Purification and Characterization of Active and Latent Forms of Recombinant Plasminogen Activator Inhibitor 1 Produced in *Escherichia coli*

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ABSTRACT: Plasminogen activator inhibitor 1 (PAI-1), the principal physiological inhibitor of tissue plasminogen activator (tPA), is a protein of 379 amino acids and belongs to the SERPIN family of serine protease inhibitors. We have previously described methods to express [Sisk et al. (1990) *Gene 96*, 305–309] and purify [Reilly et al. (1990) *J. Biol. Chem. 265*, 9570–9574] a highly active form of the protein in substantial amounts, from *Escherichia coli*. Further analyses of this material showed the presence of small but significant amounts of latent rPAI-1. The present paper describes for the first time purification of latent and active forms of rPAI-1 from a single preparation, as well as the functional and structural characteristics of the two forms. Latent rPAI-1, which has properties similar to the latent forms described by other groups, was separated from active rPAI-1 by high-resolution ion-exchange chromatography or by affinity chromatography using immobilized anhydrotrypsin. It had low intrinsic activity (<5% of active rPAI-1) and was partially reactivated by guanidine hydrochloride treatment or by incubation with vitronectin. Conversion of the active rPAI-1 to the latent form was influenced by temperature and additives including sucrose, EDTA, and arginine. Active and latent rPAI-1 did not show any obvious differences in their primary structures and displayed remarkably similar secondary structures as determined by circular dichroism spectral analyses. However, they did exhibit differences in tryptophan fluorescence, suggesting tertiary structural differences between the two forms.

Plasminogen activator inhibitor 1 (PAI-1) is a specific and fast acting inhibitor of both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Chmieleweska et al., 1983, 1988; Kruithof et al., 1984, 1987; Alessi et al., 1988; Hekman & Loskutoff, 1988; Lawrence et al., 1989). The protein consists of 379 amino acid residues and belongs to the SERPIN superfamily of serine protease inhibitors (Carrell & Boswell, 1986; Ny et al., 1986; Pannekoek et al., 1986; Sprengers & Kluft, 1987; Ye et al., 1987; Boswell & Carrell, 1988). Like other SERPINS, PAI-1 has a reactive center located on a highly strained exposed loop near the C-terminus of the molecule (Carrell & Boswell, 1986; Boswell & Carrell, 1988; Huber & Carrell, 1989). The "reactive center" of PAI-1 contains the "bait" peptide bond between residues R(346) and M(347), i.e., the P1-P1′ residues (Andreasen et al., 1986; Boswell & Carrell, 1988). This bond mimics the R(560)–V(561) bond of plasminogen, which is the bond cleaved by the plasminogen activators during the activation of plasminogen to plasmin (Robbins et al., 1967; Wiman & Wallen, 1973). On the basis of the mechanism of action of other SERPINS, it has been postulated that PAI-1 binds specifically to the plasminogen activators like a substrate (Carrell & Travis, 1985). However, unlike a normal substrate, the formation of the enzyme–substrate complex does not lead to the formation and release of products. Instead, a covalent bond is formed between the active-site Ser of the plasminogen activators and the P1 residue (Arg-347), resulting in an SDS-stable complex and irreversible inactivation of the enzymes.

Native PAI-1 has been obtained from several cell lines (van Mourik et al., 1984; Nielsen et al., 1986; Wagner & Binder, 1986; Booth et al., 1987; Wun et al., 1989), while recombinant PAI-1 has been obtained from yeast (Gardell et al., 1990) and *Escherichia coli* (Franke et al., 1990; Reilly et al., 1990) using recombinant DNA techniques. The PAI-1 obtained from these sources is either in the "active" or in the "latent" form. The active form of the protein is spontaneously converted to the "latent" form (Lawrence et al., 1989; Franke et al., 1990; Reilly et al., 1990), which can in turn be partially reactivated by treatment with a detergent or with guanidine hydrochloride (Hekman & Loskutoff, 1985; Erickson et al., 1986). Carrell et al. (1991) have postulated that latent PAI-1 has a greater number of residues from the strained reactive center loop inserted between sheets 3 and 5 in the deduced structure of PAI, leading to a collapse of the strained loop and loss of inhibitory activity. The recently solved crystal structure of latent rPAI-1 shows that this postulate is essentially true (Mottonen et al., 1992). The structure reveals that residues on the N-terminal side of the primary recognition site are inserted as a central strand of the largest β-sheet, while the residues C-terminal to the recognition site occupy positions on the surface of the molecule. Although this interconversion has been fairly well documented and studied in the case of rPAI-1, latent structures have been reported in the case of other SERPINS also (Carrell et al., 1991), suggesting that it may be a general phenomenon associated with SERPIN-type protease inhibitors.

Development of methods to purify active and latent forms of PAI-1 from a single preparation would be an important step in obtaining a detailed structure for active rPAI-1 and for further studies on the nature and physiological relevance of the interconversion between the two forms of rPAI-1. We have previously described methods to express (Sisk et al., 1990) and purify (Reilly et al., 1990) active PAI-1 from *E. coli* in substantial quantities. Further analyses of this material indicated the presence of small but significant amounts of "latent" PAI-1. In the present report, we describe the separation of the active and latent forms of rPAI-1, as well as the functional and structural characteristics of the two forms.

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EXPERIMENTAL PROCEDURES

Materials. Natural PAI-1, purified from human fibrosarcoma, was purchased from American Diagnostica and was activated by SDS treatment as described (Heckman & Loskutoff, 1985). One-chain tPA, two-chain tPA, and the low molecular weight form of urokinase were also purchased from American Diagnostica. Vitronectin was obtained from Calbiochem, La Jolla, CA. Precast gels manufactured by Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan, and other reagents for electrophoresis were obtained from ISS Enprotech (Hyde Park, MA). Prestained and unstained molecular weight markers were obtained from BRL (Gaithesburg, MD). CNBr and TFA as well as anhydrotrypsin (supplied as part of a kit) were obtained from Pierce Chemical Co., Rockford, IL. Acetonitrile was obtained from E. M. Scientific, Gibbstown, NJ. Water was obtained from a Nanopure II system (Barnstead, Boston, MA). All other reagents were of analytical grade.

Bacterial Expression and Purification of Recombinant PAI-1. The expression of the rPAI encoded by the plasmid pCE 1200 in *E. coli* host TAP106 was carried out as described previously (Sisk et al., 1990). The bacteria expressing rPAI-1 were cultivated at 34 °C until they reached mid-log phase, and production of rPAI-1 was initiated at this point by raising the temperature as rapidly as possible to 42 °C. After 3–7 h at this temperature, the culture was chilled to less than 20 °C, and the cells were harvested by centrifugation. The cell paste was then stored at −70 °C until further processing.

Cell lysates and supernatants were prepared as described (Reilly et al., 1990) except that the cells were disrupted in 150 mM sodium phosphate buffer, pH 6. Purification of rPAI-1 from the supernatants was carried out at 4 °C by a modification of the previously published procedure (Reilly et al., 1990). The fractions containing rPAI-1 were identified using activity measurements, analytical SDS–PAGE, and HPLC as described in the appropriate sections. The supernatant was passed through a QAE anion-exchange cartridge (Cuno Inc.) previously equilibrated with 150 mM sodium phosphate buffer, pH 6. The flow-through from the QAE cartridge which contained the rPAI was pumped onto a SP cation-exchange cartridge (Cuno Inc.) previously equilibrated with 150 mM sodium phosphate, pH 6. The SP cartridge was washed with the same buffer until the effluent showed the starting UV absorbance at 220 nm, and the rPAI was eluted from the column using 150 mM sodium phosphate, pH 8.6. The pH of the rPAI-containing pool was adjusted to 6 and loaded onto a 6 × 45 cm S-Sepharose HP column (Pharmacia) previously equilibrated with 150 mM sodium phosphate, pH 6. The column was washed with additional amounts of the same buffer until the starting UV absorbance at 220 nm was reached. The rPAI-1 bound to the S-Sepharose column was then eluted using a NaCl gradient in 150 mM phosphate buffer, pH 6.

Protein Estimation. Protein concentration was determined either by using the BCA assay (Pierce, Rockford, IL) or by amino acid analyses after vapor-phase hydrolysis (6 N HCl for 90 min at 165 °C) (Dupont et al., 1988), using a Beckman 6300 analyzer (Beckman Instruments).

Assay of PAI-1 Activity. tPA inhibitory activity of rPAI-1 was determined according to the method of Verheijen et al. (1982) as described previously (Reilly et al., 1990).

SDS–PAGE. Analytical SDS–PAGE was carried out using precast 10–20% gradient gels. Electroblotting onto poly(vinylidine difluoride) (PVDF) was carried out as described by Matsudaira (1987).

Activation of Latent rPAI-1. The activation of latent rPAI-1 (purified peak L material) was carried out essentially as described by Erickson et al. (1986). rPAI-1 samples were diluted to a concentration of 0.1 mg/mL with 175 mM sodium phosphate, pH 8.6, dialyzed against 6 M guanidine hydrochloride at 37 °C for 4.5 h, and then dialyzed against 10 mM sodium acetate, pH 5.5, containing 10 mM NaCl and 0.01% lubrol-PX at 4 °C.

Analytical HPLC. Reverse-phase HPLC was carried out using either a C4 (for protein separations) or a C18 (for peptide separations) Vydac column (2.1 or 4.6 × 250 mm) (Separation Systems, Hesperia, CA) connected to an HP1090M HPLC (Hewlett Packard, Avondale, PA). The column was equilibrated in solvent A, i.e., 0.1% TFA (v/v) in water. Material was eluted from the column using increasing concentrations of solvent B, i.e., 0.1% TFA (v/v) in acetonitrile at 150 μL/min for the 2.1-mm columns and at 1 mL/min for the 4.6-mm columns. The effluent was monitored at 212 nm. The peaks were collected manually and stored at −70 °C.

Ion-exchange HPLC was performed using an HR5/5 Mono Q (strong anion exchanger) obtained from Pharmacia connected to an HP 1090M HPLC. The Mono Q column was equilibrated in 50 mM ammonium bicarbonate, pH 9.0, at 2 mL/min. About 25–100 μg of rPAI-1 was loaded on the column. The effluent was monitored at 280 nm, and the rPAI-1 was eluted from the column by increasing the concentration of NaCl in the equilibration buffer from 0 to 300 mM in 20 min.

Affinity Chromatography Using Immobilized Anhydrotrypsin. The procedure used for anhydrotrypsin-based affinity chromatography of rPAI-1 was essentially similar to the one using immobilized anhydrourokinase (Wun et al., 1989). The anhydrotrypsin column (1-mL bed volume) was equilibrated with 150 mM sodium phosphate, pH 7.8; 0.15 mg of rPAI-1 in 0.5 mL of 150 mM sodium phosphate, pH 8.6, was then loaded onto the column, which was eluted sequentially with 10 mL each of ammonium bicarbonate, pH 8.6, 150 mM sodium phosphate, pH 7.8, and 0.5 M L-Arg·HCl in 150 mM sodium phosphate, pH 6.5. The eluted material was analyzed by analytical HPLC and SDS–PAGE and assayed for tPA inhibitory activity.

Protein Sequencing. N-Terminal sequence analyses were carried out using 0.05–1 nmol of rPAI-1 by automated Edman degradation chemistry with a Porton PI2090E gas-phase sequencer (Porton Instruments, Tarzana, CA). The respective phenylthiohydantoin derivatives of the amino acids were analyzed in an on-line fashion. The Edman degradations as well as the phenylthiohydantoin amino acid analyses were performed using the protocols recommended by the manufacturer.

Tryptic, N-Chlorosuccinamide, and CNBr Digestion of rPAI-1. These digestions were carried out with 0.1–1 nmol of rPAI-1 essentially as described by Lee and Shively (1990). Active rPAI-1 and latent rPAI-1 were digested with trypsin in 50 mM ammonium bicarbonate, pH 8.6, at 37 °C, using an enzyme to substrate ratio of 1:100. After the desired length of digestion, the sample was subjected to analytical SDS–PAGE to determine the extent of digestion.

CNBr digestions were carried out with 5 mg of CNBr in 50 μL of 70% v/v TFA at 25 °C for 4 h. After digestion, the samples were dried down in a Speed Vac (Savant Instruments, Hicksville, NY), redissolved in an appropriate volume of 70% TFA, and subjected to reverse-phase HPLC.

The *N*-chlorosuccinamide digestions were carried with 0.15 M *N*-chlorosuccinamide at 25 °C after adjustment of the pH

of the rPAI-1 to 4.0 with acetic acid. The digests were precipitated with 0.1 volume of 50% TCA containing 2 mg/mL deoxycholate (to act as a carrier during precipitation) at 0 °C for 20 min prior to SDS–PAGE or were loaded directly onto a C4 Vydac column.

Oxidation of rPAI-1 with N-Chlorosuccinamide. A 0.1–1-nmol aliquot of rPAI-1 was treated with a 170-fold molar excess of *N*-chlorosuccinamide at 25 °C for 30 s. The reaction was quenched by the addition of excess methionine and stored frozen at −70 °C until further analyses. The oxidized rPAI-1 generated was completely resistant to CNBr digestion and partially resistant to *N*-chlorosuccinamide cleavage, indicating extensive oxidation of the Met residues and partial oxidation of the tryptophan residues.

Matrix-Assisted Laser Desorption Time of Flight Mass Spectroscopy. These were carried out using a Lasermat mass spectrometer (Finnigan Mat, San Diego, CA). rPAI-1 samples in the S-Sepharose HP buffer, i.e., 150 mM phosphate, pH 6.0, with 400–500 mM NaCl at a concentration of 1–1.2 mg/mL, were used directly. Sinapinic acid was used as the matrix, and the mass analyses were performed using the procedures and protocols recommended by the manufacturer.

Circular Dichroism Spectroscopy. CD spectra of the active and latent forms of rPAI-1 were obtained on a JASCO-600 spectropolarimeter. The samples were placed in a 0.05-cm path-length cylindrical quartz cell. The samples were prepared in a 44 mM phosphate buffer, pH 7.4, with a protein concentration of 100 μg/mL. The final spectra were an average of nine scans, and the base lines were corrected by subtracting the contribution of the buffer. Before the CD experiments, the samples were stored on ice; however, the spectra were recorded at room temperature.

Fluorescence Spectroscopy. Fluorescence measurements were made on an SLM-8000 spectrofluorometer. The samples were prepared in 44 mM phosphate buffer, pH 7.4, with protein concentrations of 90 μg/mL. The samples were placed in a 1.0-cm path-length quartz cuvette for recording the emission spectra. The excitation wavelength was set at 295 nm in order to preferentially excite the tryptophan residues. To correct for any fluctuation in the light source, the emission intensity was measured as a ratio of the photon counts at the photomultiplier tube (PMT) of the emission channel to the photon counts at the PMT of a reference channel (Dwivedi et al., 1991). The spectra were corrected for any contribution from the buffer.

RESULTS AND DISCUSSION

Separation of Active and Latent rPAI-1. S-Sepharose HP column chromatography of the rPAI-1 peak eluted from a SP-cartridge yielded two minor and two major peaks (Figure 1). The first major peak (peak A) eluted around 510 nM NaCl, and was followed immediately by the second major peak (peak L). A single predominant band at the position expected for rPAI-1 was present in both peak A and peak L pools (Figure 1, inset). However, it is clear from Figure 1 that most of the tPA inhibitory activity is associated with peak A and that peak L has minimal inhibitory activity. All fractions corresponding to the ascending side of peak A and those fractions with tPA inhibitory activity equal to or greater than 8.5×10^5 IU/mL on the descending side of the peak A were pooled to obtain the active peak A pool. In order to obtain the peak L pool, fractions with less than 1×10^5 IU/mL were pooled and rechromatographed on the S-Sepharose HP column. The rechromatography yielded a major peak at the peak L position with a small shoulder at the peak A position.

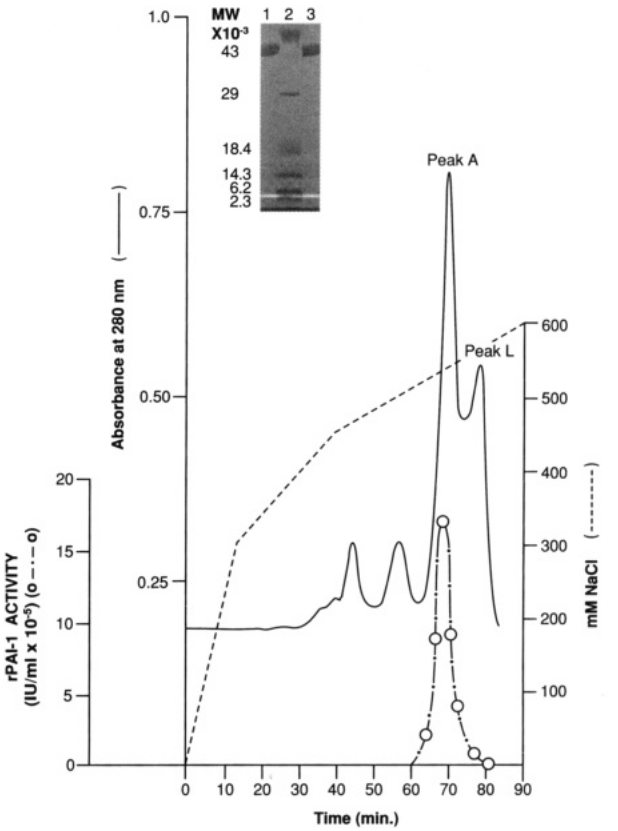


FIGURE 1: Purification of rPAI-1 by cation-exchange chromatography on S-Sepharose HP. A 6 × 45 cm column was used. The flow rate was 25 mL/min, and 1-min fractions were collected. Other conditions were as described under Experimental Procedures. The fractions were assayed for inhibitory activity against 1 IU of tPA in the S2251 assay. The inset shows the Coomassie Blue stained gel obtained on SDS–PAGE of the pooled peak A and peak L material. Lane 1, peak A pool; lane 2, prestained molecular weight standards; lane 3, peak L pool.

The fractions with activity less than or equal to 0.1×10^5 IU/mL from the second run were pooled and represent the rechromatographed peak L pool. The peak A and peak L pools thus obtained were about 90 and 98% pure, respectively, on the basis of analytical HPLC on Mono Q. Specific activity calculations yielded values of about 550 000 and 5000 IU/mg for the peak A and L pools, respectively. It is quite likely that most of the activity associated with the peak L fraction is due to small amounts of peak A contamination and that the intrinsic specific activity of peak L is significantly lower than 5000 IU/mg.

Primary Structural Studies. Mass spectral analyses of the material from peaks A and L, under identical conditions, yielded an average molecular weight of about 43 350, indicating that the two proteins do not differ significantly in their molecular weights. The two forms of rPAI-1 also gave the same N-terminal sequence up to 35 places. The peptide maps obtained after CNBr and *N*-chlorosuccinamide digestions of the two rPAI-1 forms were very similar. These data indicate that, as expected, the two proteins have remarkably similar primary structures.

Activation of Latent rPAI-1. In order to determine whether the material obtained from peak L represents latent rPAI-1, it was treated with guanidine hydrochloride which is known to activate latent rPAI-1 (Hekman & Loskutoff, 1985; Erickson et al., 1986). Such treatment restored significant inhibitory activity to the peak L material (5.6×10^3 IU/mg before treatment to 3.2×10^4 IU/mg following the treatment), while peak A material was not significantly affected.

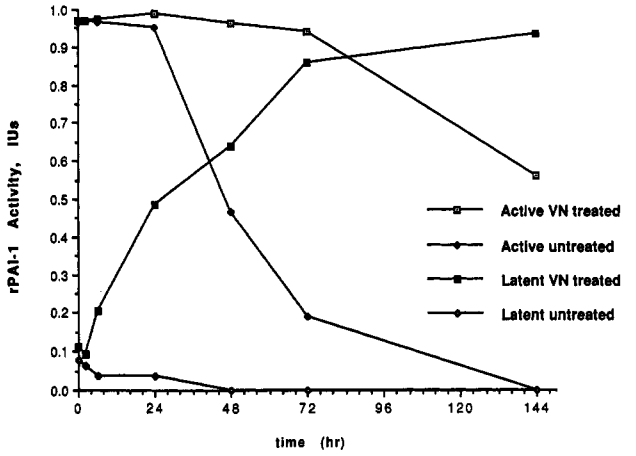


FIGURE 2: Activation of latent rPAI-1 and stabilization of active rPAI-1 by vitronectin. Active rPAI-1 and latent rPAI-1 were incubated at room temperature in the absence and presence of a 5-fold molar excess of vitronectin. At the indicated times, 10 ng of active rPAI-1 and 50 ng of latent rPAI-1 were tested for inhibitory activity against 1 IU of tPA in the S2251 assay.

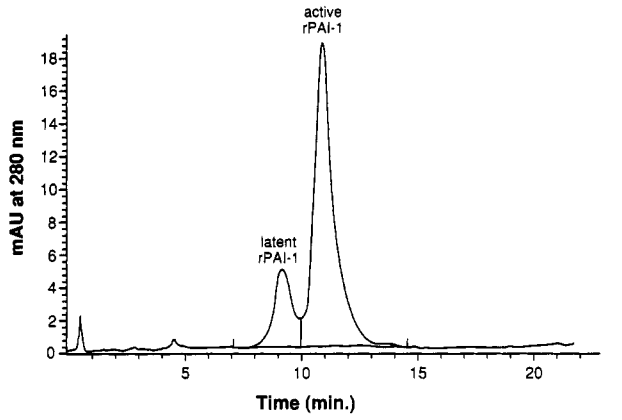


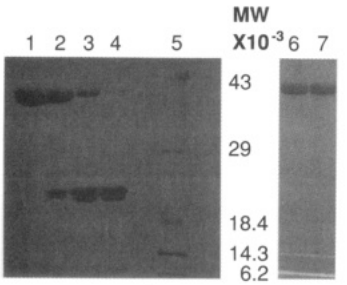
FIGURE 3: Ion-exchange HPLC separation of latent and active rPAI-1. The Mono Q ion-exchange column was equilibrated in 50 mM ammonium bicarbonate, pH 9.0, at a flow rate of 2.0 mL/min and the effluent monitored at 280 nm. rPAI-1 was loaded on the column in the same buffer and eluted from the column by increasing the concentration of NaCl in the equilibration buffer from 0 to 300 mM in 20 min.

Functionally active rPAI-1 in plasma is bound to a discrete binding protein identified as vitronectin (Mimuro & Loskutoff, 1989). Previously, we demonstrated that vitronectin stabilizes the active form of rPAI-1, and is also effective in reactivating rPAI-1 which had been converted to a latent form by prolonged incubation at 37 °C (Reilly et al., 1992). Accordingly, we determined the effect of vitronectin on the activity of the PAI-1 in peaks A and L. As shown in Figure 2, incubation with vitronectin partially restored the inhibitory activity of the peak L material and also stabilized the activities of both forms. The results with guanidine hydrochloride and vitronectin activation indicate that peak L corresponds to the latent form of rPAI-1.

Analytical HPLC of Latent and Active rPAI-1. Active rPAI-1 and latent rPAI-1 gave identical retention times when chromatographed on a C4 reverse column using acetonitrile/water/TFA. However, the two rPAI-1 forms could be separated analytically using the Mono Q ammonium bicarbonate system (Figure 3). Accordingly, rPAI-1 preparations were routinely analyzed for the relative amounts of active and latent rPAI-1 using analytical ion-exchange chromatography.

Affinity Chromatography Using Anhydrotrypsin. To further characterize active and latent forms of rPAI-1, we explored their binding affinities to anhydrotrypsin, a catalytically inert form of trypsin in which the active-site Ser (195) is converted to a dehydroalanine residue (Ishii et al., 1983). This modified trypsin derivative retains its ability to bind naturally occurring inhibitors of trypsin (Ako et al., 1974; Vincent et al., 1974). Wun et al. (1989) used the similar property of anhydrourokinase to develop a method for the affinity purification of active PAI-1. However, the amount of latent PAI-1 in their preparation was not determined, making it difficult to determine if latent rPAI-1 and active rPAI-1 can be separated by an affinity chromatographic procedure. Hence, we decided to investigate if a similar affinity procedure, using anhydrotrypsin instead of anhydrourokinase, would be suitable for the separation of latent and active rPAI-1. Under the conditions described under Experimental Procedures, latent rPAI-1 eluted unbound while active rPAI-1 bound to the column and could be eluted with phosphate buffer (150 mM, pH 6.5) containing 0.5 M Arg (data not shown). These results indicate that active rPAI-1 and latent rPAI-1 have very different binding affinities toward anhydrotrypsin. This is probably due to significant structural differences in and around their reactive centers as suggested by the crystal structure of latent rPAI-1 (Mottonen et al., 1992) and the deduced structure of rPAI-1 (Carrell et al., 1991). Since the active and latent forms of other SERPINS also have similar structural differences around the reactive center (Carrell et al., 1991), anhydrotrypsin affinity chromatography may also be useful in separating latent and active forms of other SERPINS. The active rPAI-1 and latent rPAI-1 obtained by this method were about 98% pure, demonstrating that it is very effective in resolving active from latent rPAI-1 and is suitable for obtaining active and latent rPAI-1 of extremely high purity.

Stabilization of Active rPAI. Active rPAI-1 obtained after the S-Sepharose HP could be stored at −70 °C for months with minimal loss in activity and negligible conversion to latent rPAI-1 (Reilly et al., 1990). However, the rPAI-1 lost about 50% of its activity after 2 days at 25 °C or after 2 h at 37 °C due to conversion to the latent form; significant conversion to the latent form also occurred at 4 °C (Reilly et al., 1990). This conversion represented a major challenge in the process scale-up to purify large quantities of active rPAI-1, since it was often necessary to repeat the final S-Sepharose HP ion-exchange chromatography step to obtain rPAI-1 with the desired purity and activity. These additional steps significantly reduced the purification yields. Consequently, the influence of a series of additives on the conversion of active to latent rPAI-1 was investigated. Conversion was monitored by incubating active rPAI-1 in the absence and presence of different additives at 37 °C for 2 h, reapplying the material to a Mono Q column, determining the area of the fraction eluting at the active rPAI-1 position, and comparing the value to untreated active rPAI-1 (100%). Arginine (125 mM) and EDTA (25 mM), used separately or in combination, were quite effective in stabilizing the active conformation since they decreased the formation of latent rPAI-1 by about 10–15%. Sucrose (334 mM) in combination with arginine and EDTA produced a synergistic effect and decreased the formation of latent rPAI-1 by about 30%, although it was only marginally effective when used alone. The protective effect of sucrose was quite specific in that glycerol, another polyhydroxy compound which is commonly used to stabilize proteins, was not very effective. The extent of stabilization was about the same at 25 and 4 °C. The results suggest that these additives can be used to stabilize active rPAI-1 during

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301  FRQFQADFTS LSDQEPLHVA QALQKVKIEV NESGTVASSS TAVIVSARMA

351  PEEIIMDRPF LFVVRHNPTG TVLFMGQVME P
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FIGURE 4: (Top) Trypsin digestion of active and latent rPAI-1. The tryptic digestions were carried out at 37 °C as described under Experimental Procedures, and aliquots containing 10 mg of rPAI-1 were analyzed by SDS–PAGE at different time intervals. The protein bands were visualized by staining with Coomassie Blue. The SDS gel patterns are shown in panel A. Lanes 1–4, latent rPAI-1 after digestion with trypsin for 0, 4, 12, and 24 h, respectively; lane 5, prestained molecular weight standards; lanes 6 and 7, active rPAI-1 after digestion with trypsin for 0 and 24 h, respectively. (Bottom) Alignment of the tryptic peptide sequences with the rPAI-1 sequence. The N-terminal sequences derived from the 20-kDa bands obtained by SDS–PAGE of the tryptic digests of latent rPAI-1 (shown in the top panel) have been aligned with the deduced sequence of rPAI-1 used in the present study (Sisk et al., 1990; Reilly et al., 1990).

purification, storage, and structural studies using crystallography and spectral methods.

Trypsin Digestion of Latent and Active rPAI-1. SDS–PAGE analysis of the products obtained after trypsin digestion of latent and active rPAI-1 is shown in Figure 4 (top panel). Two closely spaced bands with a MW of about 20K, along with significant amounts of full-length rPAI-1, were detected after 4 h of digestion of the latent rPAI-1. The amount of these two digestion products increased with time, and by 24 h at 37 °C, most of the full-length material was converted to the two lower molecular weight forms. The two 20K bands were extremely stable to further digestion, since they were the only detectable bands even after 24 h. This indicates that trypsin cleavage of the latent rPAI-1 is limited to only a few of the 37 possible trypsin-susceptible sites. On the other hand, oxidized rPAI-1, prepared by treatment with *N*-chlorosuccinamide, yielded only low molecular weight fragments on SDS–PAGE analyses within 12 h of trypsin digestion, indicating that it is very sensitive to trypsin digestion (data not shown). In contrast, active rPAI-1 was completely resistant to trypsin digestion under these circumstances (Figure 4, top). These results suggest that latent rPAI-1, unlike oxidized rPAI-1, does not retain some of the native structural features which confer resistance to trypsin digestion.

The size of the proteolytic fragments obtained by trypsin digestion of latent rPAI-1 indicated that the trypsin cleavage sites are localized around the middle of the molecule. This was confirmed by N-terminal sequence analyses, and the data

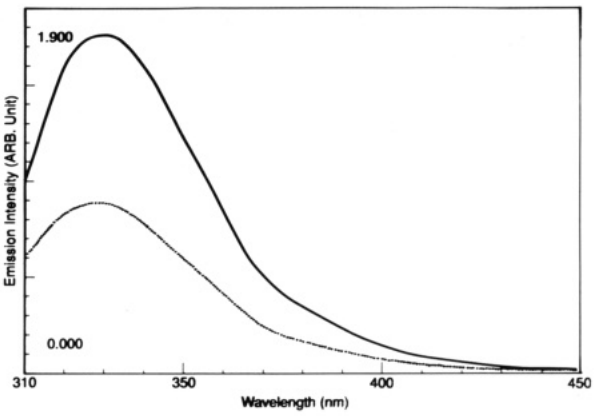

FIGURE 5: Fluorescence spectra of active and latent rPAI-1. The conditions used to record the fluorescence spectra are described under Experimental Procedures. Active rPAI-1 (—); latent rPAI-1 (···).

are summarized in Figure 4, bottom panel. The upper 20K band (band 1) gave the following two main sequences: SIVHHPPSYVAH (band 1, sequence 1), corresponding to the N-terminus of the rPAI-1, and LFHKSDGSTVS (band 1, sequence 2), corresponding to a fragment starting at residue 190 of rPAI-1. A third minor sequence, SDGSTVSV (band 1, sequence 3), corresponding to residue 194 of rPAI-1, was also obtained. The lower band (band 2) yielded the following main sequences: SDGSTVSVPMMA (band 2, sequence 1), along with minor sequences corresponding to the two main sequences from the upper band. These results indicate that the two main trypsin-susceptible bonds in latent rPAI are R(189)–L(190) and K(193)–S(194). Digestion of the R(188)–R(189) bond cannot be ruled out, since the fragment starting at L(190) could also be generated by sequential trypsin digestions after R(188) and R(189), respectively. Taken together, the trypsin digestion studies suggest that the central region of the latent rPAI-1 is exposed to the solvent while the regions containing the other trypsin-sensitive sites are relatively buried.

CD Spectra. The presence of extensive secondary structure in latent rPAI-1 was further supported by a comparison of the CD spectra of the active and latent forms of rPAI-1. The two forms of rPAI-1 gave very similar CD spectra, suggesting that their secondary structures are virtually identical. These data agree with those obtained for the latent rPAI-1 generated by incubating active rPAI at 37 °C (Strandberg et al., 1990; Dwivedi et al., 1991), but differ from those reported for latent and reactivated PAI-1 from conditioned cell culture medium (Bostrom et al., 1990). Although the reason for these structural differences between the PAI-1 obtained from different sources is not clear, glycosylation of the PAI-1 obtained from conditioned cell culture medium may be an important factor (Dwivedi et al., 1991). It is interesting to note that the secondary structure of latent rPAI-1 obtained by the scheme reported in the present study is virtually indistinguishable from that of the latent form generated by incubating active rPAI-1 at 37 °C (Dwivedi et al., 1991). This indicates that these two processes give rise to very similar secondary structures.

Fluorescence Spectra. Fluorescence emission spectra of the two forms are shown in Figure 5. Since both active rPAI-1 and latent rPAI-1 have four tryptophans per protein molecule, one would expect that the spectral intensities should be the same for two samples with the same protein concentration. On the contrary, the tryptophan emission intensity in the active form was almost twice that of the latent form. In addition, the emission maximum in the active form is red-shifted by

about 4 nm. The latent rPAI-1 obtained either by incubating active rPAI at 37 °C (Dwivedi et al., 1991) or by purification from conditioned cell culture medium (Broström et al., 1990) also showed similar fluorescence spectra. The fluorescence spectral data indicate that the tryptophans in the active and latent forms do not have the same environment. These data are further supported by the fact that tryptophan residues of active rPAI-1 are more accessible for quenching than those of the latent rPAI-1 (Dwivedi et al., 1991). These results suggest that the latent rPAI-1 has a more compact structure than active rPAI-1, an expected result based on the report that the latent form has a greater number of residues from the strained loop region inserted between the β-sheets (Carrell et al., 1991; Mottonen et al., 1992).

In summary, it is clear that the molecular events that cause the structural alterations in rPAI-1 are very complex. The purification of active and latent rPAI-1 molecules from a single preparation and the various biochemical and physical studies on these two forms as discussed in this report provide simple and rapid methods to obtain highly purified and well-characterized preparations of active and latent rPAI-1. These reagents will be very useful in obtaining a detailed structure for active rPAI-1 by crystallography and other techniques and in studying the interconversion between active and latent rPAI-1. Such information obtained may be useful in understanding the active to latent transition which has also been observed with a series of other SERPINS including antithrombin, α_1-antitrypsin, C1-inhibitor, heparin cofactor II, and α_1-antichymotrypsin (Carrell et al., 1991).

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