# Substrate Behavior of Plasminogen Activator Inhibitor-1 Is Not Associated with a Lack of Insertion of the Reactive Site Loop†

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ABSTRACT: Plasminogen activator inhibitor-1 (PAI-1) is a unique member of the serpin superfamily. In the present study, we have evaluated the effect of substitution, with a proline, at positions P5, P7, P14, P15, or P16, on the conformational flexibility and functional properties of PAI-1. These mutants (PAI-1-P5, Ile→Pro at P5; PAI-1-P7, Ala→Pro at P7; PAI-1-P14, Thr→Pro at P14; PAI-1-P15, Gly→Pro at P15; PAI-1-P16, Ser→Pro at P16) were purified and fully characterized. WtPAI-1 had a specific activity of 68 ± 10% (mean ± SD, $n = 6$) whereas PAI-1-P5, PAI-1-P7, and PAI-1-P16 had specific activities of 34 ± 9.3%, 42 ± 10%, and 36 ± 11%, respectively. PAI-1-P14 and PAI-1-P15 did not exhibit significant inhibitory activity. Conformational analysis revealed that wtPAI-1 preparations contained 12 ± 2.0% substrate, whereas PAI-1-P5, PAI-1-P7, and PAI-1-P16 were characterized with a significantly ($p < 0.001$) increased substrate behavior (i.e., 43 ± 6.1%, 42 ± 1.5% and 22 ± 1.7%, respectively). The inactive variants PAI-1-P14 and PAI-1-P15 behaved exclusively as substrates toward various serine proteinases. Heat denaturation studies revealed that cleavage of any noninhibitory substrate form of PAI-1 resulted in an insertion of the $NH_2$-terminal side of the reactive site loop. Incubation with plasmin showed the presence of a unique plasmin cleavage site (Lys191-Ser192) exclusively present in all latent forms studied. We conclude that (a) the entire P5 to P16 region in PAI-1 plays an important role in the functional and conformational properties of PAI-1; (b) the substrate behavior of serpins is not associated with a lack of insertion of the reactive site loop; (c) the identification of a plasmin cleavage site in latent PAI-1 may provide new insights in the mechanisms for the inactivation of storage pools of PAI-1.

Plasminogen activator inhibitor 1 (PAI-1)[1] is a glycoprotein with an apparent molecular weight of approximately 50 000 and does not contain cysteine residues (Van Mourik *et al*., 1984). PAI-1 rapidly inhibits tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) with second-order association rate constants of more than $2 \times 10^7$ $M^{-1}$ $s^{-1}$ (Sprengers & Kluft, 1987). Of the various plasminogen activator inhibitors, PAI-1 appears to be the principal physiological inhibitor of t-PA in plasma (Kruithof *et al*., 1984), and increased plasma levels of PAI-1 have been shown to correlate with an increased risk for cardiovascular disease [Hamsten *et al*., 1985; for a review cf. Declerck *et al*. (1994)]. PAI-1 is also present in various tissues where it is assumed to play a major role in cell-mediated proteolysis induced by u-PA (Loskutoff *et al*., 1989). PAI-1 is synthesized as an active molecule that converts spontaneously to a latent conformation that can be partially reactivated by denaturants such as guanidinium chloride, sodium dodecyl sulfate, or urea (Hekman & Loskutoff, 1985). In addition, a noninhibitory conformation with substrate properties has been identified (Declerck *et al*., 1992; Urano *et al*., 1992; Munch *et al*., 1993). PAI-1 is a member of the serine proteinase inhibitor (serpin) superfamily (Pannekoek *et al*., 1986; Ny *et al*., 1986; Ginsberg *et al*., 1986). The serpins comprise more than 40 single-chain proteins each containing 370−390 residues with an amino acid homology of approximately 35% (Huber & Carrell, 1989; Carrell & Boswell, 1986).

Inhibitory serpins interact through formation of a 1:1 stoichiometric complex with their target proteinases. The reactive site is located within a loop structure situated 30−40 amino acids from the carboxy-terminal end and providing a "bait" residue (P1 residue) that mimics the normal substrate of the target proteinase (Laskowski & Kato, 1980). The $Arg^{346}$−$Met^{347}$ bond in PAI-1 has been identified as the P1P1′ bond (Lindahl *et al*., 1990). Recent crystallographic data (Mottonen *et al*., 1992) have suggested that in latent PAI-1, the bait region (P1P1′) and secondary binding sites are not accessible to the active site of the serine proteinases.

In general, all serpins have the same highly ordered tertiary structure consisting of β-sheets A, B, and C. The conformation of the reactive site loop containing residues P16−P10′ is highly variable (Sprang, 1992). In the intact noninhibitory ovalbumin, the reactive site loop (P6−P1′) adopts an α-helical conformation (Stein *et al*., 1990). The crystal

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[1] Abbreviations: PAI-1, plasminogen activator inhibitor-1; wt, wild-type; serpin, serine proteinase inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; *E. coli*, *Escherichia coli*; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SD, standard deviation.

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structure of the active antithrombin revealed the partial insertion of the reactive site loop and $\beta$-sheet hydrogen-bonding interactions between the P16−P15 residues and the s3A and s5A strands (Schreuder *et al*., 1994; Carrell *et al*., 1994). However, an uncomplexed active antichymotrypsin mutant showed an intact reactive site loop in a distorted helical conformation, and no preinsertion into $\beta$-sheet A was observed (Wei *et al*., 1994). The crystal structure of latent PAI-1 showed that strand s4A is inserted into $\beta$-sheet A from the "hinge" at P15 to residue P4. The residues C-terminal to the cleavage site continue with an extended loop (P4−P10′) lying on the surface of the molecule (Mottonen *et al*., 1992).

In general, inhibitory serpins appear to have a more flexible reactive site loop compared to that of noninhibitory serpins (Carrell *et al*., 1991). The structures of the cleaved inhibitory serpins, cleaved at P1P1′, showed that the new N- and C-termini were separated by 70 Å, yielding a thermodynamically stable conformation (Stein & Chothia, 1991; Loebermann *et al*., 1984; Baumann *et al.*, 1991). The new C-terminal residue (P1) is at the end of $\beta$-strand s4A within a six-stranded antiparallel $\beta$-sheet structure whereas the new N-terminal end (P1′) forms the new $\beta$-strand s1C. Recent data have shown that a similar conformational rearrangement occurs upon complexation of active PAI-1 with t-PA or u-PA (Shore *et al*., 1995; Wilczynska *et al*., 1995), thereby demonstrating a correlation between inhibition and reactive site loop insertion. In contrast, the noninhibitory serpin ovalbumin has not undergone such a conformational change upon cleavage (Wright *et al.*, 1990). The presence of a charged, rather bulky, arginine residue in the reactive site loop of ovalbumin has been suggested to prevent insertion after cleavage (Stein *et al*., 1990). Taken together, these observations have led to the hypothesis that insertion of the reactive site loop into $\beta$-sheet A is a prerequisite for proteinase inhibition.

The alternative behavior of PAI-1 as an inhibitor, a noninhibitory substrate, or a nonreactive latent form has been shown to be dependent on the initial conformation of this serpin (Declerck *et al*., 1992). The substrate form of PAI-1 reacts with its target proteinases, e.g., t-PA, u-PA, plasmin, or thrombin, resulting in cleavage of the P1P1′ bond but, in contrast to the active form, without formation of a covalent complex and without inhibition of the proteinase. The characterization of PAI-1 variants carrying mutations at positions P12, P10, P8, or P6 revealed that at least the region P12−P8 contributes significantly to the substrate properties of PAI-1 (Audenaert *et al*., 1994). Preliminary data on the PAI-1 variant containing a proline at position P6 revealed that also changes at this position affect the functional properties of PAI-1. In order to further delineate functionally important positions in the reactive site loop of PAI-1, we have constructed and characterized five mutants, in which the amino acids at positions P16, P15, P14, P7, and P5 were substituted with proline. The results demonstrate that substitution of P15, P14, and P12 yields PAI-1 variants exhibiting exclusively, stable substrate properties toward a variety of serine proteinases. On the other hand, heat denaturation studies carried out on all variants (including wild-type substrate PAI-1) and their cleaved substrate derivatives demonstrate that insertion is not an exclusivity of the inhibitory pathway.

## EXPERIMENTAL PROCEDURES

*Materials.* Restriction enzymes were obtained from Pharmacia (Uppsala, Sweden) or from Boehringer Mannheim (Brussels, Belgium). $T_4$ DNA ligase, the Klenow fragment of *Escherichia coli* DNA polymerase I, and alkaline phosphatase were purchased from Boehringer Mannheim (Brussels, Belgium). The oligonucleotide-directed mutagenesis system (the pMa/c plasmids; Stanssens *et al*., 1989) was kindly provided by Corvas (Ghent, Belgium). The oligonucleotides for mutagenesis were purchased from Pharmacia. M13KO7 helper phage was obtained from Promega (Leiden, The Netherlands). The expression vector pIGE20 was kindly provided by Innogenetics (Ghent, Belgium), together with the bacterial strains *E. coli* DH1$\lambda$ for cloning and *E. coli* MC1061 for expression as well as the pAcI plasmid encoding the thermolabile repressor.

Luria broth growth medium was purchased from Life Technologies, Inc. (Ghent, Belgium). The proteinase inhibitors leupeptin, phenylmethanesulfonyl fluoride, dithiothreitol, pepstatin, benzamidine hydrochloride, and antipain were from Sigma (St. Louis, MO). SP-Sepharose was purchased from Pharmacia. The chromogenic substrate S-2403 was obtained from Chromogenix (Mölndal, Sweden). t-PA was a kind gift from Boehringer Ingelheim (Brussels, Belgium); u-PA (consisting of a mixture of high and low molecular weight u-PA in a 75:25 ratio) was a kind gift from Bournonville Pharma (Braine l'Alleud, Belgium). Plasmin and thrombin were kindly provided by Dr. R. Lijnen (University of Leuven, Belgium). t-PA-S478A, a mutant of t-PA in which the active site serine was replaced by alanine, was a kind gift from Genentech Inc. (South San Francisco, CA).

*General DNA Techniques.* DNA manipulations were performed according to the instructions of the suppliers. Plasmid DNA was isolated using a Quiagen purification protocol (provided by Westburg N. V., The Netherlands). Transformations of *Escherichia coli* were performed using the calcium chloride procedure (Sambrook *et al.*, 1989). For preparation of single-stranded DNA, the *E. coli* strain WK6 was used. Site-directed mutagenesis was performed using the pMa/c system (Stanssens *et al*., 1989) in the repair-deficient *E. coli* strain WK6 mutS. Propagation of the plasmids pMa/c or derivatives was carried out in *E. coli* WK6. For expression, wild-type PAI-1 (wtPAI-1) and PAI-1 mutants were cloned into pIGE20, a $P_L$ expression vector, and expressed in *E. coli* MC1061 after cotransformation with pAcI (encoding the heat-labile repressor). DNA sequencing was performed using the dideoxy chain termination reaction method of Sanger *et al*. (1977) and the automated laser fluorescent A. L. F. (Pharmacia).

*Construction of PAI-1 Variants.* pMc-PAI-1 was constructed as described before (Audenaert *et al*., 1994). For in vitro site-directed mutagenesis, single-stranded DNA of this construct was prepared by transformation of pMc-PAI-1 in *E. coli* WK6 and infection of an overnight culture with helper phage M13KO7. After 6 h of infection, cells were centrifuged, and single-stranded DNA was isolated from the supernatant by poly(ethylene glycol)precipitation and phenol−chloroform extraction. Subsequently, single-stranded pMc-PAI-1 was hybridized with *Hin*dIII−*Eco*RI-digested pMa and with one of the following synthetic oligonucleotides, to obtain the desired mutation: PAI-1-P16, 5′ GGC CAC CGT

GCC CGG CTC GTT CAC CTC 3′; PAI-1-P15, 5′ GGA GGC CAC CGT GGG GCT CTC GTT CAC 3′; PAI-1-P14, 5′ TGA GGA GGC CAC CGG TCC ACT CTC GTT 3′; PAI-1-P7, 5′ TGA GAC TAT GAC CGG TGT GGA TGA GGA 3′; PAI-1-P5, 5′ GCG GGC TGA GAC CGG TAC AGC TGT GGA TGA 3′.

Specific restriction sites, i.e., a *Nci*I site (CC↓G/CGG) for PAI-1-P16, a *Ban*II site (GRGCY↓C) for PAI-1-P15, and an *Age*I site (A↓CCGGT) for PAI-1-P14, PAI-1-P7, and PAI-1-P5, were simultaneously created to allow confirmation of the desired mutation. Extension reactions were carried out with the Klenow fragment of DNA polymerase as described (Sambrook *et al.*, 1989). After transformation of *E. coli* WK6 mutS, the cells were grown overnight in the presence of ampicillin. Then plasmid DNA was isolated and used to transform *E. coli* WK6, followed by selection on agar plates containing ampicillin. Subsequently, randomly selected clones were grown in small volumes, and mutations were evaluated by restriction enzyme analysis. Then, larger scale DNA preparations were made for further confirmation of the mutations by restriction analysis and by nucleotide sequencing using A. L. F.

*Construction of Expression Plasmids.* pIGE20-PAI-1 was constructed as described before (Audenaert *et al*., 1994). After mutagenesis of pMc-PAI-1 into pMa-PAI-1-P16, pMa-PAI-1-P15, pMa-PAI-1-P14, pMa-PAI-1-P7, and pMa-PAI-1-P5, *Sac*I−*Xba*I fragments were recovered from the mutant pMa-PAI-1 constructs and substituted for the wild-type *Sac*I−*Xba*I fragment in pIGE20-PAI-1.

*Expression and Purification of WtPAI-1 and Variants.* After cloning the mutant fragments in the pIGE20 expression vector, the expression plasmids were cotransformed with pAcI in *E. coli* MC1061. Transformed *E. coli* MC 1061 cultures were grown overnight at 28 °C and diluted 1:100. Diluted cultures were grown until $A^{580nm} = 0.2$, and subsequently PAI-1 synthesis was induced by increasing the temperature to 42 °C for 3 h. Cells were harvested by centrifugation for 15 min at 4000*g* at 4 °C, and cell pellets were either used immediately or frozen at −80 °C. The cell pellets were resuspended in 50 mM sodium acetate buffer, pH 5.5, containing 2 mM glutathione and 0.01% Tween 80 (20 mL for a typical 1.6 L culture). After addition of leupeptin (2.5 μg/mL), pepstatin A (0.9 μg/mL), benzamidine hydrochloride (203 μg/mL), antipain (1.3 μg/mL), PMSF (52 μg/mL), and DTT (13 μg/mL), the cell suspension was disrupted in a standard French Pressure 20K cell (Aminco, Urbana, IL) imposing a pressure of 138 MPa. The broken cell extract was centrifuged at 20 000 rpm for 20 min at 4 °C. The obtained supernatant was immediately subjected to purification.

Purifications were performed at 4 °C. The supernatant was diluted (1:2 to 1:4) with a 0.15 M phosphate buffer containing NaCl (except for PAI-1-P14 and PAI-1-P15), glutathione and Tween-80 to obtain a final concentration of 0.2 M NaCl, 2 mM glutathione, and 0.01% Tween 80, pH 6.0, and loaded onto a SP-Sepharose fast flow column (Pharmacia) (1.6 × 20 cm), previously equilibrated with a 0.15 M phosphate buffer containing 2 mM glutathione, 0.01% Tween 80, pH 6.0 (buffer 1). The column was washed with 4 volumes of buffer 1 containing 0.3 M NaCl (0.2 M NaCl for PAI-1-P14 and PAI-1-P15), and bound proteins were eluted using a sodium chloride gradient (0.3−1.3 M NaCl) (0.2−1.2 M for PAI-1-P14 and PAI-1-P15). PAI-1-containing fractions were pooled, dialyzed against buffer 1, and again loaded onto a SP-Sepharose fast flow column. The column was washed with buffer 1 and eluted using a sodium chloride gradient (0.2−1.2 M NaCl). PAI-1-containing fractions were evaluated for their purity, pooled, and stored at −20 °C in aliquots until use.

Isolation of the active form of wtPAI-1 and separation of the substrate conformation from latent wtPAI-1 or latent PAI-1 variants were performed as described previously (Declerck *et al.*, 1992). In brief, active wtPAI-1 or PAI-1 variants were incubated at 37 °C until the residual activity was <0.5% of the theoretical maximal value. Inactivated PAI-1 was applied to immobilized t-PA-S478A, the non-binding fraction was collected (= latent form), and bound PAI-1 was eluted with 1.5 M NaCl in 0.1 M acetate buffer containing 2 mM glutathione, pH 5.5 (=substrate form). Generation of cleaved substrate derivatives for heat denaturation studies was carried out by incubation of isolated substrate forms (20−100 μg/mL) with immobilized t-PA (80 μL of Sepharose 4B containing 1.4 mg of t-PA/mL of beads). Cleavage was confirmed by SDS−PAGE.

*Immunological and Functional Determination of PAI-1.* PAI-1 antigen was determined with an enzyme-linked immunosorbent assay as described previously (Declerck *et al*., 1988) and is expressed in micrograms. PAI-1 activity was determined using the method described by Verheijen (Verheijen *et al.*, 1984) by adding a fixed amount of t-PA to the PAI-1-containing samples. t-PA was calibrated versus the international reference preparation for t-PA (NIBSC 86/670). All PAI-1 activity data are expressed as a percentage of the theoretical maximal activity, i.e., ∼745 000 units/mg, calculated on the basis of a specific activity for t-PA of 500 000 units/mg and molecular masses of 67 and 45 kDa for t-PA and PAI-1, respectively.

*Characterization of Purified PAI-1 Variants.* Samples of both wtPAI-1 and PAI-1 mutants were incubated either with catalytic amounts (5%) or with a 2-fold molar excess of t-PA, u-PA, plasmin, or thrombin. PAI-1 samples were diluted with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$) to a concentration of 0.3 mg/mL and incubated for 30 min at 37 °C with the serine proteinases. The reaction was terminated by adding SDS (final concentration 1%) and heating for 30 s at 100 °C. Reaction products were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and subsequent densitometric scanning with the Imagemaster (Pharmacia, Uppsala, Sweden).

Reactivation of PAI-1 preparations was performed by incubating samples with 6 M guanidinium chloride for 25 min at 37 °C, followed by extensive dialysis at 4 °C against 50 mM sodium acetate buffer, pH 5.5, containing 1 M NaCl, 2 mM gluthatione, and 0.01% Tween 20 (Sancho *et al.*, 1994).

*Determination of the Stability of Purified WtPAI-1 and PAI-1 Variants.* Purified PAI-1 samples were diluted to a final concentration of 50−120 μg/mL using the appropiate diluent (containing Tween 80 and $Na_2HPO_4$) to obtain a buffered solution with 45 mM phosphate, 70 mM NaCl, and 0.01% Tween, pH 7.4. Samples were incubated at 37 °C, and aliquots were removed at various times and assayed for inhibitory activity against t-PA and u-PA. The specific activity obtained at time zero was assigned a value of 100%.

Table 1: Specific Activities[a] of WtPAI-1 and PAI-1 Mutants toward t-PA and u-PA

| | t-PA | u-PA |
|---|---|---|
| wtPAI-1 | 68 ± 10 | 66 ± 6.0 |
| PAI-1-P5 | 34 ± 9.3 | 34 ± 13 |
| PAI-1-P6 | 45 ± 10 | 3.9 ± 2.2 |
| PAI-1-P7 | 42 ± 10 | 13 ± 4.1 |
| PAI-1-P14 | 0.3 ± 0.2 | 1.1 ± 0.3 |
| PAI-1-P15 | 0.2 ± 0.3 | <0.01 |
| PAI-1-P16 | 36 ± 11 | 58 ± 19 |

[a] Expressed as a percentage of the theoretical maximum value; mean ± SD, $n = 3-6$.

*Heat Denaturation Studies of WtPAI-1, PAI-1 Variants and Derivatives.* Samples containing active, latent, substrate, or cleaved substrate PAI-1 were diluted to 20 $\mu$g/mL in 20 mM sodium acetate buffer, pH 5.5, containing 1 M NaCl and 0.01% Tween 80 and incubated at a constant temperature between 30 and 100 °C for 2 h. The samples were then cooled in ice and centrifuged for 15 min in a microfuge at 14 000 rpm to remove precipitated protein. The supernatants were carefully removed and either analyzed immediately or stored at −20 °C. Residual PAI-1 protein in the supernatant was determined by ELISA (Declerck *et al*., 1988). Remaining PAI-1 antigen was expressed as a percentage versus the initial value. The latter was obtained through analysis, in parallel, of control samples incubated at 0 and 30 °C.

*Other Analytical Methods.* Sodium dodecyl sulfate−polyacrylamide gel electrophoresis was performed using 10−15% gradient gels under nonreducing conditions with the Phast System (Pharmacia). Proteins were visualized by staining with Coomassie Brilliant Blue. The amount of PAI-1 protein in purified preparations was determined spectrophotometrically at 280 nm using an absorbance coefficient ($A^{1\%}_{1cm}$) of 10.

$NH_2$-terminal sequence analysis (before and after reaction with t-PA) was kindly performed by Mrs. Anja Wuyts and Dr. J. Vandamme (Laboratory of Microbiology, Rega Institute, University of Leuven, Belgium) using an Applied Biosystems 477A protein sequencer, with identification of phenylthiohydantoins by high performance liquid chromatography.

The statistical significance of differences was evaluated using Student's *t*-test; *p*-values >0.05 were considered nonsignificant.

## RESULTS

*Determination of Specific Inhibitory Activity against t-PA and u-PA.* WtPAI-1 had a specific activity of 68 ± 10% (mean ± SD, $n = 6$) of the theoretical maximal value. PAI-1-P5, PAI-1-P6, PAI-1-P7, and PAI-1-P16 also exhibited inhibitory activities toward t-PA with specific activities ranging from 34 to 45% (Table 1). In contrast, both PAI-1-P14 and PAI-1-P15 were mainly inactive toward t-PA. The specific activities of wtPAI-1 and PAI-1-P5 toward u-PA were similar to those observed toward t-PA whereas the specific activity of PAI-1-P16 was slightly higher toward u-PA. PAI-1-P6 and PAI-1-P7 were significantly less active ($p < 0.001$) toward u-PA than toward t-PA. PAI-1-P14 and PAI-1-P15 were also virtually devoid of anti u-PA activity (Table 1).

Incubation of wtPAI-1 and active PAI-1 mutants for 16 h at 37 °C resulted in a loss of inhibitory activity. Except for the inactive PAI-1-P14 and PAI-1-P15, all other mutants could be reactivated up to 58−113% of the initial activity by treatment with guanidinium chloride (Table 2).

Table 2: Inactivation and Reactivation of WtPAI-1 and PAI-1 Mutants

| | specific activity[a] | | |
|---|---|---|---|
| | starting material | after inactivation | after reactivation |
| | Towards t-PA | | |
| wtPAI-1 | 71 ± 7.0 | 1.9 ± 1.6 | 56 ± 12 |
| PAI-1-P5 | 34 ± 3.5 | 0.5 ± 0.1 | 29 ± 8.3 |
| PAI-1-P6 | 40 ± 3.8 | 0.8 ± 0.6 | 26 ± 4.2 |
| PAI-1-P7 | 35 ± 19 | 2.3 ± 2.6 | 39 ± 21 |
| PAI-1-P14 | 0.3 ± 0.2 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| PAI-1-P15 | 0.1 ± 0.1 | < 0.01 | < 0.01 |
| | Towards u-PA | | |
| wtPAI-1 | 65 ± 9.3 | 2.4 ± 1.5 | 60 ± 25 |
| PAI-1-P5 | 19 ± 0.2 | 0.3 ± 0.1 | 14 ± 3.2 |
| PAI-1-P6 | 1.8 ± 0.4 | 0.1 ± 0.1 | 0.6 ± 0.7 |
| PAI-1-P7 | 12 ± 4.9 | 0.8 ± 0.1 | 8.9 ± 5.0 |
| PAI-1-P14 | 0.7 ± 0.4 | 0.4 ± 0.2 | 0.3 ± 0.1 |
| PAI-1-P15 | <0.01 | <0.01 | <0.01 |

[a] Expressed as a percentage of the theoritical maximum value; mean ± SD, $n = 2-4$.

*Reaction Products Formed after Incubation of WtPAI-1 and PAI-1 Mutants with Various Serine Proteinases.* Incubation of wtPAI-1, PAI-1-P5, PAI-1-P6, PAI-1-P7, and PAI-1-P16 with catalytic amounts of t-PA (5%) resulted in the neutralization of t-PA and the concomitant generation of small amounts of t-PA/PAI-1 complexes and a 41 kDa degradation product, while the majority (>90%) remained intact. In contrast, incubation of PAI-1-P14 and PAI-1-P15 with catalytic amounts of t-PA revealed exclusively and to a large extent (>95%) the formation of a 41 kDa degradation product. This indicates that the noninhibitory mutants PAI-1-P14 and PAI-1-P15 behave as substrates.

Incubation of wtPAI-1 with a 2-fold molar excess of t-PA revealed the formation of t-PA/PAI-1 complexes (56 ± 0.4%, mean ± SD, $n = 4$) , small amounts of cleaved derivative (12 ± 2.0%), and residual nonreactive material (31 ± 1.9%) (Figure1A). Under these conditions, the amounts of complexes formed with PAI-1-P5, PAI-1-P6, PAI-1-P7, and PAI-1-P16 (i.e., 40 ± 1.0%, 55 ± 5.2%, 35 ± 2.5%, and 73 ± 1.0%, respectively) were compatible with their respective activity data, and in addition, cleaved derivative (43 ± 6.1%, 31 ± 4.0%, 42 ± 1.5%, and 22 ± 1.7%, respectively) and residual nonreactive material (16 ± 5.8%, 14 ± 1.1%, 23 ± 1.6%, and 5.0 ± 1.4%, respectively) were found. In contrast, PAI-1-P14 and PAI-1-P15 were virtually completely degraded (>96%) (Figure 1A).

In the presence of a 2-fold molar excess of u-PA, wtPAI-1 revealed the formation of u-PA/PAI-1 complexes (60 ± 1.5%, mean ± SD, $n = 3$), small amounts of cleaved derivative (9.9 ± 2.0%), and residual nonreactive PAI-1 (30 ± 2.8%) (Figure 1B). Under these conditions, PAI-1-P5 and PAI-1-P16 revealed a pattern comparable to that observed in the presence of t-PA, i.e., 47 ± 8.7% and 72 ± 5.4% u-PA/PAI-1 complexes, 36 ± 8.7% and 14 ± 0.7% cleaved derivative, and 17 ± 1.8% and 12 ± 6.0% nonreactive PAI-1, respectively. PAI-1-P6 and PAI-1-P7, much less functionally active toward u-PA, revealed the formation of relatively small amounts of u-PA/PAI-1 complexes (12 ± 5.2% and 26 ± 11%, respectively) and nonreactive PAI-1 (17 ± 3.1% and 27 ± 1.0%, respectively) but large amounts

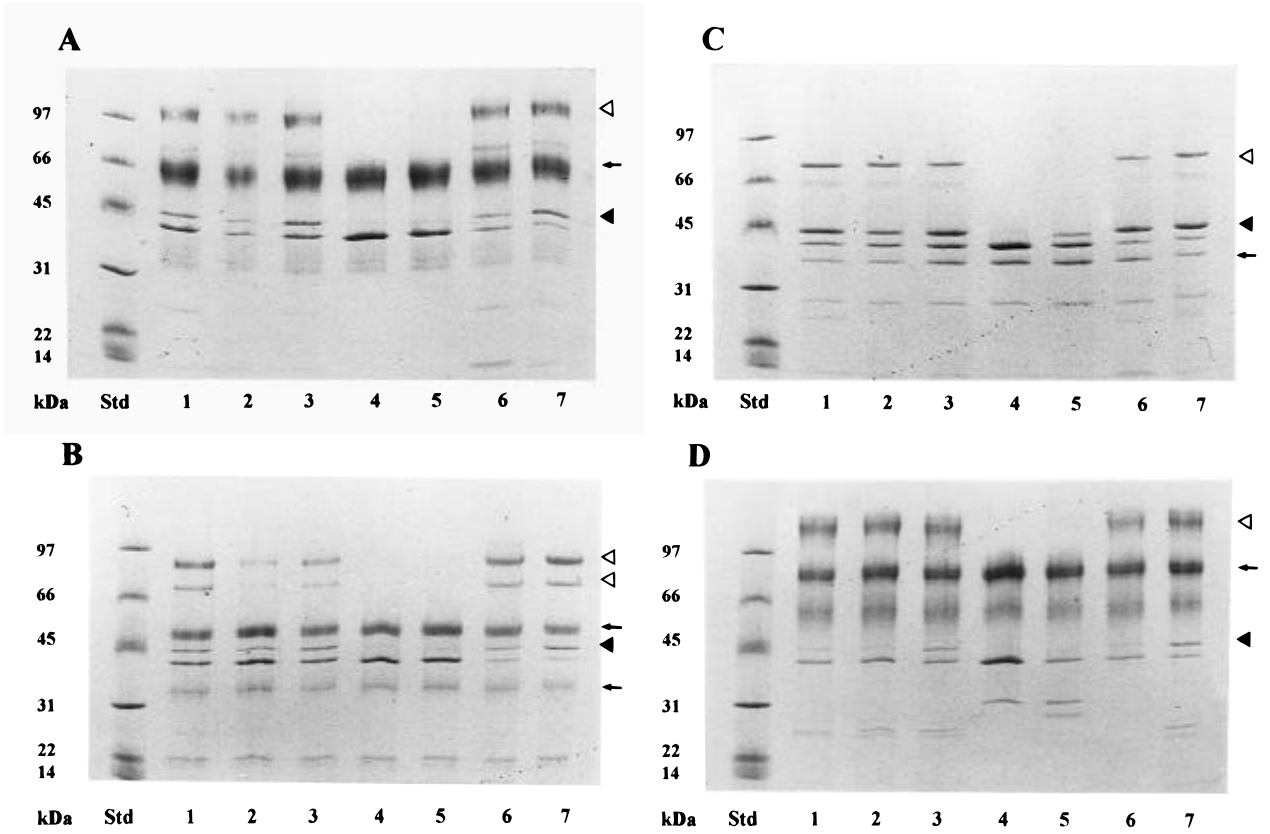


FIGURE 1: SDS−PAGE of wtPAI-1 and PAI-1 variants after addition of a 2-fold molar excess of t-PA (A), u-PA (B), thrombin (C), and plasmin (D). (1) PAI-1-P5; (2) PAI-1-P6; (3) PAI-1-P7; (4)PAI-1-P14; (5) PAI-1-P15; (6) PAI-1-P16; (7) wtPAI-1. Std represents the molecular mass standards with the indicated molecular masses: phosporylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The closed arrowhead indicates the migration position of intact PAI-1. The open arrowhead indicates the migration position of the serine proteinase/PAI-1 complex. The arrow indicates the migration position of the serine proteinase.

of the 41 kDa cleaved derivative (71 ± 3.6% and 47 ± 10%, respectively) (Figure 1B). This observation indicates that the low inhibitory activity of the latter mutants toward u-PA is mainly due to an increased substrate behavior toward u-PA as compared to t-PA. Under these conditions, PAI-1-P14 and PAI-1-P15 were virtually completely degraded (>96%), again indicative for their substrate behavior.

Incubation of wtPAI-1 with a 2-fold molar excess of plasmin revealed the formation of 58 ± 6.5% PAI-1/plasmin complex, 17 ± 0.9% of the 41 kDa cleaved derivative material, 15 ± 5.3% nonreactive material, and small amounts (9.6 ± 2.3%) of two additional degradation products migrating with a molecular mass of approximately 24 kDa (Figure 1D). Under these conditions, PAI-1-P5, PAI-1-P6, PAI-1-P7, and PAI-1-P16 yielded a pattern comparable to that observed for wtPAI-1, whereas incubation of PAI-1-P14 and PAI-1-P15 with a 2-fold molar excess of plasmin yielded predominantly the 41 kDa cleavage product. Surprisingly, incubation of the latent forms of wtPAI-1, PAI-1-P5, PAI-1-P6, and PAI-1-P7 with either an excess or catalytic amounts of plasmin resulted in the quantitative generation of the 24 kDa derivatives (i.e., 69 ± 5.7%, 94 ± 2.6%, 88 ± 3.4%, and 74 ± 1.3%, respectively, mean ± SD, $n = 3$) (Figure 2).

Addition of a 2-fold molar excess of thrombin to wtPAI-1 or PAI-1 variants yielded a pattern comparable to that observed with t-PA (Figure1C).

*Stability of PAI-1 Mutants at 37 °C.* WtPAI-1 and PAI-1 mutants were incubated at 37 °C, and PAI-1 activity was determined at various time intervals (Figure 3). WtPAI-1

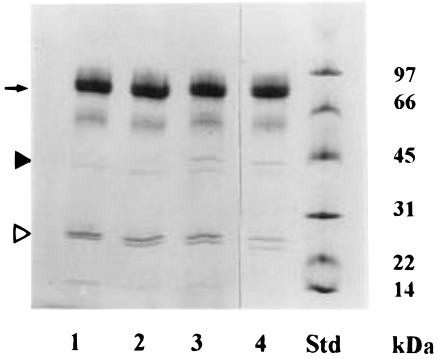


FIGURE 2: SDS−PAGE of latent PAI-1 forms after incubation with a 3-fold molar excess of plasmin. (1) PAI-1-P5; (2) PAI-1-P6; (3) PAI-1-P7; (4) wtPAI-1. Std represents the molecular mass standards (see legend to Figure 1). The open arrowhead indicates the migration position of the degradation products with mass ≈24 kDa. The closed arrowhead and the arrow indicate the migration positions of intact PAI-1 and plasmin, respectively.

and PAI-1-P5 had comparable functional half-lifes (i.e., $t_{1/2}$ = 80 ± 23 min and 106 ± 57 min, respectively). The half-life of PAI-1-P6 and PAI-1-P7 was slightly increased ($t_{1/2}$ = 151 ± 32 and 132 ± 36 min, $p < 0.02$ and $p < 0.05$, respectively, versus wtPAI-1) whereas PAI-1-P16 was 4 times more stable compared to wtPAI-1 ($t_{1/2}$ = 325 ± 225 min, $p < 0.002$). The substrate behavior of PAI-1-P14 and PAI-1-P15 after incubation for 24 h at 37 °C remained unchanged.

*Heat-Induced Denaturation of Different Conformations of WtPAI-1 and PAI-1 Variants.* The heat denaturation profiles are shown in Figure 4. As expected, the active form of

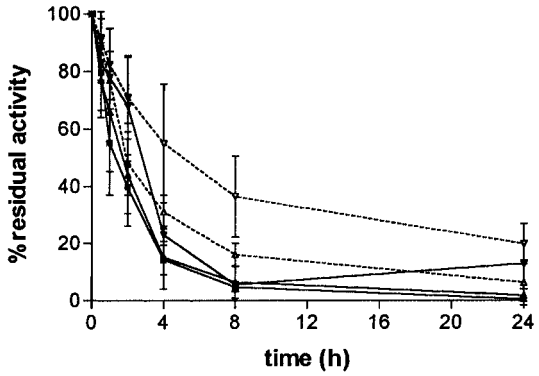


FIGURE 3: Functional stability of active PAI-1 variants at 37 °C. WtPAI-1 (■); PAI-1-P5 (▲); PAI-1-P6 (▼); PAI-1-P7 (△); PAI-1-P16 (▽). All data represent mean ± SD, $n = 3-12$.

wtPAI-1 was more sensitive to heat denaturation ($T_{m_{50\%}} = 51 \pm 2.0$ °C, mean ± SD, $n = 5$) than the latent form whereas the substrate form of wtPAI-1 exhibited an intermediate heat denaturation profile ($T_m = 60 \pm 2.3$ °C) (Figure 4A). The substrate forms of the PAI-1 variants exhibited slightly different profiles with the following $T_m$ values: 66 ± 2.2 °C, 68 ± 2.3 °C, 63 ± 3.2 °C, 51 ± 4.4 °C, 67 ± 8.5 °C, and 66 ± 4.8 °C (mean ± SD, $n = 3-6$) for substrate forms of PAI-1-P5, PAI-1-P6, PAI-1-P7, PAI-1-P14, PAI-1-P15 and PAI-1-P16, respectively (Figure 4B,C). Strikingly, in all cases studied, the cleaved substrate variants exhibited an extremely high resistance to heat denaturation (i.e., $T_m > 90$ °C, Figure 4).

*Identification of the Cleavage Site in PAI-1-P14, PAI-1-P15, and in Latent PAI-1 Forms.* Amino-terminal sequence analysis of PAI-1-P14 and PAI-1-P15 following incubation with catalytic amounts of t-PA revealed the known amino-terminal sequence of PAI-1 (i.e., Val-His-His-Pro-Pro) and in addition the equimolar generation of a new sequence, Met-Ala-Pro-Glu-Glu-Ile, indicative for cleavage at the $Arg^{346}$-$Met^{347}$ (P1P1′) position.

Amino-terminal sequencing of latent wtPAI-1 and latent PAI-1-P5, PAI-1-P6 and PAI-1-P7 after incubation with plasmin revealed the generation of a new sequence (corresponding to the lower band in Figure 2), Ser-Asp-Gly-Ser-Thr-Val-Ser-Val, indicative for a plasmin cleavage site at $Lys^{191}$-$Ser^{192}$ in latent PAI-1 forms.

## DISCUSSION

The serpins can be divided into two groups: the active inhibitory serpins (e.g., antithrombin III, $\alpha_1$-antitrypsin, C1-inhibitor, $\alpha_2$-antiplasmin) and the noninhibitory serpins (e.g., ovalbumin, angiotensinogen, maspin) (Huber & Carrell, 1989; Pemberton *et al.*, 1995). The unique serpin PAI-1 can occur in (a) a labile active inhibitory conformation, (b) a nonreactive latent conformation that can be reactivated, and (c) a noninhibitory but cleavable substrate conformation (Declerck *et al.;* 1992; Urano *et al.*, 1992; Munch *et al.*, 1993). The P12−P9 region of the inhibitory serpins consists mainly of aliphatic small alanines and appears to be highly conserved in the inhibitory serpins (Carrell *et al.*, 1991) whereas the P12−P9 region of the noninhibitory serpins consists of rather bulky or charged residues. Several naturally occurring mutants of inhibitory serpins have been described where substitutions of small aliphatic residues by bulky or charged residues at position P10 or P12 (Levy *et al.*, 1990; Skriver *et al.*, 1991; Perry & Carrell, 1989) as

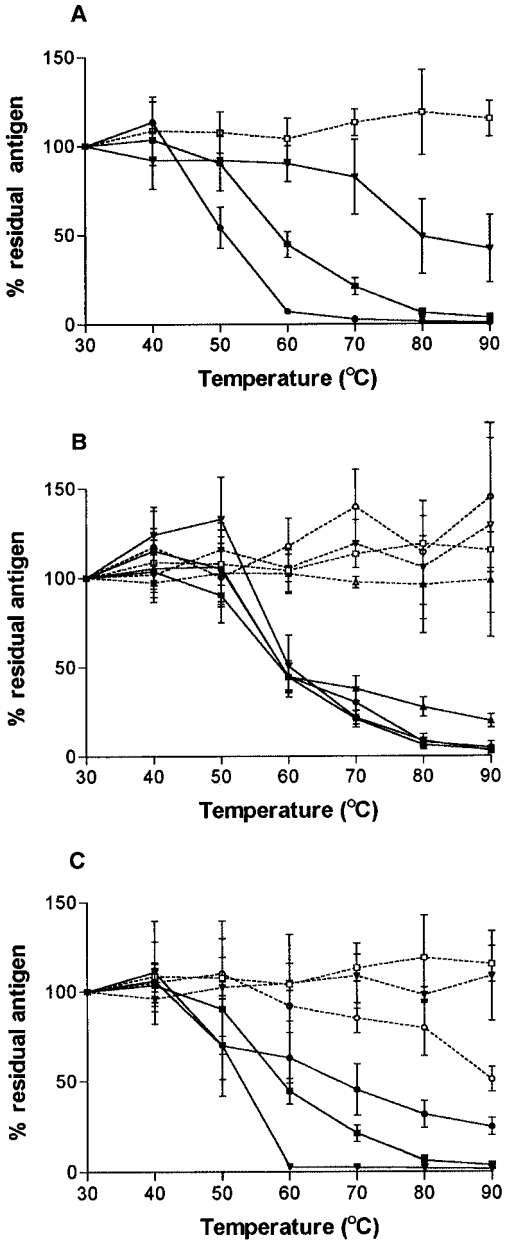


FIGURE 4: Heat denaturation profiles of wtPAI-1, PAI-1 variants, and their derivatives. (A) WtPAI-1: active (●), substrate (■), latent (▼), and cleaved substrate (□). (B) Substrate (closed symbols) and respective cleavage products (open symbols) of wtPAI-1 (■,□), PAI-1-P5 (▼,▽), PAI-1-P7 (●,○) and PAI-1-P16 (▲, △). (C) Substrate (closed symbols) and respective cleavage products (open symbols) of wtPAI-1 (■,□), PAI-1-P14 (▼,▽), and PAI-1-P15 (●,○). All data represent mean ± SEM, $n = 3-6$.

well as insertion of an alanine in the P11−P8 region (Holmes *et al.*, 1987) resulted in inactive serpins with substrate properties. On the other hand, two recent studies (Lawrence *et al.*, 1994; Tucker *et al.*, 1995) suggested that the charge [rather than the size (Lawrence *et al.*, 1994)] of the residue at position P14 [and not at P12, P10, P8, P6, or P4 (Tucker *et al.*, 1995)] plays an important role in the functional behavior of PAI-1. The current study, together with our previous study (Audenaert *et al.*, 1994), reveals that the entire P5−P16 region contributes to the functional properties of PAI-1. Substitution with a proline at position P5, P6, P7, or P16 yields inhibitory PAI-1 variants but with an increased and stable substrate behavior. These data are compatible with suggestions that, at least in part, the kinetics of insertion may govern the reaction pathway (Gettins *et al.*,

1993; Hood *et al.*, 1994). Whereas substitution with a proline at P8 or P10 (Audenaert *et al*., 1994) yields PAI-1 variants with predominantly substrate characteristics, substitution at P12 (Audenaert *et al*., 1994), P14, or P15 yields PAI-1 variants with exclusively, stable substrate properties, indicating that the P12−P15 region is the "substrate"-determining region. Interestingly, for all of the mutants exhibiting inhibitory as well as substrate properties (i.e., PAI-1-P5, PAI-1-P6, PAI-1-P7, and PAI-1-P16), a functionally stable (i.e., no transition to a latent state) substrate form could be separated from the inhibitory form. These data are in agreement with those observed for native PAI-1 (Declerck *et al*., 1992), thereby confirming that the functional behavior of PAI-1 as well as PAI-1 mutants is predetermined by the initial conformation; i.e., two distinct populations of molecules (active and substrate) exist *prior* to interaction with the target proteinase. This is in contrast to the hypothesis put forward for other serpins in which it is believed that during interaction with the target proteinase a common intermediate exists but that the ratio of substrate versus inhibitory reaction is determined by the kinetics of insertion and/or by conformational changes induced *during* interaction with the proteinase (Gettins *et al*., 1993; Hood *et al*., 1994; Huntington *et al*., 1995). This difference between serpins in general and PAI-1 illustrates again the uniqueness of PAI-1 within the serpins. Our hypothesis, that the underlying molecular basis responsible for the substrate behavior of PAI-1 is different from that for the substrate reaction observed in other serpins, is supported by the observation that incubation of $\alpha_1$-antitrypsin or antithrombin III with a peptide corresponding to the respective P1−P14 sequence induces a substrate behavior (Carrell *et al*., 1991; Schulze *et al.*, 1990; Björk *et al*., 1992) whereas similar experiments with PAI-1 resulted in an inactivation without induction of a substrate reaction (Eitzman *et al*., 1995).

Crystallographic studies demonstrated that upon cleavage of an inhibitory serpin, the N-terminal side of the active site loop inserts as a new $\beta$-strand s4A forming a more compact protein (Carrell *et al.*, 1991; Stein & Chothia, 1991; Loebermann *et al.*, 1984; Baumann *et al.*, 1991) whereas cleavage of the noninhibitory serpin ovalbumin into plakalbumin does not result in the insertion of the N-terminal side of the active site loop (Wright *et al.*, 1990). These typical differences are also reflected in heat denaturation studies of cleaved inhibitory serpins (Carrell *et al*., 1991; Gettins & Harten, 1988; Pemberton *et al*., 1989) revealing a large increase in heat stability compared to their intact active form whereas heat denaturation profiles of plakalbumin (Carrell *et al.*, 1991), cleaved maspin (Pemberton *et al.*, 1995), and the cleaved noninhibitory substrate antithrombin III Ala(P10)→Pro mutant (Carrell *et al*., 1991) revealed no increased heat stability. In one study (Hopkins *et al*., 1993), it was suggested that the increased heat stability observed for the cleaved noninhibitory $\alpha_1$-antitrypsin Gly(P10)→Pro mutant formed an exception on the widely accepted hypothesis regarding insertion and inhibitory activity. Our current data, obtained through heat denaturation studies, revealed a large increase in heat stability upon cleavage of any of the substrate forms studied ($T_m$ = 50−68 °C vs $T_m$ > 90 °C, for intact substrate forms vs cleaved substrate forms, respectively), thereby indicating that the insertion of the P16−P1 residues occurs upon cleavage of all of these noninhibitory substrate variants. Although in contrast to the general hypothesis (Carrell *et al.*, 1991), our findings are in agreement with recent conformational studies on cleaved antithrombin III Ala(P12)→Thr (Wright *et al.*, 1994), cleaved $\alpha_1$-proteinase inhibitor Thr(P14)→Arg (Hood *et al*., 1994), cleaved PAI-1 Thr(P14)→Arg (Lawrence *et al.*, 1994) (cleaved with elastase at the P4−P3 bond), and cleaved PAI-1 Ala(P12)→Pro (Sancho *et al.*, 1995). In addition, the three-dimensional structure of the cleaved substrate mutant PAI-1-P12 (Aertgeerts *et al*., 1995) indeed clearly demonstrated the insertion of residues P16−P3 into $\beta$-sheet A. Importantly, it should be stressed that the data from our current study indicate a similar insertion upon cleavage of the noninhibitory substrate form of wild-type PAI-1. To the best of our knowledge, this is the first report on insertion occurring upon cleavage of a nonmutated substrate-type serpin. Overall, the information now available from a wide variety of substrate-type serpin variants and our native substrate serpin provides accumulating evidence that the substrate behavior of noninhibitory serpins is not associated with a lack of insertion of the reactive site loop.

Another important observation in the present study is the identification of a, previously unknown, plasmin cleavage site exclusively present in latent forms of wtPAI-1 or PAI-1 variants. $NH_2$-terminal sequence analysis revealed that this cleavage site is located at the $Lys^{191}$−$Ser^{192}$ peptide bond. From the comparison with the 3-D structure of cleaved and latent PAI-1 (Aertgeerts *et al.*, 1995), it is clear that this plasmin cleavage site, exclusive for latent forms, is situated in a region of the molecule ($His^{185}$−$Pro^{200}$) that constitutes a major difference between latent and reactive forms of PAI-1. This is the first biochemical evidence for this conformational difference and the first report revealing a proteolytic cleavage site preferentially exposed in latent PAI-1. Only one other study, using cathepsin D, also revealed additional cleavage sites outside the reactive site loop of PAI-1 (Simon *et al.*, 1995). In the latter, a preferential susceptibility was observed for active PAI-1 whereas latent PAI-1 was much more resistant.

In conclusion, we have shown that insertion of the reactive site loop is not directly associated with the functional properties of the serpin PAI-1 and provided further evidence that the initial conformation of PAI-1, prior to its interaction with its target proteinases, determines the reaction pathway. In addition, we have identified a previously unrecognized plasmin cleavage site in latent PAI-1. The latter observation may have implications in the irreversible inactivation of latent PAI-1 under pathophysiological situations.

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## REFERENCES

Aertgeerts, K., Debondt, H. L., De Ranter, C. J., & Declerck, P. J. (1995*) Nat. Struct. Biol. 2*, 891−897.

Audenaert, A. M., Knockaert, I., Collen, D., & Declerck P. J. (1994) *J. Biol. Chem. 269*, 19559−19564.

Baumann, U., Huber, R., Bode, W., Grosse, D., Lesjak, M., & Laurell, C. B. (1991) *J. Mol. Biol. 218*, 595−606.

Björk, I., Ylinenjärvi, K., Olson, S. T., & Bock, P. E. (1992) *J. Biol. Chem. 267*, 1976−1982.

Carrell, R. W., & Boswell, D. R. (1986) in *Proteinase Inhibitors* (Barret, A. J., & Salvesen, G., Eds.), pp 403−425, Elsevier Scientific Publishing Co., Amsterdam, The Netherlands.
Carrell, R. W., Evans, D. L., & Stein, P. (1991) *Nature 353*, 576−578.
Carrell, R. W., Stein, P. E., Fermi, G., & Wardell, M. R. (1994) *Structure 2*, 257−270.
Declerck, P. J., Alessi, M. C., Verstreken, M., Kruithof, E. K. O., Juhan-Vague, I., & Collen, D. (1988) *Blood 71*, 220−225.
Declerck, P. J., De Mol, M., Vaughan, D. E., & Collen, D. (1992) *J. Biol. Chem. 267*, 11693−11696.
Declerck, P. J., Juhan-Vague, I., Felez, J., & Wiman, B. (1994) *J. Int. Med. 236*, 425−432.
Eitzman, D. T., Fay, W. P., Lawrence, D. A., Francis-Chmura, A. M., Shore, J. D., Olson, S. T., & Ginsburg, D. (1995) *J. Clin. Invest. 95*, 2416−2420.
Gettins, P., & Harten, B. (1988) *Biochemistry 27*, 3634−3639.
Gettins, P., Patston, P. A., & Schapira, M. (1993) *Bioessays 15*, 461−467.
Ginsberg, D., Zeheb, R., Young, A. Y., Rafferty, U. M., Andreasen, P. A., Nielsen, L., Danø, K., Lebo, R. V., & Gelehrter, T. D. (1986) *J. Clin. Invest. 78*, 1673−1680.
Hamsten, A., Wiman, B., de Faire, U., & Blombäck, M. (1985) *N. Engl. J. Med. 313*, 1557−1563.
Hekman, C. M., & Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581−11587.
Holmes, W. E., Lijnen, H. R., Nelles, L., Kluft, C., Nieuwenhuis, H. K., Rijken, D. C., & Collen, D. (1987) *Science 238*, 209−211.
Hood, D. B., Huntington, J. A., & Gettins, P. G. W. (1994) *Biochemistry 33*, 8538−8547.
Hopkins, P. C. R., Carrell, R. W., & Stone, S. R. (1993) *Biochemistry 32*, 7650−7657.
Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951−8966.
Huntington, J. A., Patston, P. A., & Gettins, P. G. W. (1995) *Protein Sci. 4*, 613−621.
Kruithof, E. K. O., Tran-Thang, C., Ransijn, A., & Bachmann, F. (1984) *Blood 64*, 907−913.
Laskowski, M., & Kato, I. (1980) *Annu. Rev. Biochem. 49*, 593−626.
Lawrence, D. A., Olson, S. T., Palaniappan, S., & Ginsburg, D. (1994) *J. Biol. Chem. 269*, 27657−27662.
Levy, N. J., Ramesh, N., Cicardi, M., Harrison, R. A., & Davis, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 265−268.
Lindahl, T. L., Ohlsson, P. I., & Wiman, B. (1990) *Biochem. J. 265*, 109−113.
Loebermann, H., Tokuoka, R., Deisenhofer, J., & Huber, R. (1984) *J. Mol. Biol. 177*, 531−556.
Loskutoff, D. J., Sawdey, M., & Mimuro, J. (1989) *Hemostasis Thromb. 9*, 87−115.
Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K.F., Gerard, R. D., & Goldsmith, E. J. (1992) *Nature 355*, 270−273.
Munch, M., Heegaard, C. W., & Andreasen, P. A. (1993) *Biochim. Biophys. Acta 1202*, 29−37.
Ny, T., Sawdey, M., Lawrence, D., Milan, J. L., & Loskutoff, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6776−6780.
Pannekoek, H., Veerman, H., Lambers, H., Diergaarde, P., Verweij, C. L., Van Zonneveld, A. J., & Van Mourik, J. A. (1986) *EMBO J. 5*, 2539−2544.
Pemberton, P. A., Harrison, R. A., Lachmann, P. J., & Carrell, R. W. (1989) *Biochem. J. 258*, 193−198.
Pemberton, P. A., Wong, D. T., Gibson, H. L., Kiefer, M. C., Fitzpatrick, P. A., Sager, R., & Barr, P. J. (1995) *J. Biol. Chem. 270*, 15832−15837.
Perry, D. J., & Carrell, R. W. (1989) *Mol. Biol. Med. 6*, 239−243.
Sambrook, J., Fritsch, E. F., Maniatis, T. (1989) *Molecular cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sancho, E., Tonge, D. W., Hockney, R. C., & Booth, N. (1994) *Eur. J. Biochem. 224*, 125−134.
Sancho, E., Declerck, P. J., Price, N. C., Kelly, S. M., & Booth N. A. (1995) *Biochemistry 34*, 1064−1069.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Schreuder, H. A., deBoer, B., Dijkema, R., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., & Hol., W. G. J. (1994) *Nat. Struct. Biol. 1*, 48−54.
Schulze, A. J., Baumann, U., Knof, S., Jaeger, E., Huber, R., & Laurell, C. (1990) *Eur. J. Biochem. 194*, 51−56.
Shore, J. D., Day, D. E., Francis-Chmura, A. M., Verhamme, I., Kvassman, J., Lawrence, D. A., & Ginsburg, D. (1995) *J. Biol. Chem. 270*, 5395−5398.
Simon, D. I., Xu, H., & Vaughan, D. E. (1995) *Biochim. Biophys. Acta 1268*, 143−151.
Skriver, K., Wikoff, W. R., Patston, P. A., Tausk, F., Schapira, M., Kaplan, A. P., & Bock, S. C. (1991) *J. Biol. Chem. 266*, 9216−9221.
Sprang, S. R. (1992) *Trends Biochem. Sci. 17*, 49−50.
Sprengers, E. D., & Kluft,C. (1987) *Blood 69*, 381−387.
Stanssens, P., Opsomer, C., McKeown, Y., Kramer, W., Zabeau, M., & Fritz, M. J. (1989) *Nucleic Acids Res. 17*, 4441−4454.
Stein, P., & Chothia, C. (1991) *J. Mol. Biol. 221*, 615−621.
Stein, E. P., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J., & Carrell, R. W. (1990) *Nature 347*, 99−102.
Tucker, H. M., Mottonen, J., Goldsmith, E. J., & Gerard, R. D. (1995) *Nat. Struct. Biol. 2*, 442−445.
Urano, T., Strandberg, L., Johansson, L. B., & Ny, T. (1992) *Eur. J. Biochem. 209*, 985−992.
Van Mourik, J. A., Lawrence, D. A., & Loskutoff, D. J. (1984) *J. Biol. Chem. 259*, 14914−14921.
Verheijen, J. H., Chang, G. T. G., & Kluft, C. (1984) *Thromb. Haemostasis 51*, 392−395.
Wei, A., Rubin, H., Coopermans, B. S., & Christianson, D. W. (1994) *Nat. Struct. Biol. 1*, 251−258.
Wilczynska, M., Fa, M., Ohlsson, P., & Ny, T. (1995) *J. Biol. Chem. 270*, 29652−29655.
Wright, H. T., Qian, H. X., & Huber, R. (1990) *J. Mol. Biol. 213*, 513−528.
Wright, H. T., & Blajchman, M. A. (1994) *FEBS Lett. 348*, 14−16.

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