Mutational and Immunochemical Analysis of Plasminogen Activator Inhibitor 1*

Huda E. Shubeitaz, Theresa L. Cottey, Arthur E. Frankel, and Robert D. Gerard

From the Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038 and Pfizer Central Research, Groton, Connecticut 06340

(Received for publication, May 15, 1990)

We have undertaken a structural and functional analysis of recombinant plasminogen activator inhibitor type 1 (PAI-1) produced in Escherichia coli using site-directed mutagenesis and immunochemistry. Expression of recombinant PAI-1 yielded an inhibitor that was functionally indistinguishable from PAI-1 made in human endothelial cells. Mutations in both the reactive center Pl and Pl' residues (Arg-Met) and a putative secondary binding site for plasminogen activators on PAI-1 have been engineered to assess their functional effects. The inhibition of a panel of serine proteases, including plasminogen activators, trypsin, elastase, and thrombin, has been studied. Substitution of the P1 arginine residue with lysine or the P1' residue with either valine or serine had no detectable effect on the rate of inhibition of plasminogen activators. However, replacement of both P1 and P1' by Met-Ser produced a variant with no detectable plasminogen activator inhibitor activity. Mutations introduced into either Asp102 or Lys104 in the second site did not affect the rate of inhibition of plasminogen activators. Complementary immunochemical experiments using antibodies directed against the same two regions of the PAI-1 protein confirm that the reactive center is the primary determinant of inhibitory activity and that the putative second site is not a necessary functional region.

Tissue-type plasminogen activator (tPA) and urokinase (uPA) are serine proteases that catalyze the proteolytic cleavage of the inactive precursor plasminogen to the active protease plasmin. Plasminogen activation is regulated through several mechanisms, including the controlled synthesis and secretion of plasminogen activators and the modulation of their enzymatic activity by specific inhibitors (Gerard and Meiddell, 1989). Both plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2) are members of the serine protease inhibitor (serpin) superfamily (Carrel et al., 1987; Ny et al., 1986; Sprengers and Kluft, 1987; Pannekoek et al., 1986; Ye et al., 1987). By analogy with other serpins (Carrell and Travis, 1985), the best current model for the inhibition of plasminogen activators by PAI-1 postulates that a loop of amino acid residues in the serpin molecule (termed the reactive center) resembling the normal substrate binds to the active site of the protease. However, instead of proteolytic cleavage and release of the products, a covalent bond forms between the catalytic serine residue of the plasminogen activator and the P1 residue of PAI-1 and irreversibly inactivates the enzyme.

Members of the chymotrypsin family of serine proteases demonstrate selectivity toward their cognate inhibitors that is determined in part by surface loops of amino acid residues which interact with the inhibitor (Bode et al., 1989; Carrell et al., 1987; Madison et al., 1989, 1990). Proteins of the serpin superfamily are most divergent in sequence within the reactive center (Ye et al., 1987), suggesting that these residues are important determinants of the specificity toward their cognate proteases. In support of this hypothesis, a change in the Met-Ser reactive site of α1-antitrypsin to Arg-Ser alters the specificity of the serpin for elastase and converts it into an effective inhibitor of thrombin and trypsin-like enzymes (Carrell and Travis, 1985). Similarly, mutagenesis of α2-antiplasmin by deletion of the reactive center P1 arginine residue to yield a Met-Ser reactive site increases its rate of inhibition of elastase (Holmes et al., 1987). While PAI-1 and PAI-2 share 38% amino acid sequence identity and probably have similar tertiary structures (Carrell et al., 1987) sequence divergence in their reactive centers may account for rate constants of inhibition of both uPA and tPA by PAI-1 that are 10-100-fold faster than those of PAI-2 (Sprengers and Kluft, 1987). Consequently, PAI-1 is the principal plasminogen activator inhibitor in plasma, even in the presence of high levels of PAI-2 (Jorgensen et al., 1987).

In the present study, the functional significance of the reactive center P1 and P1' residues of PAI-1 was assessed by mutational analysis. A "second site" on PAI-1 which contains the sequence Asp102-Leu103-Lys104 was also chosen for mutagenesis. By alignment of the primary structures of PAI-1 and α1-antitrypsin and examination of the three-dimensional structure of the antitrypsin molecule (Loebelmann et al., 1984), these residues have been localized to one end of the molecule between sheet 2A and helix E (Ye et al., 1987). Although located in the nonhomologous carboxyl-terminal extension of α2-antiplasmin, an identical tripeptide sequence binds to the active site of plasmin (Sasaki et al., 1988). Since plasminogen activators also contain kringle domains, second site substitution mutants in the Asp102 and Lys104 residues may be important in their interaction with plasmin.

* This research was supported by grants from Pfizer and the American Heart Association-Texas Affiliate and by an Established Investigatorship (to R. D. G.) from the American Heart Association-National Heart, Lung, and Blood Institute (to R. D. G.) from the American Heart Association-Texas Affiliate and by an Established Investigatorship (to R. D. G.) from the American Heart Association-Texas Affiliate. 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.

† Present address: Dept. of Medicine, University of California, San Diego, La Jolla, CA 92033.

‡ To whom correspondence should be addressed.

1 The abbreviations used are: tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator; PAI, plasminogen activator inhibitor; rPAI, recombinant PAI; KLH, keyhole limpet hemocyanin.

2 E. Madison, E. J. Goldsmith, M.-J. Gething, J. F. Sambrook, and R. D. Gerard, submitted for publication.
residues of the PAI-1 protein were constructed in an attempt to disrupt any potential charged-pair interactions between these residues and those in plasminogen activator.

To assess the functional significance of the reactive center and second site sequences as determinants of the inhibitory activity and protease specificity of PAI-1, wild-type and mutant PAI-1 molecules have been expressed in *Escherichia coli* and used both in enzyme inhibition assays to measure the rate constants of inhibition of the serine proteases tPA, uPA, and thrombin, and in complementary immunochromatographic experiments using anti-peptide antibodies. The results of these studies show that the reactive center sequences of PAI-1 are a primary determinant of inhibitor specificity and activity and suggest that other unidentified determinants outside of the P1 and P1' residues play an important role in the inhibition of serine protease activity.

**EXPERIMENTAL PROCEDURES**

*Proteins and Reagents—*Lysozyme (chicken egg white) was purchased from United States Biochemical. Human Lys-plasminogen, des-Asp fibrinogen (DESAFIB), and the chromogenic substrates Spectrozyme PL (H-D-Nle-HHT-Lys-pNA-2AcOH), Spectrozyme TH (H-H-H-H-L-A-L-Ag-pNA-2AcOH), and Spectrozyme Try (O-Tyr-Gly-d-Ala-Arg-pNA-2AcOH) were purchased from American Diagnostica. Succinyl-(Ala)-paramethoxyanilide was purchased from Behring Diagnostics.

Human uPA was obtained from Behring Diagnostics and single-chain tPA was a gift from Knoll Pharmaceuticals via Dr. James T. Willerson. Human thrombin and porcine pancreatic trypsin were obtained from United States Biochemical. Specific activities of all enzymes were determined by active-site titration using tritiated diisopropyl-fluorophosphate obtained from New England Nuclear.

*DNA Technology—*DNA technology was employed throughout (Sambrook et al., 1989). pPAIST7HS was derived from pPAIST7 (Franke et al., 1990) to include the Klenow fragment of *E. coli* DNA polymerase I, and ligation. Deletion of sequences in pPAIST7 down-stream of the HindIII site was accomplished by sequential partial *Sal*I digestion, complete HindIII digestion, and blunt-ending with the Klenow fragment of *E. coli* DNA polymerase I, and ligation.

*Construction of the Expression Vector—*Standard recombinant DNA technology was employed throughout (Sambrook et al., 1989). The mature, secreted form of PAI-1 was expressed in *E. coli* TG-1 cells using the prokaryotic expression vector, pPAISTTHS. The PAI-1 cDNA was introduced into *E. coli* TG-1 cells as pPPAISTTHS (Frankel et al., 1990) to facilitate the exchange of mutagenic oligonucleotides, SalI and HindIII fragment PAI-1 coding sequences as follows. pPAIST7 was partially digested with HindIII to linearize the plasmid, blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I and ligated to eliminate the upstream HindIII site. Deletion of sequences in pPAIST7 downstream of the PAI-1 coding sequences between the HindIII and SalI sites and elimination of the SalI site was accomplished by sequential partial SalI digestion, complete HindIII digestion, blunt-ending with the Klenow fragment of *E. coli* DNA polymerase I, and ligation.

*Construction of PAI-1 Variants—*For *in vitro* site-directed mutagenesis of the reactive center, the SalI-HindIII fragment of PAI-1 (encoding amino acids Val26 through Pro39) of *E. coli* TG-1 cells was subcloned from pPAIST7 into the pUC118 phagemid. Site-directed variants were produced by mutagenesis of the SalI-SalI fragment of PAI-1 (encoding amino acids Glu8 through Asp39) cloned in pUC118. The following synthetic complementary oligodeoxynucleotides were used as mutagenic primers:

- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-M4'-S
- 5'-CGGGGGGACATTTTCTGCTGAGA 3' R3'-G4'-K
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-V
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-V
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-M4'-M, S
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-G4'-G
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-G4'-R
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-K
- 5'-CTTGGGCGCGAGGGCGCTG 3' R3'-K

Extension reactions were carried out with the Klenow fragment of DNA polymerase using double-stranded DNA as template. Mutant colonies were identified by DNA filter hybridization using radiolabeled mutant oligonucleotides. Double-stranded DNA preparations from hybridizing colonies were transformed into *E. coli* TG-1 cells and rescreened by oligonucleotide hybridization. Dideoxynucleotide chain-termination sequencing of the entire SalI-HindIII and SalI-SalI DNA fragments using the single strand form of the phagemid as template. Mutant colonies were confirmed by DNA filter hybridization and DNA sequencing. Willerson. Human thrombin and porcine pancreatic trypsin were obtained from United States Biochemical. Specific activities of all enzymes were determined by active-site titration using tritiated diisopropyl-fluorophosphate obtained from New England Nuclear.

**Inhibition of Thrombin—**Inhibition of thrombin by rPAI-1 in crude extracts of *E. coli* was studied under pseudo-first order conditions. The inhibition of uPA was measured under second order conditions with an equimolar concentration of inhibitor. The enzyme inhibitor mixtures were incubated in microtiter plate wells for 5-fold dilution by the addition of the substance mixture. The microtiter plates were then incubated at 37 °C for 10 min, and the absorbance at 405 nm was monitored over time to determine residual enzyme activity.

**Inhibition of Trypsin and Elastase—**The inhibition of trypsin and elastase was measured using crude *E. coli* extracts. The inhibition of trypsin with an equimolar concentration of α1-antitrypsin or with a 10-fold excess of rPAI-1 was performed in microtiter plate wells for 18 h before the addition of the chromogenic substrate to dilute the reaction mixture. The plates were then incubated at 37 °C, and the absorbance at 405 nm was monitored over time.

The rate of inhibition of elastase by an equimolar concentration of α1-antitrypsin or by a 10-fold excess of rPAI-1 was determined. Elastase was preincubated with each of the inhibitors in microtiter plate wells for 18 h before the addition of the substrate casein-(Ala)-p-nitroanilide to dilute the mixture 5-fold. The increase in absorbance at 405 nm was monitored over time to determine residual enzyme activity.

**Kinetic Analysis—**Residual enzyme activity for all samples was determined by comparison with a standard curve. Under pseudo-first order conditions, the half-life (t1/2) was determined, for each inhibitor concentration, from a linear semilogarithmic plot of E0, E(t) versus time. t1/2 was then calculated by dividing the apparent rate constant (kapp = ln 2/t1/2) by the inhibitor concentration. Under second order
TABLE I

| Enzyme(s) | Buffer | [rPAI-1] | Time | Substrate(s) |
|-----------|--------|---------|------|--------------|
| uPA, 30 pm | PA, TTE | 30 pm | 1-21 min | Plasminogen, 0.15 μM |
| tPA, 60 pm | PA, TTE | 0.6 nm | 1-21 min | Spectrozyme PL, 0.3 mM |
| Thrombin, 3 nm | TTE | 60 nm | 1-5 h | Spectrozyme PL, 0.15 μM |
| Trypsin, 13 nm | TTE | 130 m | 18 h | Spectrozyme TTH, 0.35 mM |
| Elastase, 27 nm | TTE | 270 m | 18 h | Spectrozyme TRY, 0.38 mM |

* All preincubations of enzyme and inhibitor were performed for the indicated time at 23 °C.

0.1 M Tris-HCl buffer, pH 7.4, 0.1% Tween 20, 0.1 mM EDTA.

0.05 M Tris-HCl buffer, pH 7.4, 0.05% Tween 20, 0.05 mM EDTA, 0.038 M NaCl.

conditions in which equimolar active concentrations of enzyme and inhibitor were used, k, was determined as the slope of a linear plot of k/[I − E] versus time (Beatty et al., 1980).

Preparation of Anti-peptide Antibodies—The synthetic peptides CS SSTAVISARMAPEEIMD, containing the reactive center sequence, and CVQRDILKVQGF, corresponding to the second site sequence, were kindly prepared by Glenn Andrews of Pfizer, Inc. Peptides were coupled to keyhold limpet hemocyanin (KLH, Behring Diagnostics) using m-maleimidobenzoyl N-hydroxysuccinimide ester according to published methods (Russell et al., 1984). Derivatized KLH (10 mg) was reacted with 5 mg of peptide for 1 h at room temperature. Free peptide was not removed by dialysis. Female New Zealand White rabbits were immunized on days 1, 4, 7, 8, 14, 155, 197, and 214 with peptide-KLH conjugate until high titer antisera were obtained. Sera from the bleedings on day 220 were used in the experiments reported here. Normal rabbit serum was used as a negative control and polyclonal serum from rabbits immunized with purified rPAI-1 protein (injected on days 1, 24, and 66; bled on day 78) was used as a positive control. IgG fractions were prepared by precipitation with 15% sodium sulfate and protein A-Sepharose affinity chromatography. IgGs were exhaustively dialyzed versus 0.1 M Tris-HCl, pH 7.4, 0.1 mM EDTA prior to use. Antipeptide antibodies reacted only with the appropriate peptide immobilized to plastic in an enzyme-linked immunosorbant assay. Both antipeptide antibodies and polyclonal anti-PAI-1 antibody reacted with pure PAI-1 protein both on Western blots and immobilized to plastic in an enzyme-linked immunosorbant assay.

Neutralization of PAI-1 Inhibitory Activity with Antibodies—Two types of antibody binding experiments to rPAI-1 were performed to assess the capacity of the antibodies to neutralize PAI-1 activity. To determine the amount of IgG required to neutralize PAI-1 activity, a dilution of wild-type rPAI-1 extract containing 3 fmol of inhibitor was preincubated for 30 min at room temperature with increasing quantities of the purified IgGs. Urokinase (3 fmol) was then added and incubation continued for 30 min to allow PAI-1 to inhibit uPA. The residual uPA activity was determined using the indirect chromogenic assay.

In subsequent experiments using a fixed concentration of immunoglobulin, 10 μg of each IgG were preincubated with various amounts of wild-type or mutant rPAI-1 extract before addition of uPA, incubation to allow inhibition of the enzyme, and determination of residual uPA activity.

RESULTS

Production and Characterization of rPAI-1 and rPAI-1 Variants—Site-directed mutants of recombinant human PAI-1 (rPAI-1) were constructed in the reactive center and in a second site in order to assess the functional significance of these two structural regions. Specifically, the P1 arginine and the P1' methionine residues (Arg46-Met144) in the reactive center of rPAI-1 were changed to Arg-Ser to resemble the thrombin inhibitor, antithrombin III, to Met-Ser to resemble the elastase inhibitor, α1-antitrypsin, to Arg-Val to resemble the substrate plasminogen, and to Lys-Met to resemble a lyserpin. In the second site, the Arg36 residue was mutated to lysine, and Lys144 was replaced by glutamic acid. The conservative mutation from Lys144 to arginine was also conserved.

Wild-type and variant PAI-1 proteins were expressed in E. coli TG-1 cells grown under tryptophan-limited conditions. Cell lysates were prepared by a cleared lysate procedure and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Coomassie Blue staining (Fig. 1C) revealed a major protein band with an apparent molecular weight of 43,000, that comigrated with a purified human rPAI-1 standard. This band was absent from lysates prepared from cultures of E. coli TG-1 cells that had not been transformed with the rPAI-1 expression vector.

The identity of this band as rPAI-1 was verified by Western blot analysis using polyclonal anti-PAI-1 antibody. As shown in Fig. 1B, a single immunoreactive band was evident in each lane that coincided with the M, 43,000 band detected on the Coomassie Blue-stained gel. No immunoreactive band was detected in the TG-1 bacterial cell lysate. Densitometric scanning of the gel shown in Fig. 1A and comparison to the sample of purified rPAI-1 of known concentration indicated that the cell lysates contained 0.2–0.5 mg/ml rPAI-1. This suggests that rPAI-1 represents 5–10% of the total extractable protein in the cell lysates. The crude lysates were adjusted to contain equivalent amounts of rPAI-1 before analysis of their inhibitory activity.

Inhibition of uPA by Wild-type and Mutant rPAI-1—Comparison of the inhibitory functions of wild-type and mutant rPAI-1 required an accurate quantitation of the concentration of active rPAI-1 in each lysate sample. This was accomplished by preincubating increasing quantities of the rPAI-1 extracts with uPA at 23 °C for 45 min. As a control, an equivalent amount of non-PAI-1 producing E. coli TG-1 lysate was preincubated with uPA to measure any inherent uPA inhibitory activity. The fraction of uPA remaining active was then determined using the indirect chromogenic assay. Non-PAI-1-producing E. coli TG-1 cell lysates lack uPA inhibitory activity, whereas lysates prepared from cells harboring either the wild-type or mutant rPAI-1 expression vectors inhibited uPA in a dose-dependent manner (Fig. 2). Under the experimental conditions used, 5 ng of total wild-type rPAI-1 resulted in a 50% inhibition of uPA activity. The reactive center mutants rPAI-1 (R46E-K), rPAI-1 (M144S), and rPAI-1 (M144V), as well as the second site mutants rPAI-1 (R50R) and rPAI-1 (K104E) appeared to be almost as active as wild-type rPAI-1 in inhibiting uPA. However, replacement of the second site aspartic acid (D121) residue with lysine resulted in a variant that appeared to be about 40-fold less active than wild-type rPAI-1. The P4' double mutant rPAI-1 (R50M,K104M) failed to inhibit uPA under the conditions used and was indistinguishable from the non-PAI-1 producing E. coli lysate.
The apparent differences in the anti-uPA activities illustrated in Fig. 2 can be attributed either to kinetic differences in the rate of interaction of the different rPAI-1 proteins with uPA, or to differences in the fraction of active rPAI-1 present in each lysate. To resolve this question, uPA was preincubated with increasing concentrations of rPAI-1 protein at 23 °C for periods up to 20 h, rather than 45 min, to allow the uPA/PAI-1 interaction to go to completion. The fraction of active uPA remaining was determined and the active concentration of rPAI-1 present in the different extracts was obtained by extrapolation to 100% inhibition on a plot of percent inhibition versus the total amount of rPAI-1 protein preincubated with uPA. From this analysis, it was calculated that only about 15% of the total wild-type rPAI-1 was present in an active form. For all other samples except rPAI-1 (R^146→K) and rPAI-1 (R^146,M^157→M,S), the fraction of active rPAI-1 present in the lysates tested was at most 2-fold less than that for wild-type rPAI-1. However, only 0.3% of the rPAI-1 (D^190→K) was present in an active form, which is about 40-fold lower than that observed for wild-type rPAI-1. The P1,P2' variant rPAI-1 (R^146,M^157→M,S) could not be titrated since no inhibition of uPA was achieved at any rPAI-1 concentration used. The apparent differences in uPA inhibitory activities illustrated in Fig. 2, therefore, reflect differences in the fraction of active PAI-1 present in each lysate rather than differences in the rates of interaction of uPA with wild-type and mutant rPAI-1. The low percentage of active inhibitor in the variant rPAI-1 (D^190→K) is likely a result of the improper folding of this protein. Preincubation of uPA with equivalent active concentrations of wild-type and mutant rPAI-1s resulted in the same extent of inhibition of uPA.

To verify this result, the kinetics of inhibition of uPA by wild-type and mutant rPAI-1 were measured under second order conditions (Table II). Consistent with the data in Fig. 2, wild-type and all mutant rPAI-1s (except rPAI-1 (R^146,M^157→M,S)) appear to be equally rapid inhibitors of uPA.
by comparison to antithrombin III (+heparin $k_l = 3$).

Inhibitory activity of PAI-1, we have compared the abilities of tPA and uPA by rPAI-1 and human endothelial cell PAI-1 protein and the glycosylated product (Table III).

abolished inhibitory activity.

The inhibition of thrombin was measured in both the presence and absence of heparin (Table IV). Wild-type rPAI-1 is only a slow inhibitor of thrombin (+heparin $k_l = 8 \pm 1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, -heparin $k_l = 1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) by comparison to antithrombin III (+heparin $k_l = 3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, -heparin $k_l = 2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$). Although the rate enhancement of antithrombin III activity was low by comparison to what is usually obtained with high quality preparations of heparin, the effect was significant and was repeatedly observed. Interestingly, the rates of inhibition of thrombin by the rPAI-1s were also significantly increased by heparin, although the magnitude of this effect was slightly less than that observed for antithrombin III. PAI-1 contains several positively charged residues (Arg$^{26}$, Lys$^{86}$, and Lys$^{86}$) that may account for binding and stimulation by heparin that are localized to helix D of the proposed heparin-binding site of antithrombin III (Huber and Carrell, 1989). Substitution of the P$_1$ arginine residue with lysine (as in rPAI-1 (R$^{46}$-K)) or methionine (as in rPAI-1 (R$^{46}$,M$^{47}$→M,S)) abolished thrombin inhibition.

Replacement of the P$_1$ serine of antithrombin III with a residue containing a bulky side chain can significantly reduce the rate of complex formation with thrombin (Stephens et al., 1988). In agreement with this observation, the rPAI-1 (M$^{47}$→V) variant showed a 10-fold lower rate of thrombin inhibition than wild-type rPAI-1. The rPAI-1 (M$^{47}$→S) variant, which contains the same reactive center pair as antithrombin III, showed a slight increase in the rate of thrombin inhibition compared to wild-type rPAI-1, although this rate was still two orders of magnitude slower than that for antithrombin III.

The rate of thrombin inhibition by the second-site variants showed that the conservative replacement of the wild-type Lys$^{86}$ residue with arginine (as in rPAI-1 (K$^{98}$→R)) had no effect on the antithrombin activity as compared with wild-type rPAI-1, whereas replacement of the same residue with glutamic acid (rPAI-1 (K$^{98}$→E)) decreased the antithrombin activity about 10-fold.

**Inhibition of Trypsin and Elastase**—The inhibition of trypsin and elastase by wild-type and mutant rPAI-1 was tested at 23°C under pseudo-first order conditions. Even when a 10-fold molar excess of inhibitor was incubated with the enzyme for periods up to 18 h, none of the rPAI-1 lysates inhibited either trypsin or elastase (data not shown). Even rPAI-1 (R$^{46}$,M$^{47}$→M,S), which contains the same reactive center pair of residues as $\alpha_1$-antitrypsin, showed no inhibitory activity.

Neutralization of rPAI-1 Inhibitory Activity with Antibodies—Polyclonal rabbit antibodies raised against purified PAI-1 completely neutralized the inhibitory activity of a crude extract of wild-type rPAI-1 (Fig. 3). Control rabbit IgG showed no neutralization of PAI-1 activity even at a concentr-

| Table II |
|---|
| **Rate constants of plasminogen activator inhibition by wild-type and variant rPAI-1 proteins** |
| Rate constants were determined at 23°C and are expressed as mean ± S.D. in units of $M^{-1} \text{s}^{-1}$.

| rPAI-1 | uPA | tPA |
|---|---|---|
| R$^{46}$→K | $6.5 \pm 1.3 \times 10^5$ | $5.3 \pm 0.8 \times 10^5$ |
| M$^{47}$→S | $8.4 \pm 0.3 \times 10^5$ | $8.9 \pm 2.4 \times 10^5$ |
| M$^{47}$→V | $6.1 \pm 1.0 \times 10^5$ | $0.0 \pm 1.5 \times 10^5$ |
| D$^{46}$→K | $4.0 \pm 1.2 \times 10^5$ | $3.3 \pm 0.9 \times 10^5$ |
| R$^{46}$→E | $8.3 \pm 0.8 \times 10^5$ | $1.0 \pm 0.4 \times 10^5$ |
| R$^{46}$→F | $7.0 \pm 0.7 \times 10^5$ | $9.3 \pm 3.3 \times 10^5$ |
| R$^{46}$,M$^{47}$→M,S | $8.0 \pm 1.4 \times 10^5$ | $1.0 \pm 0.3 \times 10^5$ |

Values could not be determined.

| Table III |
|---|
| **Rate constants of plasminogen activator inhibition by wild-type rPAI-1 and authentic PAI-1** |
| Rate constants were determined at 25°C. Values represent means ± S.D. of several experiments and are expressed as $M^{-1} \text{s}^{-1}$.

| rPAI-1 | uPA | tPA |
|---|---|---|
| Endothelial cell PAI-1 | $3.6 \pm 1.4 \times 10^5$ | $3.0 \pm 1.1 \times 10^5$ |
| rPAI-1 | $1.0 \pm 0.6 \times 10^5$ | $2.1 \pm 0.8 \times 10^5$ |

Second-order rate constant.

Pseudo-first order rate constant.

| Table IV |
|---|
| **Rate constants of thrombin inhibition by wild-type and variant rPAI-1 proteins** |
| Rate constants were determined at 25°C and are expressed as $M^{-1} \text{s}^{-1}$.

| rPAI-1 | +Heparin | −Heparin |
|---|---|---|
| R$^{46}$→K | $\leq 1 \times 10^5$ | ND |
| M$^{47}$→S | $1 \times 10^5$ | $7 \times 10^4$ |
| M$^{47}$→V | $7 \times 10^5$ | $\leq 1 \times 10^5$ |
| D$^{46}$→K | $\leq 1 \times 10^5$ | ND |
| R$^{46}$→E | $1 \times 10^5$ | $2 \times 10^4$ |
| R$^{46}$→F | $7 \times 10^5$ | $2 \times 10^4$ |
| R$^{46}$,M$^{47}$→M,S | $8.0 \times 10^5$ | $1 \times 10^5$ |
| Antithrombin III | $9 \times 10^5$ | $2 \times 10^4$ |

ND, not done.

Values could not be determined.

![Fig. 3. Titration of rPAI-1 neutralization capacity of antibodies.](image-url)
tration 10 times higher than that required for complete neutralization by polyclonal anti-PAI-1 antibody. Titration of the neutralizing capacities of the anti-peptide antibodies against wild-type rPAI-1 revealed that only the anti-reactive center peptide antibody neutralized PAI-1 activity while the anti-second-site peptide antibody behaved identically to the control. In subsequent experiments in which a fixed amount of IgG was preincubated with increasing amounts of rPAI-1 and its variants, 10 μg of IgG was used to ensure antibody excess.

Two types of results were obtained in these experiments and representative sets of data for each type are shown in panels A and B of Fig. 4. The data presented in panel A are representative of titrations of rPAI-1 proteins which possess a wild-type reactive center sequence and include the wild-type rPAI-1 and the variants rPAI-1 (K^{146}→E), rPAI-1 (K^{146}→R), and rPAI-1 (D^{162}→K). As before, the polyclonal anti-PAI-1 IgG showed complete neutralization of the inhibitory activity of the rPAI-1 extracts, whereas the control IgG showed no neutralization of these preparations. Examination of the curves generated using the anti-peptide IgGs again demonstrated that only the anti-reactive center IgG neutralized these inhibitor preparations, while antibody directed against the second-site peptide was indistinguishable from control IgG.

However, a different set of data was obtained for the variant rPAI-1 preparations rPAI-1 (R^{146}→K), rPAI-1 (M^{347}→S), and rPAI-1 (M^{347}→V) which contain mutations in the reactive center and an example is shown in Fig. 4, panel B. Although polyclonal anti-PAI-1 antibody still showed complete neutralization of rPAI-1 activity, the anti-reactive center peptide antibody no longer neutralized these variant inhibitor preparations.

FIG. 4. Neutralization of rPAI-1 inhibitor activity by antibodies. Ten μg of purified IgG was preincubated 30 min with increasing quantities of wild-type PAI-1 (panel A) or rPAI-1 (M^{347}→S) (panel B) in a volume of 20 μl. uPA (3 fmol in a volume of 10 μl) was added and incubation continued for 30 min to allow uPA inhibition. Residual uPA activity was determined using the indirect chromogenic assay. Labels are as indicated in the legend to Fig. 3.

**DISCUSSION**

In this study, the functional significance of the reactive center and a second site on the PAI-1 molecule has been assessed by both mutational analysis and anti-peptide antibody neutralization experiments. In order to minimize the possibility of improper folding and aberrant tertiary structure, we have used substitution mutants instead of deletion mutants. A high level prokaryotic expression system was used for rPAI-1 protein production (Franke et al., 1990). Under optimal conditions about 1.0–2.5 mg of rPAI-1, constituting 5–10% of total extractable protein, were obtained per 50 ml of bacterial culture. The recombinant PAI-1 produced in the prokaryotic expression system was recognized by human endothelial cell PAI-1 antiserum in Western blot analysis and its rate constants of inhibition for both tPA and uPA were similar to those observed for authentic, human endothelial cell PAI-1. Both these results and those described previously (Franke et al., 1990) suggest that glycosylation of PAI-1 is unnecessary for inhibitory activity. As demonstrated previously for PAI-1 made by endothelial cells (Hekman and Loskutoff, 1985), PAI-1 produced in E. coli exists in two forms, one inherently active and a latent form that can be reactivated by denaturation and renaturation. By the criteria of activity, stability, and specificity, guanidine-reactivated PAI-1 is indistinguishable from the native, active form of the protein (Hekman and Loskutoff, 1988a). The latent form of rPAI-1 can also be reactivated with guanidine hydrochloride.2 The expression of PAI-1 in bacteria therefore seems to be a suitable method for the production of authentic PAI-1 activity.

Our data on the inhibition of a panel of serine proteases confirm that PAI-1 is an extremely specific inhibitor of plasminogen activators. The rate constants for the interaction between wild-type rPAI-1 and either tPA or uPA (10^{-6}–10^{-7} M^{-1} s^{-1}) are several orders of magnitude faster than that observed for the inhibition of thrombin (8.0 × 10^{-7} M^{-1} s^{-1}). Additionally, neither wild-type rPAI-1 nor any of its variants inhibited trypsin or elastase in our assays, even when a large excess of inhibitor and long incubation times were used. Our results do not agree with previously published studies in which purified, guanidine-activated bovine PAI-1 was shown to inhibit trypsin in a dose- and time-dependent manner with a second order rate constant of 1.0 × 10^{8} M^{-1} s^{-1} at 37°C (Hekman and Loskutoff, 1988b). The reasons for this discrepancy are unclear but may reflect sequence differences between the human and bovine PAI-1 proteins, our use of crude rPAI-1 preparations, or differences in the incubation temperature (23 versus 37°C).

One feature of P1 specificity in several different families of serine protease inhibitors is that replacement of lysine for arginine at the P1 position usually maintains inhibitor strength and specificity. Our mutagenesis experiments are consistent with this observation and also suggest a requirement for a basic amino acid at the P1 position of the reactive center of PAI-1 for effective inhibition of plasminogen activators. By contrast, replacement of the P1 arginine by lysine in rPAI-1 (R^{146}→K) or by histidine in antithrombin III Glasgow (Owen et al., 1988) results in a complete loss of inhibitory activity toward thrombin and suggests an absolute require-
ment for arginine at the P1 position. Examination of the three-dimensional structure of α-thrombin cocrystallized with a synthetic inhibitor shows that this stringency is due to the insertion of 9 residues into a surface loop of thrombin which forms part of the recognition site on the enzyme for the P1 residue and effectively limits both substrate and inhibitor specificity (Bode et al., 1989). By alignment of serine proteases of the chymotrypsin family, the corresponding loop in both tPA and uPA is relatively short and may account for proteolytic cleavage after both arginine and lysine residues.

It has been shown that the residue in the P1' position in most inhibitor families is less constrained; virtually any residue except proline is tolerated (Laskowski and Kato, 1980). Mutagenesis of the P1' methionine residue in the reactive center of PAI-1 to valine or serine did not affect the rate of interaction with uPA or tPA, which suggests both the tolerance of other residues and the lack of an absolute requirement for Met in the P1' position for PAI-1 to effectively inhibit plasminogen activators. A P1' methionine is also not required for the inhibition of plasmin by α1-antiplasmin (Holmes et al., 1987), and oxidation of methionine residues with N-chlorosuccinimide has no effect on the association rate of the inhibitor with a variety of serine proteases (Shieh and Travis, 1987). A P1' serine is not required for the inhibition of thrombin, although extensive mutagenesis of the P1' residue of antithrombin III has shown that there are apparently both a size optimum and hydrophobicity effects of the side chain on the rate of interaction (Stephens et al., 1988). Our results on the inhibition of thrombin by rPAI-1 and its P1' mutants also support this conclusion.

Previous results have demonstrated that even a single amino acid change in the P1 site of the reactive center of α1-antitrypsin (Owen et al., 1985; Jallat et al., 1986; Heeb et al., 1990), α2-antiplasmin (Holmes et al., 1987), or α1-antichymotrypsin (Rubin et al., 1990) can dramatically increase the rate of inhibition of other, non-cognate serine proteases by the variant serpins. Even though variants such as rPAI-1 (R46,M14+M,S) and rPAI-1 (M4+4+S) contain the same reactive center P1-P1' residues as α1-antitrypsin and antithrombin III, respectively, contrary to our prediction there was little if any increase in either the rate or specificity of inhibition of trypsin, elastase, or thrombin by these variant inhibitors. We suggest that this result is another aspect of the rigid specificity of PAI-1 for plasminogen activators. It seems that PAI-1 lacks structural characteristics aside from the reactive center P1-P1' residues that are essential for the efficient inhibition of other serine proteases. Other results have demonstrated a functional role for the P4' and P5' residues of PAI-1 in the interaction with tPA, and it is possible that residues at these positions are at least partly responsible for the strength and specificity of serine protease inhibition.

The results of anti-peptide antibody neutralization of PAI-1 inhibitory activity suggest: (i) antibody directed against the reactive center of PAI-1 can sterically block the access of this region to the active site of plasminogen activator; (ii) antibody binding to the second site located some distance from the reactive center does not block the inhibitory function of PAI-1; (iii) antibody directed against the wild-type sequence in the reactive center will not neutralize the inhibitory activity of a reactive-center variant, indicating that this anti-peptide antibody is highly specific for the wild-type reactive center sequence. Interestingly, only half of the rPAI-1 activity was usually neutralized by the anti-reactive center antibody, although this may simply reflect a low affinity of the anti-peptide antibody for the native protein.

Polyclonal antibody inhibition of PAI-1 activity is complete, even on reactive center variants of rPAI-1. One possibility is that the polyclonal antibody can still recognize and neutralize the reactive-center variant molecules due to high affinity binding. Alternatively, other epitopes on PAI-1 which are recognized and bound by the polyclonal antibody could account for the more effective neutralization of activity. The nature of these epitopes is currently unknown, although experiments are underway to test these alternatives.

Acknowledgments.—We would like to thank the Pfizer group of Glenn Andrews for peptide synthesis and Keanan Geoghegan, Ed Lee, and the Pfizer Protein Facility for monoclonal antibodies. James Willerson provided us with tPA, and Robert Maidell and Joe Sambrook were kind enough to critically read the manuscript.

REFERENCES

Beatty, K., Bieth, J., and Travis, J. (1980) J. Biol. Chem. 255, 3931-3934

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467-3475

Carrell, R., and Travis, J. (1985) Trends Biochem. Sci. 10, 20-24

Carrell, R. W., Pemberton, P. A., and Boswell, D. R. (1987) Cold Spring Harbor Symp. Quant. Biol. 52, 527-535

Fueske, A. C., Dowley, D. S., Kazanietz, M. G., Howes, J. S., Gerard, B. D., Lee, S. E., and Geoghegan, K. F. (1990) Biochem. Biophys. Acta 1037, 16-23

Gerard, R. D., and Meidell, R. S. (1989) Annu. Rev. Physiol. 51, 245-262

Heet, M. J., Bischof, R., Courtney, M., and Griffin, J. H. (1990) J. Biol. Chem. 265, 2365-2369

Hekman, C. M., and Loskutoff, D. J. (1985) J. Biol. Chem. 260, 11581-11587

Hekman, C. M., and Loskutoff, D. J. (1988a) Arch. Biochem. Biophys. 262, 199-200

Hekman, C. M., and Loskutoff, D. J. (1988b) Biochemistry 27, 2911-2918

Holmes, W. E., Lijnen, H. R., and Collen, D. (1987) Biochemistry 26, 5133-5140

Horton, G. L., Tollefsen, D. M., and Fok, K. F. (1988) J. Cell. Biochem. Suppl. 12B, 278

Huber, R., and Carrell, R. W. (1989) Biochemistry 28, 8951-8966

Jallat, S., Carvalho, D., Teisser, L. H., Roekhlui, D., Boitsch, C., Ogasaki, F., Crystal, R. G., and Courtney, M. (1980) Protein Eng. 1, 29-35

Johansen, M., Philips, M., Chauvin, S., and Phillips, J. (1987) Thromb. Haemostasis 58, 872-878

Laskowski, M., and Kato, I. (1989) Annu. Rev. Biochem. 58, 895-926

Loebenberg, H., Tokewski, R., Dethofber, J., and Huber, R. (1984) J. Mol. Biol. 177, 531-556

Madams, F., Goldsmith, E. J., Gerard, R. D., Gethin, M. J. H., and Sambrook, J. F. (1989) Nature 339, 721-725

Madison, E., Goldsmith, E. J., Gerard, R. D., Gethin, M. J. H., and Sambrook, J. F. (1989) Nature 339, 721-725

Nyu T., Sadowski, M., Lawrence, D., Millan, J. L., and Loskutoff, D. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6776-6780

Owen, M. C., Brennan, S. O., Lewis, J. H., and Carrell, R. W. (1983) New. Engl. J. Med. 309, 945-958

Owen, M. C., Beresford, C. H., and Carrell, R. W. (1988) FEBS Lett. 231, 317-320

Pinnock, H., Veerman, H., Lambers, H., Diepgaard, P., Verweij, C. L., van Zonneveld, A. J., and van Mournik, J. A. (1986) EMBO J. 5, 2539-2544

Peer, F. Y., Munford, R. S., Campbell, W. B., Reich, J. S., Chien, K. R., and Gerard, R. D. (1990) J. Immunol. 144, 3506-3512

Rubin, H., Wang Z. Nicklay, P., R. Mclauryn, S. Naidoo, N., Schoeneberg, O. L., Johnson, J. L., and Cooperman, S. (1990) J. Biol. Chem. 265, 1199-1206

Russell, D. W., Schneider, W. J., Yamamoto, T., Laskey, K. L., Brown, M. S., and Goldstein, J. L. (1984) Cell 37, 577-585

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Press, New York

Sasaki, T., Morita, T., and Iwanaga, S. (1986) J. Biochem. (Tokyo) 99, 1699-1705

Shieh, B. H., and Travis, J. (1987) J. Biol. Chem. 262, 6055-6059

Sprengers, E. D., and Kluft, C. (1987) Blood 69, 381-387

van Mourik, J. A., and van Zonneveld, A.-J., and van Mourik, J. A. (1986) EMBO J. 5, 2539-2544

Wright, H. R., and Carrell, R. W. (1989) Biochemistry 28, 8951-8966

Ye, R. D., Won, T.-C., and Sadler, J. E. (1987) J. Biol. Chem. 262, 3718-3725

Zoller, M., and Smith, M. (1985) DNA 3, 479-488
Mutational and immunochemical analysis of plasminogen activator inhibitor 1.
H E Shubeita, T L Cottey, A E Franke and R D Gerard

J. Biol. Chem. 1990, 265:18379-18385.

Access the most updated version of this article at http://www.jbc.org/content/265/30/18379

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/30/18379.full.html#ref-list-1