Saturation Mutagenesis of the Plasminogen Activator Inhibitor-1 Reactive Center*

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Plasminogen activator inhibitor-1 (PAI-1) is a specific inhibitor of the serine proteases tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). To systematically investigate the roles of the reactive center P1 and P1' residues in PAI-1 function, saturation mutagenesis was utilized to construct a library of PAI-1 variants. Examination of 177 unique recombinant proteins indicated that a basic residue was required at P1 for significant inhibitory activity toward uPA, whereas all substitutions except proline were tolerated at P1'. P1-Lys variants exhibited lower inhibition rate constants and greater sensitivity to P1' substitutions than P1-Arg variants. Alterations at either P1 or P1' generally had a larger effect on the inhibition of tPA. A number of variants that were relatively specific for either uPA or tPA were identified. P1-Lys-P1'Ala reacted 40-fold more rapidly with uPA than tPA, whereas P1-Lys-P1'Trp showed a 6.5-fold preference for tPA. P1-P1' variants containing additional mutations near the reactive center demonstrated only minor changes in activity, suggesting that specific amino acids in this region do not contribute significantly to PAI-1 function. These findings have important implications for the role of reactive center residues in determining serine protease inhibitor (serpin) function and target specificity.

Tissue-type plasminogen activator (tPA)† and urokinase-type plasminogen activator (uPA) are serine proteases that convert the zymogen plasminogen to the active protease plasmin (1). In addition to its critical importance in fibrinolysis (2), the regulation of plasminogen activation is considered essential for a variety of other biological processes (1). Although four plasminogen activator inhibitors have been identified, plasminogen activator inhibitor-1 (PAI-1) is the major physiological inhibitor of tPA and uPA in plasma (3).

PAI-1 is a single-chain glycoprotein consisting of 402 amino acids, including a 23-residue signal peptide at its NH2 terminus. PAI-1 is a member of the serine protease inhibitor (serpin) superfamily (4–6), a group of inhibitor and noninhibitor proteins believed to have similar tertiary structures (7–9). The reactive center of inhibitory serpins has been localized to an exposed loop of amino acids ("strained loop") (10) that may resemble a structure within the natural substrate of the serpin's target protease. Serpins are thought to inhibit their target proteases via a common mechanism involving formation of a Michaelis complex, nucleophilic attack by the protease on the serpin's P1-P1' reactive center peptide bond, and generation of an equimolar sodium dodecyl sulfate (SDS)-stable complex (8). However, the precise nature of this enzyme-inhibitor complex is not clear (8, 11).

Several studies have investigated the contributions of the reactive center region to serpin activity and target specificity. A critical role for the P1 residue in inhibitor function has been demonstrated for a number of family members (12–17). Similarly, amino acid substitutions at P1 (18), P3 (19), and P1' (20, 21) have been shown to affect the activity or specificity of a variety of serpins. Analyses of several PAI-1 reactive center region variants have also been reported. A large set of P1, P3, and P1' mutants (22), and a PAI-1 variant with residues P1 through P1' replaced by P1-P1' of antithrombin III (ATIII) (23), demonstrate the relative importance of these reactive center residues for inhibitor activity and specificity. However, studies of specific PAI-1 mutants with changes at P1 and P1' (24, 25), and of variants with substitutions at residues P3 through P1' (26), suggest that the strained loop residues other than P1 may play only a minor role in PAI-1 function.

In order to systematically examine the roles of the P1 and P1' residues in PAI-1 function, saturation mutagenesis was employed to construct a library of PAI-1 mutants containing all 400 combinations of amino acids at these two positions. Analyses of 177 unique mutant proteins indicate that the P1 and P1' positions contribute to the interaction of PAI-1 with its target proteases.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Wild-type PAI-1 in Escherichia coli—The coding sequence for mature wild-type human PAI-1 protein was subcloned into the expression vector pET3a (gift of F. W. Studier) (27) by standard methods (28). The amino-terminal Val of mature PAI-1 is designated amino acid +1, and the adenine of the PAI-1 mRNA initiation codon is designated nucleotide +1 (4). The sequence from nucleotide +70 (amino acid +1) to the NcoI site at nucleotide +322 was derived by the polymerase chain reaction.
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(PCR) (29) using oligonucleotides A and B as primers (Table I). This fragment contained a synthetic NdeI site at the 5′ end, introduced by PCR, which provided the start codon for PAI-1 expression in E. coli. The remaining sequence, from the NcoI site at nucleotide +323 to the BglII site at +1352, was isolated from the PAI-1 cDNA clone PAI 6.3A (4). These fragments were recloned into the NdeI and BamHI sites of pET3a. Dideoxynucleotide sequence analysis (30) was performed through the entire PAI-1 coding sequence to exclude PCR errors or cloning artifacts.

PAI-1 expression was induced by the addition of 0.4 mM isopropyl-

thio-β-D-galactoside (IPTG) to log-phase BL21(DE3) (27) trans-
grown for 2 h and harvested by centrifugation, and PAI-1 was purified as described previously (31) with modifications (26). Protein content was quantitated by Bradford assay (Bio-Rad), and NH2-terminal sequence analysis was performed in a model 473 Pulsed-
Liquid Phase Peptide Sequencer (Applied Biosystems) using standard operating conditions.

Preparation of Affinity-purified Rabbit Anti-human PAI-1 Polyclonal Antibody—New Zealand White rabbits were immunized with purified recombinant PAI-1 by standard methods (32). IgG from the rabbit with the highest titer of anti-PAI-1 antibody was isolated by the caprylic acid method, ammonium sulfate-precipitated, and purified by immunoaffinity chromatography (32) on a recombinant PAI-1-Sepharose column. A portion of the affinity-purified anti-PAI-1 antibody was biotinylated with N-hydroxysuccinimide biotin according to manufacturer’s instructions (Boehringer Mannheim).

Construction of Library of PAI-1 Reactive Center Mutants—The coding sequence for mature wild-type human PAI-1 (P,Arg,P,Met) was subcloned into the EcoRI and BamHI sites of M13mp18, in-frame with the β-galactosidase α-peptide sequence, as described above for pET3a except that PCR primer C (Table I) was used to create an in-frame EcoRI site at the 5′ end. The resulting construct was termed M13PAI-1. DNA sequence analysis confirmed wild-type PAI-1 coding sequence (30).

Site-directed mutagenesis was performed on single-stranded M13PA1-1 template using the Mutagene M13 in vitro Mutagenesis kit (Bio-Rad). Oligonucleotide D (Table I) was used to prepare the library of random P, P, P-PAI-1 variants. Oligonucleotides E, F, G, H, I, J, K, and L (Table I) were used to create the variants P,Arg,P,Cys, P,Arg,P,His, P,Arg,P,Phe, P,Arg,P, Thr, P,Lys,P,Met, P,Lys,P,Leu, P,Lys,P,Phe, and P,Thr,P,Met, respectively. Electroporation (Gene Pulser, Bio-Rad) was utilized to transform host strain XL1-Blue (Stratagene) with the DNA heteroduplexes.

Screening of PAI-1 Variant Library by Oligonucleotide Hybridization—Initial analysis of random phage in the mutant M13PA1-1 library revealed that approximately half contained wild-type PAI-1 sequence. To identify these background sequences, filter plaque lifts were hybridized with a32P-end-labeled probe M (Table I) by standard methods (28), followed by a final wash at 70°C for 10 min in 5 × SSPE (750 mM NaCl, 50 mM Na2HPO4, 5 mM EDTA, pH 7.4) containing 0.1% SDS. Mutant PAI-1 phage, identified by failure to hybridize with the wild-type probe M, were selected and subjected to an additional round of plaque purification prior to further analyses.

Specific P, P, P, P, P, and O, P, Lys, variants, and oligonucleotide P (Table I) were used to select for P,Arg, P, and Lys variants, and oligonucleotide P (Table I) was used to select for P,Arg,P,Met mutants. Conditions were identical to those above, except the final washes were at 64°C for oligonucleotides N and O and 63°C for oligonucleotide P.

Expression and Sequence Analysis of PAI-1 Mutants—Twenty-five milliliter of a 1:100 dilution of overnight XL1-Blue culture was inoculated with mutant M13PA1-1 phage and grown at 37°C for 3 h. Recombinant PAI-1 expression was induced by the addition of 0.4 mM IPTG to 2.5 mM, and the bacterial cells were harvested 2 h later by centrifugation at 2200 × g for 10 min at 4°C. The supernatants from this step were saved for isolation of single-stranded M13 DNA (see below). Bacterial pellets were resuspended in 0.4 ml of lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.2 mM phenylmethyl-

sulfonyl fluoride), lysozyme was added to 300 μg/ml, and the cells were kept on ice for 30 min with periodic vortex mixing. DNase I was added to 10 μg/ml, and the suspension was incubated for 1 additional h at 25°C. Cell debris was removed by centrifugation at 16,000 × g for 5 min, and the resulting crude lysates were used for all subsequent analyses.

Isolation of single-stranded phage DNA from the culture super-
natants was by standard methods (28). This DNA was used as template for dideoxynucleotide sequence analysis (30) of nucleotides +1055 to +1140 using oligonucleotide Q as primer (Table I).

Analysis of Recombinant M13PAI-1 Protein—A specific PAI-1 enzyme-linked immunosorbent assay (ELISA) was developed to quantitate PAI-1 protein in the crude lysates. Immulon 2 (Dynatech) microtiter plates were coated with affinity-purified rabbit anti-human PAI-1 antibody at 750 ng/ml. Following blocking with 3% powdered milk (Carnation), the plates were incubated with serial dilutions of purified recombinant PAI-1 and crude M13PA1-1 lysates diluted in filtered ELISA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.01% polyoxyethyleneorbitan monooleate (Tween 80), 0.1% powdered milk). The plates were then incubated sequentially with biotinylated affinity-purified anti-PAI-1 antisera and horseradish peroxidase-conjugated streptavidin (Boehringer Mannheim). Ortho-phenylenedi-

amine (Sigma) was used as a substrate, and plates were read at 492 nm with an MR650 spectrophotometer (Dynatech). The concentra-
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tion of PAI-1 in the crude lysates was determined from a standard curve.
curve constructed using purified recombinant PAI-1. Dilution of recombinant PAI-1 in crude lysate or in buffer resulted in identical standard curves.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels under reducing conditions by the method of Laemmli (33). Western blotting was performed using a semi-dry Sartoblot apparatus (Sartorius), anti-human PAI-1 antibody, alkaline phosphatase-conjugated strept-avidin (GIBCO-Bethesda Research Laboratories), and 3-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Promega) as a substrate (28). Reverse fibrin autography was performed as described previously (34). Detection of PAI-1/uPA complex and cleaved PAI-1 was achieved by incubating purified PAI-1 or PAI-1 in crude lysate with an equimolar amount of high molecular weight uPA for 30 min at 25 °C, followed by nonreducing SDS-PAGE and Western blotting. Assay for PAI-1 Activity—The variant PAI-1 proteins were screened for their ability to inhibit uPA in a direct chromogenic assay using the synthetic substrate S-2444 (KabiVitrum) as described previously (31). Briefly, 50 μl of crude lysate serially diluted in assay buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, 0.01% Tween 80, 100 μg/ml bovine serum albumin), or 50 μl of activity assay buffer alone, was incubated with 50 μl high molecular weight uPA (25 IU/ml activity concentration; American Diagnostica) for 90 min at 37 °C in microtiter plates (Falcon Microtest III). After the addition of 100 μl of 0.5 mM S-2444 to each well, the absorbance at 410 nm was recorded at 15-min intervals with an MR650 spectrophotometer (Dynatech). PAI-1 activity was quantitated from the amount of residual uPA activity.

Kinetic Analyses—Recombinant two-chain high molecular weight uPA (Abbott Laboratories) was active site-titrated with fluorescein mono-p-guanidinobenzoate hydrochloride (35). Recombinant single-chain tPA (Genentech) was dialyzed overnight against 1 M HEPES, 0.5 mM NaCl, 1 mM EDTA, 0.02% Tween 80, pH 4.0, and converted to the two-chain form by incubation with plasmin-Sepharose gel as described (36). The concentration of active tPA was determined by titration with purified recombinant PAI-1 which had been standardized against uPA. tPA was not titrated directly since a reliable titrant for determining microgram quantities of tPA has not been reported. The concentration of active PAI-1 protein in the lysates and purified recombinant PAI-1 preparation was determined by titration with uPA. Briefly, varying amounts of PAI-1 were incubated with 10 or 50 nm uPA and 1 mg/ml bovine serum albumin in a volume of 100 μl for times sufficient to allow for complete enzyme inhibition based on measured rate constants. Samples were diluted with 1 ml of 50 μM fluorescent substrate solution (see below), and the residual enzymatic activity was measured from the linear rate of substrate hydrolysis as detailed below for the kinetic experiments. The amount of enzyme used (in pmol) divided by the volume of PAI-1 required to completely inhibit the enzyme yielded the concentration of active PAI-1 in each sample.

Rate constants for PAI-1 inhibition of uPA or tPA were determined by the method of Tian and Tsou (37). The fluorogenic substrates Glu-Gly-Arg-7-amido-4-methyl-coumarin (Bachem Bioscience) and tosyl-Gly-Pro-Arg-7-amido-4-methyl-coumarin (Sigma) were used for uPA and tPA, respectively. The second-order rate constants (k) of the most active mutants (k > 2 × 10^4 M⁻¹ s⁻¹) were measured with the following continuous assay under pseudo first-order conditions. Enzyme, PAI-1, and substrate in a total volume of 1.1 ml of assay buffer (0.1 M HEPES, 0.1 mM NaCl, 1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4) were added to an acrylic cuvette, coated with polyethylene glycol 20,000 to minimize protein absorption (38), at 22 °C. Enzyme concentrations were typically 0.5 mM, PAI-1 concentrations were usually 10 times that of the enzyme, and the substrate concentration was 50 μM. Mutants with rate constants >3.5 × 10^4 M⁻¹ s⁻¹ required PAI-1/tPA ratios of 5:1 or 4:1 to achieve measurable reactions. The fluorescence intensity versus time (<1% substrate consumption) was recorded with either SLM 8000 or Perkin-Elmer 650-10s fluorescence spectrophotometers with excitation at 380 nm and emission at 440 nm. Using the nonlinear regression program DNRPS5 (39), data were fitted to the equation, \( F_t = A (1 - e^{-kt}) + Ct + D \), where \( F_t \) is the observed fluorescence at time \( t \), and \( A, B, C, \) and \( D \) are fitted parameters. \( A \) is the amplitude of the exponential term, \( B \) is the observed pseudo first-order rate constant (kobs) for the exponential, \( C \) is the rate of background substrate hydrolysis, and \( D \) is the initial fluorescence at \( t = 0 \). The background substrate hydrolysis rates were negligible with purified PAI-1, but were observable in lysates, possibly due to endogenous proteinases. kobs was divided by the PAI-1 concentration to yield k. The rate constants of most mutants were measured in triplicate at each of three PAI-1 concentrations. For some rapid-reaction inhibitors, only one or two PAI-1 concentrations were used in the reaction with tPA, with more than three replicates performed at these concentrations. kobs was proportional to the PAI-1 concentration in all cases. k values obtained for uPA reactions were corrected for the competitive effect of the substrate by multiplying by the factor 1.6, calculated from the expression, \( 1 + [S]/K_m \), using the measured \( K_m \) of 81 μM. No such corrections of k were necessary for the tPA reactions, since the substrate concentrations used were well below \( K_m \). Standard errors were ±10% for all PAI-1 proteins except wild-type and those mutants which yielded rate constants >3.5 × 10^4 M⁻¹ s⁻¹ with tPA. In these cases, errors were as high as ±50% due to the low signal amplitude. Less active mutants (k < 2 × 10^4 M⁻¹ s⁻¹) were measured with a discontinuous assay. PAI-1, enzyme, and 1 mg/ml bovine serum albumin were incubated in a 100-μl volume for varying times (up to ~6 h) and then diluted with 1 ml of 50 μM substrate in assay buffer for measurement of residual enzyme activity. Linear substrate hydrolysis rates (corrected for background hydrolysis) were plotted versus incubation time on semi-log paper. The slope of this curve divided by the PAI-1 concentration yielded k. A 10-fold molar excess of PAI-1 over enzyme was used. The slowest detectable rate constant was 10^4 M⁻¹ s⁻¹. Under these conditions, no more than 30% of the wild-type inhibitor was converted to the latent form.

RESULTS

Expression and Purification of Recombinant Wild-type PAI-1—Purification of recombinant PAI-1 protein produced using the pET3a expression vector (27) yielded up to 20 mg of PAI-1/liter of E. coli culture. Analysis of the final preparation by SDS-PAGE showed that the PAI-1 was >99% homogeneous, since potential contaminants represented less than 0.1 μg/10 μg total protein (Fig. 1). Protein sequence analysis identified only the NH₂ terminus of mature PAI-1 (Val-His-His-Pro), indicating that the initiator Met had been efficiently removed (>90%). Since PAI-1 can exist in an active or a latent conformation (40, 41), titration of the purified PAI-1 with uPA was performed. Results showed that as much as 90% of the PAI-1 was in the active form (data not shown).

Expression of PAI-1 Protein in M13PAI-1—Upon induction by IPTG, M13PAI-1 should produce PAI-1 protein with seven vector-derived amino acids (Met-Thr-Met-Ile-Thr-Asn-Ser) fused to the NH₂ terminus. SDS-PAGE analysis of M13PAI-1 lysates identified the expected band of 43 kDa that was absent from control lysates (Fig. 2). Western blot analysis and reverse fibrin autography confirmed that this band contained functionally active PAI-1 (data not shown). One ml of M13PAI-1-infected E. coli culture produced approximately 1.2 μg of PAI-1 antigen as measured by ELISA, whereas

**FIG. 1.** Analysis of purified recombinant PAI-1. Recombinant PAI-1 was purified and the final product analyzed by SDS-PAGE. Gel was stained with Coomassie Blue. The total amount of protein in each lane and the migration of molecular mass standards are indicated.
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Saturation Mutagenesis of PAI-1 and PAI-1′—A library of PAI-1 mutants containing substitutions at the P1 and P1′ positions was constructed by site-directed saturation mutagenesis using oligonucleotide D (Table I). In this 56-mer, each of the four nucleotides was randomly incorporated at the middle six positions, corresponding to the codons for P1Arg and P1′Met. The result is a population of oligonucleotides complementary to the PAI-1 sequence flanking the reactive center but containing all possible combinations of codons at P1 and P1′. Sequence analysis of the reactive centers of nine mutant clones is shown in Fig. 3. All but three of these changes were single-base substitutions, of which 82% were G → A transitions. The aberrant oligonucleotide mutations occurred at an average frequency of 0.7%/nucleotide, but appeared most commonly at nucleotides +1103, +1114, and +1115 (at frequencies of 7, 3, and 3%/nucleotide, respectively).

Screening of Mutant Library for PAI-1 Functional Activity—Crude lysates containing mutant PAI-1 proteins were screened for functional activity with uPA in a direct chromogenic assay using the substrate S-2444 (31). Analysis of independent duplicate clones for 48 representative variants consistently yielded identical results. According to the second-order rate equation (42), mutant inhibitors with rate constants \( k_r \) should have been detected in this assay. 37 of the 177 unique mutants screened were identified as active (Fig. 4), and each contained either Arg or Lys at P1. P1Arg; P1Pro and P1Lys; P1′Pro were the only two P1Arg or P1Lys variants that were inactive in this assay. However, kinetic analyses (discussed below) indicated that P1Arg; P1′Pro had slight inhibitory activity. The remaining 138 unique PAI-1 mutants showed no detectable activity (Fig. 4). Variants marked by letters a–m (Fig. 4) contained an additional substitution(s) outside the P1–P1′ residues as a consequence of the mutagenesis procedure. All but three of these changes (i, l, m) also occurred in active mutants (Fig. 5), suggesting that these secondary alterations outside the reactive center were not responsible for the activity losses of the inactive mutants. However, an effect of mutations i, l, and m on PAI-1 activity cannot be excluded. All potential amino acid substitutions at P1 and P1′ were calculated for 204 randomly selected variants. The observed frequencies correlate well with the expected values at both positions, although Arg and Lys are somewhat over-represented, and Leu and the aromatic amino acids are somewhat under-represented. Examination of the nucleotide content of the six targeted positions in the coding sequence revealed that adenine, thymine, cytosine, and guanine comprised 41, 11, 25, and 23% of the mutations, respectively.

Additional Mutations Introduced by the Synthetic Mutagenesis Oligonucleotide—Surprisingly, 27% of the sequenced variants contained at least one additional mutation outside the two targeted codons. All but one of these mutations were found within the sequence covered by the mutagenesis oligonucleotide. However, since only nucleotides +1035 to +1140 were sequenced, additional substitutions at a greater distance cannot be excluded. No extra alterations were identified in the 108 wild-type phage that were sequenced. Although single and multiple-base deletions and insertions were observed, 77% of the additional mutations were single-base substitutions, of which 82% were G → A transitions. The aberrant oligonucleotide mutations occurred at an average frequency of 0.7%/nucleotide, but appeared most commonly at nucleotides +1103, +1114, and +1115 (at frequencies of 7, 3, and 3%/nucleotide, respectively).

Inhibition of uPA by PAI-1 mutants. Wild-type PAI-1 and PAI-1′ mutants were screened for their ability to inhibit uPA by a chromogenic assay. + and − indicate active and inactive, respectively. Mutants marked a–m contained the following additional mutations: a, P2Ala → Val; b, P1′/Pro → Ser; c, P2Ala → Val; d, P1′Glu → Val; e, P′Pro → Leu; f, P1′/Pro → Leu; g, P2Ala → Val; h, P′/Pro → Leu; i, P2Ile → Leu and P1′Glu → Val; j, P2Ala → Val; k, P′/Pro → Ser; l, P2Ala → Val and P1′/Ser → Val; and m, P′ and P′ deletions. All but three of these changes (i, l, m) also occurred in active mutants with no apparent loss of functional activity (Fig. 5).
acid substitutions were represented at the P, and P' positions at least once in the screened variants, and all lysates contained similar levels of recombinant PAI-1 as measured by the ELISA. Titration of the active mutant proteins with uPA indicated that 20–50% of each mutant was in the active conformation.

**Kinetic Analyses of PAI-1 Mutants**—The second-order rate constants (k) for the interactions of wild-type PAI-1 or selected PAI-1 mutants with uPA and two-chain tPA were determined (Table II). Control lysates prepared from M13-infected E. coli showed no inhibition of either uPA or tPA. Wild-type M13PAI-1 protein in crude lysate and purified recombinant wild-type PAI-1 yielded similar k, values with both plasminogen activators (PAs), indicating that neither the additional amino-terminal fusion of M13PAI-1 nor the other components of the crude bacterial lysate significantly affected PAI-1 functional activity. Consequently, all subsequent analyses of the mutant PAI-1 proteins were performed using crude lysates.

When Arg was at the P, position, all P', substitutions except Pro resulted in mutants having detectable rate constants with both PAs (Table II). Although there was little change from the wild-type k, values when P, Met was replaced by many of the amino acids, several P', substitutions resulted in significant k, reductions, most notably P, Arg-P', Glu (24-fold decrease in k, with uPA and 63-fold decrease with tPA). Although substitution of an acidic residue (Asp, Glu) at P', with both uPA and tPA, the presence of a basic residue (Arg, Lys) at P', had small effects on the k, values of both PAs (Table II). The PIArg-P', Pro mutant, which appeared inactive in the screening assay, had strikingly reduced k, values of 4.4 x 10^5 M^-1 s^-1 with uPA and <10^6 M^-1 s^-1 with tPA.

All P,Lys mutants had slower rate constants with both PAs and were generally more sensitive to substitutions at P' than the corresponding P,Arg mutants (Table II). Relative to the P,Arg variants containing the identical P', amino acid, the k, values for the P,Lys mutants were reduced from 2.6-fold (P', Cys) to 190-fold (P', Asp) with uPA and from 2.4-fold (P', Cys) to 240-fold (P', Glu) with tPA. P,Lys mutants containing a charged amino acid (Arg, Lys, Asp, Glu) at P', were >20-fold less reactive than the P,Arg counterpart with both uPA and tPA. Similar to the P,Arg mutants, the slowest-reacting P,Lys variants contained an acidic residue at P'.

The effect of P', substitutions on the rates of inhibition by P,Arg and P,Lys variants was more dramatic when tPA was the target protease. For the interaction with uPA, the difference in k, values between the slowest and most rapid P', variants (excluding Pro) was 20-fold when Arg was at P, and 320-fold when Lys was at P,. With tPA as the target enzyme, these differences increased to 200- and 3300-fold for P,Arg and P,Lys mutants, respectively.

A number of variants differed markedly in their relative specificities for uPA and tPA (Table II). The most uPA-specific mutants had k, values that were 23-fold (P, Lys, P', Gly), 28-fold (P, Lys, P', Glu), and 40-fold (P, Lys, P', Ala) higher with uPA than with tPA. Wild-type PAI-1 was slightly uPA-specific, having a 2.4-fold higher k, value with uPA. The P',Trp variants were the most tPA-specific, having k, values that were approximately 6-fold higher with tPA than with uPA. Several other variants with aromatic residues at P,' also showed a preference for tPA (Table II).

Selected PAI-1 variants were examined for complex formation with uPA by SDS-PAGE and Western blot analysis using anti-PAI-1 antibody (data not shown). Stable complex and a cleaved form of PAI-1 were observed with wild-type and all active mutants screened. Analysis of six inactive mutants (P, Ser, P', Met, P, Glu, P', Met, P, Val, P', Ser, P, Leu, P', Ser, P, Ala, P', Arg, and P, Cys, P', Val) showed no PAI-1/uPA complex formation, consistent with loss of inhibitor activity. In addition, there was no evidence for proteolytic cleavage of these inhibitors, indicating that the loss of mutant inhibitor function was not the result of conversion to substrate.

**DISCUSSION**

Expression of PAI-1 in E. coli—A number of groups have expressed functional human PAI-1 in E. coli and demonstrated that its properties were similar to those of natural PAI-1 and recombinant PAI-1 produced by mammalian cells (22–24, 31, 43). In contrast to several of these earlier reports (22–24), the E. coli expression system employed here resulted in recovery of recombinant PAI-1 which was predominantly in the active form (up to 90%), permitting direct study without requiring reactivation of the latent form by denaturants (40). This is consistent with a previous report describing the production of highly active PAI-1 from E. coli (31). The short induction times or the similar methods of isolation used in these two studies may explain the improved recovery of PAI-1 activity. The elevated yields of PAI-1 obtained from the pET3a expression vector represent an additional significant advantage of this system.

Compared with the recombinant PAI-1 expressed using pET3a, a lower proportion of the PAI-1 produced from the M13PAI-1 expression vector was in the active conformation. Although the reason for this difference is not clear, the narrow range of active-form PAI-1 observed among the active mutants (20–50%) suggests that the P, P', residues do not significantly influence the conversion of PAI-1 from the active to the latent form. Similarly, replacement of the PAI-1...
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Table II
Second-order rate constants for the interactions of PAI-1 mutants with plasminogen activators

For each mutant, the second-order rate constants (k) for the interactions with uPA and two-chain tPA are shown. The ratio of the value with uPA to the value with tPA (uPA/tPA) is given at the right. P1Arg mutants are listed in order of their k values with uPA, with the most rapid inhibitor at the top. P1Lys mutants are paired with the P1Arg mutant containing the same P1' substitution. ND indicates not determined, and NA indicates not applicable. wt, wild-type.

<table>
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<th>P1</th>
<th>P1'</th>
<th>k (uPA)</th>
<th>k (tPA)</th>
<th>uPA/tPA</th>
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Saturation Mutagenesis—Saturation mutagenesis strategies have been used by several laboratories to investigate the structural determinants of protein function (22, 44, 45). These methods provide a more complete and unbiased view of the requirements for protein function than can generally be obtained from the study of individually designed mutants. PAI-1 is particularly well suited for this approach, since it lacks cysteine residues, can be stably expressed in E. coli, and is active in crude bacterial lysates. In the current study, we employed the bacteriophage M13 to express mutant PAI-1 protein for a saturation analysis of the P1 and P1' positions. Although M13 is not generally used as a bacterial expression vector, its application in the system described here facilitated efficient mutagenesis, sequence analysis, and mutant protein expression from a single construct.

Although oligonucleotide D (Table I) was designed to generate random amino acid substitutions at P1 and P1', a bias for or against particular amino acids was evident. This observation can be explained by a higher incorporation of thymine (41%) compared with adenine (11%) at the six targeted positions during oligonucleotide synthesis. Thus, differences in oligonucleotide synthesizer function and synthesis chemistry are variables that can influence the preparation of random oligonucleotide mixtures for saturation mutagenesis.

Over one-quarter of the PAI-1 variants contained at least one mutation outside the P1 and P1' codons. These additional mutations appear to be derived almost exclusively from the mutagenesis oligonucleotide, since all but one occurred within the oligonucleotide sequence. Similar errors within synthetic oligonucleotides, occurring at frequencies of 0.3–0.7%/nucleotide, have been described previously (46, 47). These reported mutations were also primarily G → A transitions at frequencies ranging from 50 to 95% of the base substitutions and are thought to have arisen during the oligonucleotide synthesis capping reaction (48). Although these background errors might escape detection in many oligonucleotide applications, they are an important consideration in the construction of a large library of mutants.

Role of the P1 Residue—Mutations at the P1 position of many serpins can drastically alter activity and target protease specificity (12–17). Our data confirm the critical role of the P1 residue in PAI-1 function. Although all possible P1 residues were present in the screened PAI-1 mutants, only P1Arg or P1Lys variants displayed significant rates of inhibition with uPA. This is consistent with the specificity of trypsin-like proteases, such as uPA, which preferentially attack substrates with an Arg or Lys at P1 (49). Our study also demonstrated that P1Arg PAI-1 mutants inhibited both PAs more rapidly than the corresponding P1Lys mutants. In contrast, nonserpin serine protease inhibitors, including members of the pancreatic and soybean trypsin inhibitor families, can tolerate either Lys or Arg at P1, with little change in inhibitor activity or specificity (50). The requirement for a basic residue at P1 of PAI-1 was also observed by York et al. (22) in their survey of P1, P2, and P3 mutants. Additionally, Shubeita and co-workers (24) examined four specific P1-P1' PAI-1 mutants (Arg-Ser, Arg-Val, Lys-Met, and Met-Ser), all contained within our panel, and obtained second-order rate constants consistent with the data reported here.

The P1-P1' residues of serpins not containing Arg or Lys at P1 appeared in our panel of PAI-1 mutants, P1Leu-P1'Ser, with the reactive center of α-antichymotrypsin and hirapin cofactor II, showed no detectable activity in our uPA screening assay. P1Met-P1'Ser, with the reactive center of α-antitrypsin, demonstrated a markedly reduced k of 3.4 x 10^6 M^-1 s^-1 with uPA. Thus, non-Arg/Lys amino acids present at P1 of other serpins are not well tolerated at P1 of PAI-1 for the inhibition of uPA. Interestingly, although a k of <10^-7 M^-1 s^-1 was recently reported for the interaction between...
Role of the P1' Residue—Ser is highly conserved at the P1' position of many serpin, Bowman-Birk, and potato type II inhibitors (8, 50). PAI-1 lacks this conserved Ser, suggesting a possible functional role for its P1'-Met. However, in contrast to the narrow range of amino acids allowed at P1, all amino acids except Pro were tolerated at the P1' position, and many (including Ser) had only minimal effects on k_i. Thus, neither size nor hydrophobicity appears to be critical parameters at P1', suggesting that the side chain of the P1' residue does not play a significant role in PAI-1 function. The flexibility of PAI-1 at P1' differs with both the size and hydrophobicity constraints of ATIII (20) and the size limitations and methylene group requirement of Bowman-Birk soybean trypsin inhibitor (52).

Only the substitution of Pro at P1' of PAI-1 resulted in markedly reduced or absent inhibitory activity. This intolerance for Pro may be due to a distortion of the secondary structure in the vicinity of the reactive center bond rather than a direct effect of the Pro side chain. As with PAI-1, substitution of P1'Ser by Pro in ATIII resulted in loss of reactivity (51). Interestingly, specific mutations near the reactive center bond along the strained loop is crucial for PAI-1 inhibition of uPA. This is consistent with the observations of York and co-workers (22) at P2 and P3. Similarly, insertion of an alanine in the vicinity of the reactive center has little effect on activity (52).

The presence of a negative charge at P1', reduced the reactivity of PAI-1 with both uPA and tPA. For example, although Glu and Gln are comparable in size, P1'-Arg-P1'-Glu had an 11- and 70-fold lower k_i than P1'-Arg-P1'-Gln with uPA and tPA, respectively. Although the reason for this decreased activity is unknown, a negative charge at P1' may directly interfere with or somehow destabilize the formation of a PAI-1/PA Michaelis complex. Alternatively, potential intramolecular interactions between the negative charge and other regions of PAI-1 may alter the configuration of the PAI-1 reactive center.

Although a wide variety of amino acids were tolerated at P1', their quantitative effects on PAI-1 activity were dependent on both the P1 residue and the target protease. P1'Arg mutants were both less sensitive to amino acid changes at P1' and more active than P1'Lys mutants. Similarly, uPA was both less sensitive to the P1' amino acid and more efficiently inhibited by most PAI-1 mutants than tPA. Thus, it appears that the degree to which the P1' residue modulates PAI-1 activity is decreased by favorable interactions between the P1 residue and the target protease.

Although the activity of P1'-Arg-P1'-Ala was similar to that of wild-type, a "shift" of this reactive center one amino acid toward the COOH terminus in P1'Ala-P1'-Arg-(P2'Ala) resulted in no detectable activity (Table II). This observation suggests that the precise positioning of the reactive center bond along the strained loop is crucial for PAI-1 inhibition of uPA. This is consistent with the observations of York and co-workers (22) and P3 and P2. Similarly, insertion of an alanine between residues P3 and P1 of the naturally occurring mutant α2-antiplasmin (α2-AP) Enschede, resulting in displacement of the reactive center one position toward the COOH terminus, also results in loss of α2-AP inhibitory function (54). In contrast, deletion of the P1'/Met residue of α2-AP, shifting the P' residues toward the NH2 terminus without moving the location of the reactive center, has little effect on activity (13). Interestingly, specific mutations near the reactive centers of CI-inhibitor, ATIII, and α2-AP have been reported to change these proteins from inhibitors to substrates for their target proteases (54-59). The analysis of six inactive P1',P'- mutants in this study revealed no evidence for the conversion of PAI-1 to a uPA substrate as a mechanism for loss of inhibitory function.

Role of Amino Acids Outside of P1, and P1'—The functional importance of residues adjacent to the reactive center of PAI-1 is controversial. The substitution of residues P3, P3', P4', and P5' of PAI-1 with the homologous amino acids of ATIII has been reported to result in a 40-fold reduction of the second-order rate constant with tPA (23). In contrast, replacement of residues P1' through P5' of PAI-1 by the corresponding region of PAI-2, ATIII, or a serpin consensus sequence has been shown to result in only minimal effects on PA inhibition (26). A recent analysis of a large set of P1', P2, and P3 PAI-1 mutants has also suggested a generally minor role for the P3, P4, and P5 residues in PAI-1 activity (22). Our data are consistent with these latter observations. Mutations outside of P1', generated in several P1'Arg and P1'Lys variants as a consequence of errors in oligonucleotide synthesis, had only minor effects on PAI-1 activity (Fig. 5). Of particular note, even the nonconservative substitution of Ser for P1'Pro in P1'Arg-P1'-Ala resulted in just a 2.7-fold reduction of the k_i value with uPA (to 3.4 × 10^6 M^-1 s^-1) relative to P1'Arg-P1'-Ala. Relative Specificities for uPA and tPA—Wild-type PAI-1 inhibited uPA 2.4-fold more rapidly than it inhibited tPA. Most of the variants also preferred uPA to tPA, with relative specificities for uPA as high as 40-fold (P1'Lys-P1'-Ala). However, 10 mutants, including all those with P1' aromatic substitutions, either preferred tPA or reacted equally well with both PAs. Thus, the P1 and P1' residues play an important role in determining the relative specificity of PAI-1 for its target PAs. Interestingly, York and co-workers (22) noted that the majority of their P1 and P3, P4, P5-PAI-1 mutants were specific for tPA, having rate constants that were up to 23-fold more rapid with tPA than uPA. By combining the observations from these two studies, it may be possible to generate novel PAI-1 mutants with even more marked target specificities. Such variants could provide important new tools for the study of PAI-1 function in complex systems, both in vivo and in cell culture.

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REFERENCES