Selective Screening of a Large Phage Display Library of Plasminogen Activator Inhibitor 1 Mutants to Localize Interaction Sites with Either Thrombin or the Variable Region 1 of Tissue-type Plasminogen Activator*

(Received for publication, November 20, 1995)

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Phage display technology has been exploited to study in detail the interaction between plasminogen activator inhibitor 1 (PAI-1) and either thrombin or an essential positively charged "loop" of tissue-type plasminogen activator (t-PA), denoted variable region 1 (VR1). For this purpose, a PAI-1 mutant phage library was used that served as a reservoir of PAI-1 proteins potentially deficient in the interaction with either VR1 or thrombin. A stringent two-step selection procedure was developed. (i) A negative selection was performed by incubating the pComb3/PAI-1 mutant library with an excess of a thrombin mutant with its VR1 domain substituted with that of t-PA (thrombin-VR1). (ii) The remaining phages were complexed with t-PA (positive selection) and selected by panning with an immobilized anti-t-PA monoclonal antibody. Four consecutive panning rounds yielded an enrichment of pComb3/PAI-1 mutant phages of ~50-fold. Sequence analysis of 16 different cDNAs, encoding PAI-1 mutants that are hampered in the binding to thrombin-VR1, revealed the following mutations. Four independent variants share a mutation of the P4' residue (Glu350 → Lys). Nine independent PAI-1 variants share a substitution of P1' (Met247 → Lys), whereas three others share a P2 substitution (Ala245 → Asp). Kinetic analysis of representative PAI-1 mutants provides evidence that the P4' residue is essential for the interaction with the VR1 domain, consistent with the data of Madison et al. (Madison, E. L., Goldsmith, E. J., Gething, M. J., Sambrook, J. F., and Gerard, R. D. (1990) J. Biol. Chem. 265, 21423–21426), whereas the P1' and P2 residues confer thrombin specificity. Concordant with the design of the selection procedure, mutants were obtained that inhibit thrombin-VR1 at least 100-fold slower than wild-type PAI-1, identifying residues that are central to the interaction with either thrombin or VR1. This study demonstrates that phage technology can be used to analyze large numbers of mutants defective in their interaction with other (domains of) proteins, provided an adequate selection scheme is devised.

Plasminogen activator inhibitor 1 (PAI-1)1 is the main inhibitor of the serine proteases tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator and therefore a major physiological regulator of the fibrinolytic system (reviewed in Ref. 1). A number of studies have delineated regions or amino acids of PAI-1 that are involved in the interaction with t-PA: first, the reactive-site P1 residue (Arg246) of PAI-1 that interacts with the catalytic center of t-PA; second, the region between amino acids 110 and 145 of PAI-1 that binds to an unknown domain on t-PA (2); and third, presumably negatively charged residues on PAI-1 that are involved in an interaction with the positively charged variable region 1 (VR1) on the protease domain of t-PA (3–5). Initially, evidence for the importance of the VR1 "loop" of t-PA for the interaction with PAI-1 was provided by mutagenesis experiments: deleting the VR1 loop or replacement of the positively charged amino acids of VR1 by negatively charged residues reduced the second-order rate of inhibition (k2) for PAI-1 by at least 3 orders of magnitude (from 2 × 107 to ≤104 M−1 s−1) (4). Consistent with these data, we demonstrated that replacement of the VR1 domain of thrombin by the corresponding region of t-PA created a protein (denoted thrombin-VR1) that acts as an efficient "target" serine protease for PAI-1, in contrast to native thrombin (k2 = 2 × 106 versus ≤104 M−1 s−1, respectively) (6). Although native thrombin is virtually not inhibited by PAI-1, we and others recently reported that, in the presence of either high molecular weight heparin or vitronectin, the rate of thrombin inhibition by PAI-1 is increased 2–3 orders of magnitude (7–9). The binding of PAI-1 to these cofactors has been extensively studied, and the interaction sites on the PAI-1 protein have been precisely localized (10–13).

In this paper, we employ a large library of PAI-1 mutants that are displayed on the surface of phages (14). This library serves as a reservoir of tools to further delineate structure-function relationships of this multifunctional protein. We devised a stringent two-step selection procedure to isolate mutants of PAI-1 that are hampered in the binding to either the VR1 domain or thrombin. For this purpose, the PAI-1 mutant phage library was incubated with an excess of thrombin-VR1, and subsequently, nonbinding phages were captured by complexing with t-PA and an immobilized anti-t-PA monoclonal antibody. The selection procedure was specifically devised to obtain PAI-1 mutants that inhibit thrombin-VR1 at a rate that is at least 100-fold slower than that of wild-type PAI-1. Accord-

*This work was supported by Grant 902-26-132 from the Netherlands Organization for Scientific Research and by Grant 92.003 from the Netherlands Thrombosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator; VR1, protease domain variable region 1; mAb, monoclonal antibody.
ingly, amino acid residues were identified that are central to the interaction with either the VR1 domain or thrombin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The construction of the phage-displayed pComb3/PAI-1 mutant library (containing 1.7 × 10^9 independent colonies) has been previously described (14). *Escherichia coli* strain XL1-Blue (F') and VCSM13 helper phages were from Stratagene (La Jolla, CA). The *E. coli* expression plasmid pMBL11 was donated by Dr. B. E. Enger-Valk (Medical Biological Laboratory TNO, Rijswijk, The Netherlands). *E. coli* K12 (strain MG1655) was from the Bacterial Collection (University of Utrecht, Utrecht, The Netherlands). Restriction and DNA-modifying enzymes were from Gibco BRL (Paisley, United Kingdom). The antibodies ampicillin, tetracycline, and kanamycin were purchased from Sigma. The phosphorylated oligonucleotides 5'-TCGACT-\( \text{GAGAAGCTTTCTAGATAGTGAG-3'} \) and 5'-GATCCTCATATCTA-GAAGACTTGAG-3' to modify plasmid pMBL11 were obtained from Pharmacia (Rosendaal, The Netherlands). α-32P-DATP was from the Radiochemical Center (Amersham, United Kingdom). Sequences and Version 2.0 sequencing kits were purchased from U. S. Biochemical Corp.

Unfractionated heparin from porcine intestinal mucosa (H-3125, grade I, Lot 29F-0314; specific activity of 178 units/mg; average \( M_r \) of 15,000–15,000) was obtained from Sigma. The chromogenic substrates CH3SO2-D-hexahydrotyrosine-Gly-Arg-\( \text{-D-Phe-Pip-Arg-} \) and \( \text{-nitroanilide (where Pip is pipecolyl; S2238) were obtained from Pentapharm (Basel, Switzerland) and Chromogenix (Molndal, Sweden), respectively. CM-Sephadex C-50 and Sephadex G-50 superfine were from Pharmacia (Uppsala, Sweden).**

**Proteins**—Human thrombin was kindly donated by Dr. G. Tans (StAPH, Maastricht, The Netherlands). The thrombin mutant, designated thrombin-VR1 (containing the VR1 domain of t-PA), was constructed and purified as described previously (6). Both thrombin and thrombin-VR1 were active site-titrated with hirudin (6). Human thrombin was kindly donated by Dr. G. Tans (StAPH, Maastricht, The Netherlands). The thrombin mutant, designated thrombin-VR1 (containing the VR1 domain of t-PA), was constructed and purified as described previously (6). Both thrombin and thrombin-VR1 were active site-titrated with hirudin (6). Human thrombin was kindly donated by Dr. G. Tans (StAPH, Maastricht, The Netherlands). The thrombin mutant, designated thrombin-VR1 (containing the VR1 domain of t-PA), was constructed and purified as described previously (6). Both thrombin and thrombin-VR1 were active site-titrated with hirudin (6).

**Interaction of PAI-1 with t-PA and Thrombin**—Interaction of PAI-1 with t-PA or thrombin-VR1 was incubated for 1 h at 37°C with a 100-fold dilution of thawed phospholipid vesicles in either HBST buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 80) or HBST buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 80) at 4°C for several weeks without loss of activity. Aliquots of PAI-1 were desalted by chromatography on 1-ml Sephadex G-50 superfine columns, equilibrated in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 80, just before kinetic analysis. The concentration of active PAI-1 (mutant) in each sample was determined by titration of increasing amounts of the PAI-1 preparations with two-chain Bowes melanoma t-PA using a chromogenic assay (17), followed by Coomassie Brilliant Blue staining.

**Inhibition of t-PA or Thrombin-VR1 by pComb3/PAI-1 Phage Libraries**—Inhibition of t-PA or thrombin-VR1 by PAI-1 phages derived from the preselected library (i.e., selected for PAI-1 mutants active toward t-PA) or from the library obtained after four rounds of selection was performed as described (14). Briefly, an increasing amount of freshly grown PAI-1 phages (10^13 colony-forming units/ml) were incubated for 90 min at 37°C with either 0.5 nM t-PA or thrombin-VR1 in 25 μl of HBS buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, and 0.1% (v/v) polyethylene glycol 6000). Residual t-PA or thrombin-VR1 activity was determined at 37°C by continuous measurement of the absorbance at 405 nm in a Titertek Twinreader (Flow Laboratories, Irvine, United Kingdom) after adding 25 μl of 4 mM chromogenic substrate Pefachrome tPA or S2238, respectively, and 150 μl of HBS buffer.

**Inhibition of t-PA or Thrombin-VR1 by Purified Individual PAI-1 Mutants Obtained after Four Rounds of Selection**—Wells of microtiter plates (Nunc Maxisorp, Gibco BRL) were pretreated for 1 h at 37°C with 0.1% (v/v) polyethylene glycol 20,000, and the reaction was started by the addition of 4 μl of 125 nM thrombin (total volume of 50 μl; final concentration of 5 nM) or thrombin-VR1 (total volume of 250 μl; final concentration of 1 nM). The reactions contained varying concentrations of PAI-1 (mutants) and, as indicated, either 1 unit/ml heparin or 400 nM vitronectin. At various times intervals, 5-μl samples (or 5 μl in the case of thrombin-VR1) were taken, and residual thrombin (or thrombin-VR1) activity remaining after determination by measurement of the absorbance at 405 nm in a Titertek Twinreader after addition to a mixture of 150 μl (or 100 μl) of HBST buffer and 25 μl of 4 mM S2238. Progress curves were linear for at least 1 h, indicating that the reaction was sufficiently quenched by the chromogenic substrate. Control experiments, with thrombin or thrombin-VR1 alone, indicated that no inactivation of the chromogenic substrate occurred. To determine order rate constants for inhibition of t-PA by PAI-1 (mutants) were determined at 37°C in HBST buffer in a Beckman DU 70 spectrophotometer. Cuvettes were pretreated with 1% (v/v) polyethylene glycol 20,000. The inhibitors were preincubated for 2 min at 37°C in HBST buffer using polyethylene tubes that had been pretreated with 1% (v/v) polyethylene glycol 20,000, and the reaction was started by the addition of 4 μl of 125 nM thrombin (total volume of 50 μl; final concentration of 5 nM) or thrombin-VR1 (total volume of 250 μl; final concentration of 1 nM). The reactions contained varying concentrations of PAI-1 (mutants) and, as indicated, either 1 unit/ml heparin or 400 nM vitronectin. At various times intervals, 5-μl samples (or 5 μl in the case of thrombin-VR1) were taken, and residual thrombin (or thrombin-VR1) activity remaining after determination by measurement of the absorbance at 405 nm in a Titertek Twinreader after addition to a mixture of 150 μl (or 100 μl) of HBST buffer and 25 μl of 4 mM S2238. Progress curves were linear for at least 1 h, indicating that the reaction was sufficiently quenched by the chromogenic substrate. Control experiments, with thrombin or thrombin-VR1 alone, indicated that no inactivation of the chromogenic substrate occurred. To determine order rate constants for inhibition of t-PA by PAI-1 (mutants) were determined at 37°C in HBST buffer in a Beckman DU 70 spectrophotometer. Cuvettes were pretreated with 1% (v/v) polyethylene glycol 20,000. The inhibitors were preincubated for 2 min at 37°C in a “corner” of a cuvette. The reaction was started by the addition of 10 μl of prewarmed t-PA. Final concentrations were 3 nM PAI-1 (mutant) and 3 nM t-PA at 37°C. At various times intervals, the reaction was quenched by a 40-fold dilution in 0.7 mM Pefachrome tPA in HBST buffer. Residual t-PA activity was measured by determining the absorbance for 5 min at
Interaction of PAI-1 with t-PA and Thrombin

The library of freshly grown pComb3/PAI-1 phages (PAI-1 φ), which had been previously selected for active PAI-1 mutants, was incubated for 1 h at 37 °C with an excess of thrombin-VR1 (Ila-VR-1) to form complexes with PAI-1 mutants that have retained the ability to bind thrombin-VR1 ("negative selection" (A)). Subsequently, t-PA was added to the mixture to form t-PA/PAI-1 phenotype complexes with PAI-1 mutants that do not bind to thrombin-VR1 ("positive selection" (B)). Finally, the mixture was transferred to a well that was coated with an anti-t-PA mAb (CLB-16) to capture the t-PA/PAI-1 phenotype complexes (C). Binding of phages to a well coated with an irrelevant mAb (CLB-Cag 69) served as a negative control, whereas incubation of phages with t-PA only served as a positive control. This selection and panning procedure was repeated three times.

405 nm at 10-s intervals. The second-order rate constants of inhibition (k1) were determined as follows. To determine rate constants greater than 10⁶ M⁻¹ s⁻¹, enzyme and inhibitor were incubated at equimolar concentrations (E₀ = I₀), and k₁ was calculated from the following equation: (1/E₀) - (1/Eₜ) = k₁ × t. Rate constants smaller than 10⁵ M⁻¹ s⁻¹ were obtained under pseudo first-order conditions, i.e. at inhibitor concentrations (E₀) 25-200-fold higher than enzyme concentration (E₀). The k₁ was calculated from a linear plot of log percent remaining enzyme versus time (t) according to the following equation: tₕ₅₀ = In(2/k₁ × I₀). The lowest detectable rate constant was ~6 × 10⁵ M⁻¹ s⁻¹.

RESULTS

Design of a Selection Scheme for pComb3/PAI-1 Phage Mutants Defective in Their Interaction Either with the VR1 Domain of t-PA or with Thrombin—Our rationale for selecting PAI-1 mutants defective in the interaction either with the VR1 domain of t-PA or with thrombin is based on the following considerations. First, the second-order rate constant of inhibition (k₁) between PAI-1 and t-PA lacking the VR1 domain is ~1000-fold lower (4) than that of intact t-PA (10⁷ M⁻¹ s⁻¹) (18, 19). Second, PAI-1 is a poor inhibitor of thrombin in the absence of cofactors (k₁ < 10⁶ M⁻¹ s⁻¹) (20). In contrast, the association rate of PAI-1 with a thrombin mutant (thrombin-VR1) having its VR1 domain substituted with that of t-PA is at least 2000-fold faster (2 × 10⁹ M⁻¹ s⁻¹) (6), emphasizing the vital role of the VR1 domain of t-PA in the interaction with PAI-1. Third, it is assumed that PAI-1 mutants with an altered VR1-binding site will be virtually unable to inhibit thrombin-VR1, whereas such mutants would still be capable of inhibiting t-PA, albeit at a diminished rate. Fourth, PAI-1 mutants that are defective in their interaction with thrombin will also be unable to inhibit thrombin-VR1. Based on these considerations, we employed a large library of PAI-1 mutants (1.7 × 10⁹) that are functionally displayed on the surface of phages and used a stringent selection protocol to ultimately identify amino acid residues that play a dominant role in the interaction with either thrombin or the VR1 domain of t-PA. Previously, we extensively characterized this library, which consists of 46% single and 34% double mutants and 20% wild-type PAI-1 proteins (14). Prior to the actual selection procedure, phages that either do not carry a PAI-1 protein or display a nonfunctional protein (due to, for example, a translational termination codon) were discarded. For this purpose, amplified pComb3/PAI-1 phages (3.8 × 10¹⁰) were incubated with an equimolar amount of t-PA, and complexes were captured by panning with the immobilized anti-t-PA mAb CLB-16 as described (14). Maximum binding of ~5.6 × 10⁹ phages/well was obtained. Incubation of t-PA with pComb3 phages, devoid of PAI-1, resulted in the binding of 9.7 × 10⁷ phages/well. After amplification of the active pComb3/PAI-1 mutants, a negative selection was carried out by incubating the prescreened pComb3/PAI-1 mutant phage library for an extended period (1 h at 37 °C) with an excess of thrombin-VR1 (Fig. 1A), followed by a positive selection of the remaining phages with t-PA (Fig. 1B). Complexes of pComb3/PAI-1 phages with t-PA were again selected by panning with the immobilized anti-t-PA mAb CLB-16 or with an irrelevant mAb (Fig. 1C). Four consecutive panning rounds revealed an enrichment of ~50-fold (Table I). Incubation of the pComb3/PAI-1 phages with t-PA alone demonstrated that maximal binding per well was achieved (~10⁷ phages/well). Subsequently, the phage library obtained after four rounds of panning was compared with the prescreened phage library with respect to inhibition of t-PA or thrombin-VR1 by using chromogenic assays. Clearly, both libraries show a similar t-PA inhibition, whereas the ability to inhibit thrombin-VR1 is significantly decreased after four rounds of selection (Fig. 2).

Expression and Purification of PAI-1 Mutants—To characterize the properties of single PAI-1 mutants displayed on the surface of phages, we inserted PAI-1 cDNAs, obtained after expression and purification of PAI-1, resulted in the binding of 9.7 × 10⁷ phages/well. After amplification of the active pComb3/PAI-1 mutants, a negative selection was carried out by incubating the prescreened pComb3/PAI-1 mutant phage library for an extended period (1 h at 37 °C) with an excess of thrombin-VR1 (Fig. 1A), followed by a positive selection of the remaining phages with t-PA (Fig. 1B). Complexes of pComb3/PAI-1 phages with t-PA were again selected by panning with the immobilized anti-t-PA mAb CLB-16 or with an irrelevant mAb (Fig. 1C). Four consecutive panning rounds revealed an enrichment of ~50-fold (Table I). Incubation of the pComb3/PAI-1 phages with t-PA alone demonstrated that maximal binding per well was achieved (~10⁷ phages/well). Subsequently, the phage library obtained after four rounds of panning was compared with the prescreened phage library with respect to inhibition of t-PA or thrombin-VR1 by using chromogenic assays. Clearly, both libraries show a similar t-PA inhibition, whereas the ability to inhibit thrombin-VR1 is significantly decreased after four rounds of selection (Fig. 2).

Expression and Purification of PAI-1 Mutants—To characterize the properties of single PAI-1 mutants displayed on the surface of phages, we inserted PAI-1 cDNAs, obtained after four rounds of selection, into plasmid pMBL11-N to allow expression of soluble protein. Subsequently, randomly chosen individual PAI-1 mutant proteins were purified from E. coli extracts (16). The yield of PAI-1 mutants after purification from 10-ml cultures was 0.5 ml with a concentration ranging from 1.5 to 4 μm active PAI-1, as determined by titration with standard amounts of t-PA. Typically, the preparations consist of ~50% active PAI-1 protein. Initially, the purified PAI-1 mutants were analyzed for their ability to inhibit t-PA or thrombin-VR1 using a chromogenic assay. Analysis of 92 inde-
pended proteins revealed that 76 displayed an inhibition of t-PA and thrombin-VR1 that is comparable to that of wild-type PAI-1 (data not shown). In contrast, 16 PAI-1 mutant proteins have a decreased capacity to inhibit thrombin-VR1 compared with wild-type PAI-1, whereas the inhibition of t-PA is similar. Results of a typical inhibition experiment are shown in Fig. 3. Neutralization of t-PA or thrombin-VR1 by increasing amounts of wild-type PAI-1 (Fig. 3A) or a PAI-1 mutant (Fig. 3B) is presented. Clearly, inhibition of t-PA is similar for both PAI-1 proteins, whereas inhibition of thrombin-VR1 by the PAI-1 mutant is substantially reduced.

Sequence and Kinetic Analysis of PAI-1 Mutants—To characterize the effect of mutations on PAI-1 specificity in more detail, cDNA sequence analysis was performed, and the second-order rate constants for inhibition of t-PA or thrombin-VR1 by a set of representative PAI-1 mutants were established. Furthermore, to discriminate whether these PAI-1 mutants were disturbed in their interaction with the VR1 domain of t-PA or with thrombin, the mutants were monitored both for thrombin-VR1 inhibition and thrombin inhibition in the presence of either heparin or vitronectin (Table II). Sequence analysis of these 16 PAI-1 mutants, with reduced activity toward thrombin-VR1, demonstrates that four mutants share a P4’ mutation (Glu350 → Lys). Furthermore, nine mutants contain a Lys residue at the reactive-site P1’ position (Met347 → Lys). Finally, the three remaining mutants harbor a mutation at the P2 position (Ala345 → Asp). It should be noted that next to single P4’, P1’, and P2 mutants, also other, mostly silent mutations were encountered. This finding illustrates that independent mutants had been selected.

Kinetic analysis of wild-type PAI-1 used in this study demonstrated a second-order rate constant for t-PA inhibition of 8.3 × 10^6 M^{-1} s^{-1}, comparable with published data (18, 19). Rate constants for inhibition of thrombin-VR1 or thrombin in the presence of one of the cofactors by wild-type PAI-1 were somewhat lower than described previously (6, 20) due to the use of a different storage buffer of PAI-1. All PAI-1 mutants reveal a slightly reduced rate constant for thrombin-VR1 relative to t-PA (at least 4.7-fold). Interestingly, the P4’ mutant (Glu350 → Lys) displays similar rate constants for thrombin in the presence of either one of the cofactors. In addition to the P4’ mutant, variants were selected that are deficient in the interaction with both thrombin-VR1 and native thrombin. The P1’ mutant (Met347 → Lys) exhibits markedly lower rate constants for inhibition of thrombin-VR1 or thrombin in the presence of either heparin or vitronectin, whereas t-PA inhibition is only slightly reduced. Results obtained with the P1’ mutants are in good agreement with published data: replacement of P1’ Met347 by a Lys residue results in a slight reduction of the second-order rate constant for t-PA inhibition (21). Similarly to the P1’ mutant (Met347 → Lys), t-PA inhibition by the P2 mutant (Ala345 → Asp) is slightly reduced, consistent with the observation of York et al. (22). By contrast, inhibition of thrombin-VR1 or thrombin in the presence of one of the cofactors by the P2 mutant (Ala345 → Asp) is virtually abolished ($k_1 ≤ 6 × 10^5 M^{-1} s^{-1}$).

**Fig. 2.** Inhibition of t-PA or thrombin-VR1 by the preselected PAI-1 mutant library or by the library obtained after four consecutive panning rounds. Increasing numbers of phages, derived from the preselected pComb3/PAI-1 library (selected only for active PAI-1 variants) or from the library obtained after four panning rounds, were incubated with t-PA or thrombin-VR1 for 90 min at 37 °C in a volume of 25 μl as described under “Experimental Procedures.” Residual amidolytic activity of t-PA or thrombin-VR1 (indicated as percentage) was assayed by the addition of 150 μl of HBST buffer and 25 μl of 4 mM Pefachrome tPA or S2238, respectively, followed by continuous recording of the absorbance at 405 nm in a Titertek Twin reader. ○, t-PA inhibition by preselected pComb3/PAI-1 phage library; □, thrombin-VR1 inhibition by preselected pComb3/PAI-1 phage library; ●, t-PA inhibition by pComb3/PAI-1 phage library obtained after four consecutive panning rounds; ■, thrombin-VR1 inhibition by pComb3/PAI-1 phage library obtained after four consecutive panning rounds.

**Fig. 3.** Inhibition of t-PA or thrombin-VR1 by wild-type PAI-1 or PAI-1 Mut347 → Lys. Increasing amounts of purified wild-type PAI-1 (A) or purified PAI-1 Met347 → Lys (B) were incubated in microtiter wells for 1 h at 37 °C in HO buffer, employing a total volume of 25 μl containing 3.3 nM t-PA or thrombin-VR1. The residual activity of t-PA or thrombin-VR1 was measured as described under “Experimental Procedures.” ○, t-PA inhibition by wild-type PAI-1; □, thrombin-VR1 inhibition by wild-type PAI-1; ●, t-PA inhibition by PAI-1 Met347 → Lys; ■, thrombin-VR1 inhibition by PAI-1 Met347 → Lys.
t-PA P1 polymerase chain reaction and contains an average of two
involved in a particular interaction. The PAI-1 mutant library
combined with an appropriate selection procedure and DNA
resulting from a single base pair change. Obviously, our re-
portion of inactive proteins. It should be emphasized that the
expected that positively charged residues at the P1
mutations, amino acids are altered solely by single base pair substitu-
stringed mutagenesis procedure restricts the range of amino
proposed by Madison et al. (5). Initially, these investigators
PAI-1 are involved in this interaction. Further assessment of
n’t tolerate for thrombin inhibition (23) due to ionic repulsion

described under “Experimental Procedures.” Values represent the
second-order rate constants of inhibition \(k_1\) were determined at 37 °C as described under “Experimental Procedures.” Values represent the

| PAI-1 species | t-PA\(^a\) | Thrombin-VR1\(^a\) | Thrombin + hep\(^b\) | Thrombin + VN\(^b\) | t-PA/thrombin-VR1\(^b\) | t-PA/thrombin + hep\(^b\) | t-PA/thrombin + VN\(^b\) |
|--------------|---------|----------------|----------------|----------------|----------------|----------------|----------------|
| Wild-type    | 8.3 × 10^4 | 3.5 × 10^5 | 6.4 × 10^4 | 2.4 × 10^4 | 1.0 | 1.0 | 1.0 |
| P1 Met\(^{347}→\) Lys | 5.7 × 10^4 | 5.1 × 10^5 | 9.6 × 10^3 | 7.7 × 10^2 | 4.7 | 4.6 | 2.1 |
| P2 Ala\(^{345}→\) Asp | 1.1 × 10^5 | ≤6.0 × 10^2 | ≤6.0 × 10^2 | ≤77.3 | ≥14.1 | ≥5.3 |
| P4' Glu\(^{350}→\) Lys | 3.1 × 10^5 | 2.3 × 10^3 | 7.6 × 10^4 | 4.6 × 10^4 | 56.8 | 0.3 | 0.2 |

\(^a\) Second-order rate constants of inhibition \(k_1\) were determined at 37 °C as described under “Experimental Procedures.” Values represent the average of two independent experiments.

\(^b\) The ratio of the \(k_1\) value for t-PA to the value for thrombin-VR1 (t-PA/thrombin-VR1), thrombin in the presence of heparin (t-PA/thrombin + hep), or thrombin in the presence of vitronectin (t-PA/thrombin + VN) is given. Ratios are expressed relative to that exhibited by wild-type PAI-1. The values for the inhibition of thrombin-VR1 and thrombin in the presence of heparin by wild-type PAI-1 represent the average of five independent assays.

**DISCUSSION**

This study illustrates that phage display of a mutant library, combined with an appropriate selection procedure and DNA sequencing, may rapidly provide the location of amino acids involved in a particular interaction. The PAI-1 mutant library employed in this study has been constructed by error-prone polymerase chain reaction and contains an average of two mutations/unit length of PAI-1 cDNA (1137 base pairs), corresponding to 46% single and 34% double mutants and 20% wild-type proteins (14). Due to the moderate extent of mutations, amino acids are altered solely by single base pair substitutions. Consequently, only a limited set of amino acid substitutions will be encountered as exemplified by, for example, the PAI-1 P1’ (Met\(^{347}→\) Lys) and P2 (Ala\(^{345}→\) Asp) mutants, resulting from a single base pair change. Obviously, our restricted mutagenesis procedure restricts the range of amino acid alterations, whereas a more rigorous random mutagenesis procedure would result in a library containing a relatively large portion of inactive proteins. It should be emphasized that the design of the selection protocol will dictate the properties of the selected mutants. The PAI-1 mutant phage library was incubated with a 100-fold excess of thrombin-VR1, corresponding to pseudo first-order reaction conditions that allow the following calculations. To capture ~97% of a specific PAI-1 mutant, the selected incubation period (1 h) should be equal to five times the half-time of the reaction \((t_{\text{1/2}} = 12\ \text{min})\). From the equation \(t_{\text{1/2}} = \ln 2/\left(k_1 \times E_0\right)\), where \(E_0\) is the concentration of thrombin-VR1 (150 mU), it is deduced that PAI-1 mutants with a second-order rate constant for thrombin-VR1 inhibition exceeding 6.4 \(× 10^4 \text{ M}^{-1} \text{s}^{-1}\) will be complexed. Consequently, our selection procedure was devised to specifically select mutants that reacted at least 100-fold slower with thrombin-VR1 than wild-type PAI-1 \((k_1 = 2 \times 10^6 \text{ M}^{-1} \text{s}^{-1})\) (6). Clearly, this expectation is borne out by the experimental data: the second-order rate constants for inhibition of thrombin-VR1 by the selected PAI-1 P1’ (Met\(^{347}→\) Lys), P2 (Ala\(^{345}→\) Asp), and P4’ (Glu\(^{350}→\) Lys) mutants are 5.1 \(× 10^3\), ≤6 \(× 10^2\), and 2.3 \(× 10^2\) \text{ M}^{-1} \text{s}^{-1}, respectively (Table II).

The dominant contribution of the P1’ Met, P2 Ala, and P4’ Glu residues of PAI-1 to the interaction with thrombin-VR1 can be assigned either to binding to the VR1 loop of t-PA or to binding to the thrombin moiety. This distinction could be made upon measuring the inhibition of native thrombin in the presence of a cofactor (heparin or vitronectin). In this respect, the rate of inhibition of thrombin is decreased 50–100-fold for the P1’ (Met\(^{347}→\) Lys) and P2 (Ala\(^{345}→\) Asp) mutants as compared with wild-type PAI-1, whereas the inhibition rate for the PAI-1 P4’ mutant (Glu\(^{350}→\) Lys) is unaltered. Similarly, the rate of inhibition of t-PA by the PAI-1 P1’ (Met\(^{347}→\) Lys) and P2 (Ala\(^{345}→\) Asp) mutants is decreased 14- and 8-fold, respectively, whereas the rate of t-PA inhibition by the PAI-1 P4’ mutant (Glu\(^{350}→\) Lys) is only slightly reduced (2.6-fold). Nevertheless, altering the P1’ or P2 residue preferentially compromises the interaction of PAI-1 with thrombin rather than the inhibition of t-PA. This conclusion agrees with a recent report stating that positively charged residues at the P1’ position are not tolerated for thrombin inhibition (23) due to ionic repulsion of the S1’ subsite of thrombin (23, 24). Taken together, it is concluded that efficient thrombin inhibition requires uncharged amino acid residues at both the P1’ and P2 positions.

Substitution of P4’ Glu\(^{350}\) with Lys does not affect thrombin inhibition, but severely diminishes binding to the VR1 domain of t-PA. The selection of the PAI-1 P4’ mutant (Glu\(^{350}→\) Lys), created by a single base pair substitution, was anticipated on the basis of our selection strategy combined with the data reported by Madison et al. (5). Initially, these investigators replaced the positively charged residues of the VR1 loop of t-PA by Glu residues, rendering the protein fairly resistant to PAI-1 \((k_1 = 10^4 \text{ M}^{-1} \text{s}^{-1})\) (4). Subsequently, they showed that substitution of the negatively charged P4’ Glu\(^{350}\) of PAI-1 with the positively charged Arg residue virtually restored the inhibition of this t-PA variant (5). These observations have now received substantial support by the preferential selection of the P4’ mutant (Glu\(^{350}→\) Lys), demonstrating the dominant contribution of Glu\(^{350}\) in the interaction with the VR1 domain of t-PA. However, it is thought that several positively charged residues of the VR1 loop contribute to the interaction with PAI-1 and, consequently, that several negatively charged residues on PAI-1 are involved in this interaction. Further assessment of PAI-1 residues involved in the interaction with the VR1 loop probably requires an adjustment of the selection protocol to isolate mutants with a second-order rate of inhibition for thrombin-VR1 between 6.4 \(× 10^5\) \text{ M}^{-1} \text{s}^{-1} and, as argued before, that of wild-type PAI-1. Clearly, two options are available to reduce the stringency of the selection. First, the time allowed for complex formation between thrombin-VR1 and phage-displayed PAI-1 mutants can be decreased, ultimately resulting in mutants with higher inhibition constants. Second, assuming that the interaction between the VR1 domain and PAI-1 is predominantly of an ionic nature, the ionic strength during complex formation can be raised. Currently, these options are being assessed to optimally explore the phage display technology for analyzing the structure and function of PAI-1. The approach described in this report is applicable to many other proteins, provided these proteins can be functionally displayed on the surface of phages and if an adequate selection procedure is designed.

Acknowledgment—We thank A. E. Smilde for technical assistance.
REFERENCES
1. van Meijer, M., and Pannekoek, H. (1995) Fibrinolysis 9, 263–276
2. Keijer, J., Linders, M., van Zonneveld, A. J., Ehrlich, H. J., de Boer, J. P., and Pannekoek, H. (1991) Blood 78, 401–409
3. Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M. J., and Sambrook, J. F. (1989) Nature 339, 721–724
4. Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M. J., Sambrook, J. F., and Bassel-Duby, R. S. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3530–3533
5. Madison, E. L., Goldsmith, E. J., Gerard, M. J., and Gething, M. J., and Gerard, R. D. (1990) J. Biol. Chem. 265, 21423–21426
6. Horrevoets, A. J. G., Tans, G., Smilde, A. E., van Zonneveld, A. J., and Pannekoek, H. (1993) J. Biol. Chem. 268, 779–782
7. Ehrlich, H. J., Keijer, J., Preissner, K. T., Gebbink, R. K., and Pannekoek, H. (1991) Biochemistry 30, 1021–1028
8. Naski, M. C., Lawrence, D. A., Mosher, D. F., Podor, T. J., and Ginsburg, D. (1993) J. Biol. Chem. 268, 12367–12372
9. Ehrlich, H. J., Gebbink, R. K., Keijer, J., Linders, M., Preissner, K. T., and Pannekoek, H. (1990) J. Biol. Chem. 265, 13029–13035
10. Ehrlich, H. J., Klein Gebbink, R., Keijer, J., and Pannekoek, H. (1992) J. Biol. Chem. 267, 11606–11611
11. Lawrence, D. A., Berkpenas, M. B., Palaniappan, S., and Ginsburg, D. (1994) J. Biol. Chem. 269, 15223–15228
12. van Meijer, M., Klein Gebbink, R., Preissner, K. T., and Pannekoek, H. (1994) FEBS Lett. 352, 342–346
13. Padmanabhan, J., and Sane, D. C. (1995) Thromb. Haemostasis 73, 829–834
14. Pannekoek, H., van Meijer, M., Schleef, R. R., Loskutoff, D. J., and Barbas, C. F. (1993) Gene (Amst.) 128, 135–140
15. van Zonneveld, A. J., Veerman, H., Brakenhoff, J. P. J., Aarden, L. A., Cajot, J. F., and Pannekoek, H. (1986) Thromb. Haemostasis 57, 82–86
16. Sancho, E., Tange, D. W., Hockney, R. C., and Booth, N. A. (1994) Eur. J. Biochem. 224, 125–134
17. Lasemmi, U. K. (1970) Nature 227, 680–685
18. Kruithof, E. K., Tran-Thang, C., Ransijn, A., and Bachmann, F. (1984) Blood 64, 907–913
19. Thorsen, S., Philips, M., Selmer, J., Lecander, I., and Åstedt, B. (1988) Eur. J. Biochem. 175, 33–39
20. Keijer, J., Linders, M., Wegman, J. J., Ehrlich, H. J., Mertens, K., and Pannekoek, H. (1991) Blood 78, 1254–1261
21. Sherman, P. M., Lawrence, D. A., Yang, A. Y., Vandenbush, E. T., Paielli, D., Olsen, S. T., Shore, J. D., and Ginsburg, D. (1992) J. Biol. Chem. 267, 7588–7595
22. York, J. D., Li, P., and Gardell, S. J. (1991) J. Biol. Chem. 266, 8495–8500
23. Sherman, P. M., Lawrence, D. A., Verhamme, I. M., Paielli, D., Shore, J. D., and Ginsburg, D. (1995) J. Biol. Chem. 270, 9301–9306
24. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475