 form.

Hypothetically, MA-33B8 could bind to an epitope on PAI-1 in the vicinity of the reactive center and thereby change the P9-NBD fluorescence and prevent proteinases from attacking...
the RCL. The fact that antibody binding to active PAI-1 required displacement of the RCL does not prove in itself that this results in latency. However, it is highly unlikely that displacement of the RCL of P9-NBD PAI-1, without insertion into sheet A, would result in changes of the fluorescence of the environmentally sensitive NBD-probe that are within experimental error identical to those resulting from formation of the latent labeled PAI-1.

The experiments that determined the relative rates of binding to MA-33B8 of PAI-1 forms other than active P9-NBD PAI-1 provided additional insights. Wild type PAI-1 at a concentration equal to that of the P9-NBD mutant decreased the amplitude of the antibody-induced fluorescence enhancement by 55%. This indicates that P9 NBD and wild type PAI-1 bind to MA-33B8 at similar rates, consistent with the observation that the antibody inactivated the two PAI-1 forms at comparable rates. The loop-inserted forms of PAI-1, however, including latent PAI-1, elastase-cleaved PAI-1, and the tPA-PAI-1 complex, bound much more rapidly to MA-33B8 than active PAI-1, indicating that loop insertion leads to a greater exposure of the epitope. The fact that the octapeptide-complexed PAI-1 and the P14 Thr → Arg mutant, which cannot rapidly insert the loop, reacted poorly or not at all with MA-33B8 is consistent with this assumption.

A significant aspect of the antibody effect is the speed with which it causes conversion to latency. At 25 °C, the spontaneous active-to-latent conversion of wild type and P9-NBD-labeled PAI-1 has a $t_{1/2}$ of 9.5 and 23.5 h respectively, whereas in the presence of 4.2 $\mu$M MA-33B8 the $t_{1/2}$ was reduced to 9 s for both forms. No evidence for saturation was seen in the antibody concentration dependence of the rate of conversion to the latent form, indicating that inactivation of PAI-1 could be substantially faster at saturating conditions.

To accomplish this rate enhancement, antibody binding must either devise a new and faster path for latency in PAI-1, or make the existing path faster by reducing the energy gap between the ground state of the active PAI-1 molecule and a major transition state for its conversion to the latent form. In the first alternative (Scheme 1), the epitope is fully exposed in active PAI-1 (I) and no binding energy is reserved for changing the conformation of the inhibitor or supporting a transition state. Antibody binding, however, generates a species of the inhibitor (I′Ab), the molecular dynamics of which are different from that of the parent molecule and which is less stable at the input of the same amount of thermal energy.

$$\text{Ab} + I \rightarrow I′Ab \rightarrow I_{\text{lat}}Ab$$

**Scheme 1**

If the epitope were fully exposed in active PAI-1, as implicated by the mechanism in Scheme 1, MA-33B8 would bind free active PAI-1 (I) as fast as it binds the loop insert PAI-1 forms. The data in Fig. 5 and 6, however, show that whereas binding of MA-33B8 to loop-inserted forms of PAI-1 occurred at a rate comparable to that typically reported for antibody antigen reactions (i.e., $10^{8} \text{ M}^{-1} \text{ s}^{-1}$), the second order rate constant for MA-33B8 binding to active PAI-1 was reduced approximately 1000-fold. To account for an apparent second order rate constant for the overall process in Scheme 1 as low as $10^{4} \text{ M}^{-1} \text{ s}^{-1}$, we would have to assume that the second step is much slower than the reversal of the biomolecular binding step. If this were the case, however, obvious signs of rate saturation would be observed at concentrations of MA-33B8 corresponding to the typical antibody-antigen dissociation constant, which is lower than 4.2 $\mu$M (15, 16). For these reasons, the mechanism in Scheme 1 can be considered unlikely.

In the alternative mechanism, presented in Scheme 2, the dominant form of active PAI-1 (I) obscures the epitope for MA-33B8. The epitope becomes completely exposed in a conformation (I′) that is also a transition state for formation of the latent species. Antibody binding traps this transition state and reduces its energy relative to the active inhibitor, resulting in an enhanced rate of PAI-1 inactivation. Scheme 2 is analogous to that used to explain the rate enhancement caused by binding a substrate (I) to the surface of an enzyme (Ab) (25).

The spontaneous formation of latent PAI-1 was negligible for the duration of our antibody binding experiments. The documented strong binding of MA-33B8 to latent PAI-1 is logical in view of the mechanism in Scheme 2 but does not explain the rate enhancement. Although one of the two paths leading to I′Ab may be preferred kinetically, they are thermodynamically equivalent.

According to Scheme 2, the maximal rate enhancement factor ($f_{\text{max}}$) is linked thermodynamically to the relative affinities of I′ and I for the antibody and is given by $f_{\text{max}} = K_{IAb}/K_{I′Ab}$, where $K_{IAb}$ and $K_{I′Ab}$ are the equilibrium constants for dissociation of IAb and I′Ab, respectively. Our results indicate that $f_{\text{max}}$ exceeds 4000, which means that MA-33B8 binds I′ more than 4000 times stronger than it binds I. The rate enhancement was not saturated at 4.2 $\mu$M antibody binding sites, which precludes the possibility that at this or lower concentrations, MA-33B8 will exhibit significant binding to PAI-1 without changes, which would bring the conformation of I closer to that of I′. Not indicated in Scheme 2 but possibly suggested by the weak binding of MA-33B8 to the loop exposed octapeptide blocked PAI-1 is binding of the antibody in several discrete steps to an increasingly exposed epitope. Other modifications to Scheme 2 may include slightly different rates for the collapse of I′ and I′Ab into the latent species. None of these modifications, however, will significantly alter the conclusions based on the mechanism depicted in Scheme 2.

$$\text{Ab} \leftrightarrow K_{\text{Ab}} \text{Ab} \leftrightarrow K_{IAb} \text{IAb} \leftrightarrow K_{I′Ab} \text{I′Ab} \leftrightarrow K_{I_{\text{lat}}Ab} \text{I}_{\text{lat}}Ab$$

**Scheme 2**

In Scheme 2, exposure of the epitope in I′ occurs by an unfavorable equilibrium. This predicts that the second order rate constant measured for formation of latent PAI-1 should be greatly reduced compared with that measured for antibody-antigen interactions in general, and for binding of MA-33B8 to the fully exposed epitope in PAI-1 in particular, a prediction in agreement with our results. This reduction is independent of whether I′Ab is formed via binding of Ab to I′ or I. Scheme 2 also explains why at an antibody binding site concentration as high as 4.2 $\mu$M, the rate of inactivation is not saturated. The overall equilibrium dissociation constant of I′Ab is the product of the two equilibria of each path and involves either tight binding to an epitope exposed in an unfavorable equilibrium or unfavorable binding to a partly exposed epitope, coupled to a compensatory more favorable equilibrium for its complete exposure.

In keeping with Scheme 2, our results show that MA-33B8 binds at a fast rate to latent, tPA-complexed, and elastase-cleaved PAI-1; at a significantly reduced rate to active PAI-1; and not at all or only weakly to the P14 Arg and peptide-complexed PAI-1 variants. The first group is characterized by
complete or partial insertion of strand 4 into sheet A; active PAI-1 has its RCL exposed but the potential for loop insertion; and the RCL of the substrate PAI-1 forms is exposed and cannot insert at a significant rate. Taken together, this suggests that the conformational change that exposes the epitope for MA-33B8 is associated with some insertion of the RCL into sheet A. Because the spontaneous formation of latent PAI-1 is virtually irreversible and because antibody binding increases the rate of this process by a factor larger than 4000, the epitope must be exposed in an intermediate (I*) that, in the absence of antibody, has greater tendency to revert to the active loop extracted conformation (I) than to proceed to form latent PAI-1, as indicated in Scheme 2. Because insertion of strand 4A should be increasingly prone to proceed to completion for each additional residue inserted, it is probable that insertion of only a few of the 14 residues of strand 4A is sufficient to expose the epitope. A tentative prediction is that the epitope for MA-33B8 is exposed in an intermediate that has inserted the proximal part of the RCL into sheet A to a point such that further insertion would require complete displacement of $\beta$-strand 1C at its distal end.

Our results do not enable us to identify specifically the epitope for MA-33B8. Loop insertion can unmask an epitope that is directly shielded by the extracted RCL, or it could be associated with a conformational change that exposes an epitope elsewhere on the molecule. To start with, however, the epitope cannot involve residues that are not exposed in the antigen, the tPA:PAI-1 complex. This should exclude residues in the P15–P1 portion of the RCL, which are either inserted into sheet A, covered by the helix F loop, or in close contact with the proteinase. Also excluded are residues covered by the proteinase at its final locking site. The fact that latent P9-NBD PAI-1 binds MA-33B8 without changes in NBD fluorescence argues against the epitope being within 10 Å of the label on P9. This would exclude residues in the loop that carries helix F, which has been shown to harbor the epitopes for the murine antibodies that convert PAI-1 to a substrate for tPA (26, 27). Our data also indicate that the vitronectin and heparin binding sites on PAI-1 do not overlap the epitope for MA-33B8.

Binding of a multimeric form of vitronectin to PAI-1 has been reported to reduce the rate of latency by a factor 2 (24). This effect, however, has not been documented with monomeric vitronectin, which was used in this study. Direct measurements of the inactivation of native and P9-NBD PAI-1 in the presence of monomeric vitronectin, at a concentration that was saturating based both on our own observations and on data presented in the literature (28), revealed a stabilizing effect on native PAI-1 of only 1.3, whereas no significant effect on P9-NBD PAI-1 was observed. The absence of an effect of monomeric vitronectin on the rate of change of the P9-NBD PAI-1 fluorescence induced by antibody binding is consistent with these results.

In summary, we have found that binding of MA-33B8 induces at least a 4000-fold decrease of the half-life of active PAI-1 and have provided evidence that the inactive species generated is latent PAI-1. We have presented data that suggest that binding of antibody depends on partial insertion of the RCL into $\beta$-sheet A of the inhibitor and that antibody binding stabilizes an intermediate that represents or is close to a transition state on the pathway to latent PAI-1. Our results should provide the basis for studies aiming at identifying the epitope for MA-33B8 and determining the mechanism for the antibody-induced, as well as the spontaneous, formation of latent PAI-1.

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