Purification and Characterization of Recombinant Plasminogen Activator Inhibitor-1 from *Escherichia coli*

(Received for publication, August 4, 1989)


From the E. I. du Pont de Nemours and Company, Medical Products Department, Experimental Station, Wilmington, Delaware 19880-0400 and the §Medical Products Department, Glasgow Site, Newark, Delaware 19719-6101

A recombinant form of plasminogen activator inhibitor-1 (rPAI-1) has been purified from lysates of pCE1200, a bacterial expression vector containing the full length PAI-1 gene, by utilizing sequential anion exchange and cation exchange chromatography on Q-Sepharose and S-Sepharose columns. Approximately 140 mg of rPAI-1, estimated at 98% purity on the basis of analytical high performance liquid chromatography, could be obtained from 200 g wet weight of cells. The purified protein exhibited a single Coomassie Blue-stainable band at the region of M, = 42,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an NH2-terminal amino acid sequence consistent with the expected translation product of the pCE1200 PAI-1 insert. The rPAI-1 rapidly inhibited single- and two-chain tissue plasminogen activators, as well as urokinase, with apparent second order rate constants in the range of 2-5 × 106 M⁻¹s⁻¹. A specific activity measurement of 250,000 units/mg was calculated for the rPAI-1 based on its ability to inhibit the enzymatic activity of a single-chain tissue plasminogen activator. Stability studies showed that the activity of the rPAI-1 was very stable when stored at temperatures of 25 °C or lower, but decayed within hours when stored at 37 °C. Sodium dodecyl sulfate treatment, which partially activates the latent form of natural PAI-1, inactivated rPAI-1. These results show that the purified rPAI-1 produced from pCE1200 displays many of the properties associated with the biologically active form of natural PAI-1.

Tissue-type plasminogen activator (tPA) is a serine protease which converts the proenzyme plasminogen to the fibrinolytic enzyme plasmin (1, 2). The activity of tPA is highly stimulated by fibrin (3) and specifically inhibited by plasminogen activator inhibitors (PAIs) (4). At present, four different types of PAI have been identified, including PAI-1, originally identified in plasma and in endothelial cell culture fluids (5, 6); PAI-2, first demonstrated in human placenta and in pregnancy plasma (7, 8); PAI-3, first demonstrated in urine and subsequently shown to be identical with protein C inhibitor (9); and the protease nexin, identified in the conditioned medium of fibroblasts and other cells (10). However, because of the second order rate constants for its interaction with single-chain tPA (3.7 × 107 M⁻¹s⁻¹) and two-chain tPA (1.6 × 108 M⁻¹s⁻¹), which are among the highest reported for enzyme-inhibitor interactions, it is generally accepted that PAI-1 is the principal physiological inhibitor of tPA (11, 12). Most tPA in plasma circulates complexed to PAI-1 (13, 14).

Elevated plasma PAI-1 levels are associated with an increased risk of thromboembolic disease (15) suggesting a critical role for the protein in the regulation of in vivo fibrinolysis. Increased PAI-1 levels have also been found in several other clinical situations including septicemia, a variety of malignancies and liver disease (16-18). However, critical biological and pharmacological studies with purified protein will be required to define precise roles for PAI-1 in various pathological conditions.

The concentration of PAI-1 in normal plasma is very low (approximately 20 ng/ml), making purification of large quantities of PAI-1 from whole blood a formidable task (19). Alternatively, PAI-1 has been isolated from a number of different cell lines including bovine aortic endothelial cells (6), HT 1080 human fibrosarcoma cells (20), hepatoma tissue culture rat hepatoma cells (21), and human endothelial cells (22). However, the isolated protein from these sources exists chiefly as a latent form with a very low specific activity, as determined in PA inhibition assays (6, 23). This latent form can be partially activated by treatment with denaturants such as sodium dodecyl sulfate (SDS), guanidinium hydrochloride, and urea (24), or by treatment with phospholipids (25). Recent studies suggest that PAI-1 is synthesized as an active form which is rapidly converted to the latent form by some unknown mechanism (26).

Expression of the PAI-1 cDNA in either prokaryotic or eukaryotic cells has been described by a number of groups (27-31). However, expression levels of recombinant PAI-1 (rPAI-1), where reported, have been extremely low. Furthermore, rPAI-1 from *Escherichia coli* has been characterized as lacking prelatent activity exclusively in the latent form (32).

In the present study, we have purified and characterized a recombinant form of PAI-1 expressed in *E. coli* from the expression vector pCE1200.2 Our results indicate that substantial quantities of functionally active, stable protein may be readily purified from this vector, and that this rPAI-1 shares many properties in common with the active form of natural PAI-1.
Materials—Natural PAI-1, purified from human fibrosarcoma cells, was purchased from American Diagnostica and was activated with SDS treatment as described (24). One-chain tPA, two-chain tPA, and the low molecular weight form of urokinase were also purchased from American Diagnostica.

Bacterial Expression of Recombinant PAI-1—The expression of rPAI-1 encoded by the plasmid pCE1200 in E. coli host TAP106 will be described in a separate report. Briefly, a 421 phage library from human endothelial cells was screened using oligonucleotide probes directed to the 5' end of the PAI-1 gene. A cDNA clone was isolated and described in a separate report. Briefly, a Xgtll phage library from human endothelial cells either by sonication for 5 min using a medium tip probe (Heat Systems Ultrasonics) or by pressure disruption using a Gaulin model 120C was isolated from temperature-induced E. coli pCE1200 containing the PAI-1 cDNA were prepared by sonication and resuspended in 5 volumes of starting buffer and resuspended in 10 volumes of 50 mM sodium phosphate buffer, pH 6.0, over an ice bath and disrupting the cells either by sonication for 5 min using a medium tip probe (Heat Systems Ultrasonics) or by pressure disruption using a Gaulin model 120C. The suspension containing lysed cells was centrifuged at 16,000 X g for 20 min at 4 °C, the supernatant was collected, and the pellet was resuspended in 5 volumes of buffer and resuspended as above. Supernatant collected from a 16,000 X g centrifugation of this resuspended material was pooled with the first supernatant, filtered through a 5-micron filter, and pumped onto a Q-Sepharose fast flow (Pharmacia LKB Biotechnology Inc.) column (4.4 X 30 cm), equilibrated with 50 mM sodium phosphate, pH 6.0, at a flow rate of 10 ml/min. The column was washed with the same buffer, and the effluent was collected until absorbance at 280 nm was no longer measurable (approximately 0.5 column volumes). The effluent was then loaded onto a S-Sepharose (Pharmacia) column (4.4 X 30 cm) equilibrated with 50 mM sodium phosphate, pH 6.0, the column was washed with 1 column volume of buffer, and the protein eluted using a 0–1 M sodium chloride gradient in buffer. PAI-1, which eluted at approximately 0.4 M sodium chloride, was estimated to be 85–90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by analytical reversed-phase HPLC (see below). The fraction containing PAI-1 was pooled diluted 2.5-fold with water, and loaded onto a further S-Sepharose column equilibrated with 50 mM sodium phosphate, pH 6.0. The column was washed with 2 column volumes each of starting buffer and 50 mM sodium phosphate buffer, pH 8.0, and the PAI-1 eluted using 150 mM sodium phosphate buffer, pH 8.6.

Characterization of the Purified rPAI-1—Analytical SDS-PAGE (34) was performed utilizing 8–25% gradient gels with the Pharmacia Phast system. Proteins were visualized by staining with Coomassie Blue. Analytical HPLC was performed with an HP 1090 M HPLC system. A C4 Vydac column (4.6 X 50 mm) was equilibrated with 0.1% trifluoroacetic acid in water, and the samples were applied to the column in the same solvent. Samples were eluted using a gradient of acetonitrile containing 0.1% trifluoroacetic acid.

NH2-terminal sequence analysis was obtained using automated Edman degradation chemistry. An ABI model 470 A (Applied Biosystems) or a Porton model P1 2090 (Porton Instrumenta) gas-phase sequencer was employed for the degradation (35). The respective phosphoamidite-derivatives were identified in an on-line fashion, using the HPLC standards supplied by the manufacturers. Approximately 0.5–2.0 nmol of rPAI-1 was used for the sequencing. Assay of PAI-1 Activity—PAI-1 activity of natural or recombinant protein was determined essentially as described (36). PAI-1 was incubated with single-chain tPA for 10 min at 37 °C, and the residual activity was measured with the chromogenic substrate Val-Leu-Lys-p-nitroanilide (S2251) according to the manufacturer's instructions (Kabi Vitrum). One unit of PAI-1 is defined by the amount of protein that inhibits 1 IU of tPA. For stability studies, rPAI-1 in 0.05 M Tris buffer, pH 8.3, containing 0.01% Tween 80, was preincubated for various times at either 4, 25, or 37 °C prior to activity determinations. Second order rate constants for the inhibition of one-chain tPA, two-chain tPA, and uPA by PAI-1 were calculated from the half-times of inhibition determined graphically, as previously described (37). Rate constants are expressed as equal to or more than a particular value to reflect the notion that the rPAI-1 may not be 100% functional.

RESULTS

Purification of rPAI-1—PAI-1 antigen levels in lysates prepared from temperature-induced E. coli pCE1200 containing the mature PAI-1 gene were approximately 100 μg/ml, as measured by an enzyme-linked immunosorbent assay specific for PAI-1. This value represented nearly 10% of the total protein content in the lysates. A procedure for purifying the rPAI-1 from bacterial lysates was developed which involved sequential passage over anion exchange (Q-Sepharose) and cation exchange (S-Sepharose) columns. Using a sodium chloride gradient, PAI-1 eluted from the Q column at approximately 400 mM sodium chloride and was estimated to be approximately 85–90% pure by SDS-PAGE (Fig. 1). Therefore, the PAI-1-containing fractions were further chromatographed on a second S-Sepharose column, and the PAI-1 eluted with 150 mM sodium phosphate buffer, pH 8.6. The purified rPAI-1 exhibited a single Coomassie Blue-stainable band at 42 kDa (Fig. 1). This is consistent for an unglycosylated protein of 381 residues, the predicted translation product contained within the expression plasmid pCE1200. Purity was estimated at 98% on the basis of this SDS-PAGE and analytical HPLC (Fig. 2). An NH2-terminal sequence of Ser-Ile-Val-His-His-Pro-Pro-Ser-Tyr-Val-Ala-His-Leu-Ala-Ser-Asp-Phe-Gly-Val was determined for the purified protein. This represents the predicted sequence for the pCE1200 rPAI-1 translation product, accounting for removal of the NH2-terminal Met residue by aminopeptidases in E. coli.

Inhibition of tPA by rPAI-1—The purified rPAI-1 was compared with natural PAI-1, purified from human fibrosarcoma cells, for its ability to neutralize the enzymatic activity of tPA. A fixed amount of tPA (10 IU) was incubated with increasing amounts of either natural or rPAI-1, and the plasminogen-activating activity was determined by employing an amidoassay with the chromogenic substrate S2251.

First-order rate constants for the inhibition of one-chain tPA, two-chain tPA, and uPA by PAI-1 were calculated from the half-times of inhibition determined graphically, as previously described (37). Rate constants are expressed as equal to or more than a particular value to reflect the notion that the rPAI-1 may not be 100% functional.

![Figure 1](http://www.jbc.org/article/S0021-9525(17)30752-8/DC1/fig1.png)
activity reported for any recombinant form of PAI-1; the theoretical specific activity for PAI-1 is 650,000 units/mg when using tPA as the target enzyme (12). The activity of rPAI-1 was not increased by pretreating with SDS, a procedure which has been reported to promote conversion of latent PAI-1 to a latent form (24, 38). The effect of incubation at different temperatures on the activity of rPAI-1 was therefore determined. As shown in Fig. 4, rPAI-1 activity decayed by 50% at 37°C in approximately 2 h. Analysis of subsequent lots revealed half-life values for rPAI-1 at 37°C ranging from 70 to 120 min. These values compare favorably with the 90-min incubation period at 37°C reported to promote a 50% reduction in the PAI activity in conditioned

In contrast, natural PAI-1 purified from human fibrosarcoma cells was essentially inactive without SDS treatment (Fig. 3). A specific activity of approximately 25,000 IU/mg was calculated for the denaturant-treated PAI-1. This lower activity, in comparison with the rPAI-1, may reflect irreversible inactivation of some protein during the preparation of conditioned media and the subsequent purification steps (38, 39).

**Rate Constants**—The half-times of the inhibition of one-chain tPA, two-chain tPA, and uPA by rPAI-1 were used to calculate apparent second order rate constants (Table I). These values, in the range of 2-5×10^7 M^-1 s^-1, are consistent with those reported for active, natural PAI-1 (37, 40).

**Stability of rPAI-1**—Incubation of functionally active, natural PAI-1 at 37°C inactivates the protein, perhaps by converting PAI-1 to a latent form (24, 38). The effect of incubation at different temperatures on the activity of rPAI-1 was therefore determined. As shown in Fig. 4, rPAI-1 activity decayed by 50% at 37°C in approximately 2 h. Analysis of subsequent lots revealed half-life values for rPAI-1 at 37°C ranging from 70 to 120 min. These values compare favorably with the 90-min incubation period at 37°C reported to promote a 50% reduction in the PAI activity in conditioned

**TABLE I**

**Kinetics of inactivation of uPA and tPA by rPAI-1**

Inhibition assays were performed as described under "Experimental Procedures" except that the incubation period of PA with rPAI-1 varied from 0-20 min. The t1/2 of inhibition was determined from graphs of the data; k' = 0.693/k.

<table>
<thead>
<tr>
<th>Concentration of rPAI-1</th>
<th>t1/2</th>
<th>k'/[PAI-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>s</td>
<td>M^-1 s^-1</td>
</tr>
<tr>
<td>A. uPA</td>
<td>1.5×10^-10</td>
<td>≤78</td>
</tr>
<tr>
<td>(9.2×10^-15)</td>
<td>3.0×10^-10</td>
<td>≤46</td>
</tr>
<tr>
<td>B. One-chain tPA</td>
<td>1.2×10^-10</td>
<td>≤161</td>
</tr>
<tr>
<td>(9.9×10^-12)</td>
<td>2.4×10^-10</td>
<td>≤103</td>
</tr>
<tr>
<td>C. Two-chain tPA</td>
<td>3.0×10^-10</td>
<td>≤46</td>
</tr>
<tr>
<td>(9.9×10^-12)</td>
<td>6.0×10^-10</td>
<td>≤26</td>
</tr>
</tbody>
</table>

**FIG. 4.** Stability of rPAI-1 to incubation at different temperatures. 500 μg of rPAI-1 (specific activity of 0.2 unit/mg) in 1 ml of 0.01 M Tris buffer, pH 8.0, was incubated at either 4°C (○), 37°C (C), or 37°C (×). At the indicated times, the ability of 50 ng of rPAI-1 from each temperature group to inhibit 10 IU of tPA was determined by means of an amidolytic assay. Data are expressed as the percent of tPA activity observed in the absence of PAI-1.

**TABLE II**

**Effect of incubation at 42°C on the expression and activity of rPAI-1 by the pCE1200/TAP106 bacterial expression vector**

The bacterial culture was induced by raising the temperature to 42°C, and, at the indicated time points, 1-ml samples were collected. Lysates prepared from these samples were assayed for PAI-1 levels and activity as described under "Experimental Procedures." The level of PAI-1 in the uninduced culture was negligible. All values are the mean of triplicate determinations.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>PAI-1 antigen</th>
<th>PAI-1 activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/ml</td>
<td>IU/ml</td>
<td>IU/μg</td>
</tr>
<tr>
<td>1</td>
<td>19.7</td>
<td>4,875</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>67.8</td>
<td>21,250</td>
<td>0.31</td>
</tr>
<tr>
<td>3</td>
<td>109.1</td>
<td>25,800</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>145.2</td>
<td>27,000</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>212.5</td>
<td>25,800</td>
<td>0.12</td>
</tr>
<tr>
<td>6</td>
<td>283</td>
<td>10,600</td>
<td>0.037</td>
</tr>
<tr>
<td>7</td>
<td>310</td>
<td>9,380</td>
<td>0.030</td>
</tr>
</tbody>
</table>
medium from human endothelial cells (39). At a lower temperature of 25 °C, the rPAI-1 lost 50% of its activity after incubation for 2 days. The rPAI-1 was very stable at 4 °C, with only a 15% loss in activity observed following a 7-day incubation period (Fig. 4). No decrease in specific activity has been observed following storage of rPAI-1 at −20 °C for a 1-year period.

The temperature sensitivity of rPAI-1 prompted us to examine protein expression by the pCE1200 bacterial expression vector since induction of this particular vector requires incubation at 42 °C. For this purpose, samples were collected from a bacterial culture at various time points after incubation at 42 °C, crude lysates were prepared, and PAI-1 antigen levels as well as activity levels were determined for each sample. The results of this analysis, shown in Table II, indicate that the antigen level of PAI-1 increases continuously during the course of the 7-h study. However, the specific activity of the rPAI-1, expressed as the ratio of activity to antigen, actually peaked at 2 h and declined thereafter. Apparently prolonged incubation of the pCE1200 expression system at 42 °C results in a greater quantity of rPAI-1, but with a lower degree of activity.

**DISCUSSION**

We have developed a procedure for the purification of rPAI-1 from the E. coli expression vector pCE1200 in sufficient amounts to allow biochemical characterization of the protein. Approximately 140 mg of protein could be purified from 200 g wet weight of cells over a period of 9 days, using a procedure that included sequential application of material over anion and cation exchange columns. Purity of the protein collected from a second cation column run was assessed at 98% on the basis of analytical SDS-PAGE and HPLC. The purified protein exhibited a single Coomassie Blue-stainable band at 42 kDa and an NH2-terminal amino acid sequence consistent with the unglycosylated translation product of the pCE1200. From other studies (41, 42), two forms of mature natural PAI-1 have been identified: a 379-amino acid protein with an amino terminus of Val-His-His; and a 381-amino acid protein with an amino terminus of Ser-Ala-Val-His-His. The rPAI-1 described in this report corresponds to the 381-amino acid form with the exception of an Ile substitution for Ala at residue 2.

The recombinant protein was functionally active, as assessed in studies where enzymatic activity of rPAI was measured using an amidolytic assay. Specific activity calculations yielded a value for rPAI-1 of 250,000 units/mg, making it among the highest value reported for purified PAI-1 from any source. The theoretical specific activity of PAI-1 is 650,000 IU/mg when using tPA as the target enzyme (12). Our results confirm that glycosylation is not required for functional activity of PAI-1 (29). The rPAI-1 was remarkably stable at 4 °C, with little loss in activity observed following a 7-day incubation period. Activity of the rPAI-1 did decay upon storage at higher temperatures of 25 °C and 37 °C, as has been reported for PAI-1 purified from other sources (24). However, the 2-h half-life compares favorably with the 90-min half-life reported for PAI-1 activity in conditioned medium of human endothelial cells (39).

While many procedures have been described for purification of PAI-1, in most instances the resulting protein has been functionally inactive or at best a few percent active (6, 20, 21, 22, 24). These latent PAI-1 preparations may be activated by treatment with denaturants such as SDS; however, even following such treatment, most isolated PAI-1 preparations contain less than 5% active molecules. This low degree of activation may be due to irreversible inactivation of some protein during the purification process (38, 39). Interestingly, treatment with SDS, which increased the activity of native PAI-1 purified from human fibrovascular cells, actually decreased the activity of the purified rPAI-1. These results are consistent with findings from a recent study (43) which suggested that SDS exerts dual effects on PAI-1: enhancing the inhibitory activity of essentially inactive or latent PAI-1 preparations, while partially inactivating active PAI-1 preparations.

More recently, it has been suggested that activity of PAI-1 is dependent upon its association with vitronectin, an adhesive protein found in plasma (44), platelets (45), urine (46), and the extracellular matrix (47). Functionally active PAI-1 was purified from HT 1080 cells (48) and from HepG2 hepatoma cells (43) was associated with vitronectin and/or its NH2-terminal fragments. In contrast, purified HT 1080 PAI-1 which did not contain detectable amounts of vitronectin expressed only limited inhibitory activity (43, 48). We have observed that vitronectin inhibits the decay of rPAI-1 activity following incubation at 37 °C (data not shown). These results suggest that the activity of rPAI-1, like that of the natural protein (43, 48, 49), is stabilized by vitronectin. The physiological relevance of the association between PAI-1 and vitronectin remains to be established.

An alternative approach to purifying native PAI-1 from cultured cells or plasma is to explore the properties of recombinant PAI-1. A number of groups have expressed the PAI-1 cDNA in either prokaryotic or eukaryotic cells (27–31). However, biological activity of rPAI-1 from E. coli was reported only in the reverse fibrin autography assay (28, 29), a procedure which is known to promote conversion of latent to active protein. A subsequent report has indicated that rPAI-1 from E. coli is expressed almost predominantly in the inactive form (32). PAI-1 has recently been purified from Chinese hamster ovary cells which had been transfected with cDNA of PAI-1 (31). As with PAI-1 purified from plasma, only the high Mr, fraction, most likely representing PAI-1 complexed with vitronectin from the fetal bovine serum utilized in the growth medium, was functionally active. Furthermore, the expression level of rPAI-1 in transfected Chinese hamster ovary cells was low and variable (0.1 mg/liter).

In view of these previous reports on both natural and rPAI-1s, it was surprising that the Mr = 42,000 rPAI-1 in this study displayed significant functional activity in the absence of any discrete binding protein. The unique activity of this particular protein in comparison with natural and recombinant PAI-1s from other sources may relate to temperature sensitivity of PAI-1. Since incubation of PAI-1 at 37 °C results in a loss of activity (39), prolonged incubation of PAI-1-producing cells at 37 °C or higher may actually promote inactivation of the natural or recombinant protein in the conditioned medium prior to its collection for harvesting and eventual purification. This hypothesis is supported by the results in Table II which show that, beyond 2 h, the activity of rPAI-1 decreases with increased incubation time of the pCE1200 expression vector at 42 °C. We are currently investigating whether the bulk of this inactive rPAI-1 collected at the later time points represents latent or irreversibly inactivated protein. Another important consideration for maintaining activity of purified PAI-1 may relate to the ionic strength of the buffer used for storage. We have observed a considerable amount of aggregation of purified rPAI-1 when the ionic strength of the phosphate buffer was reduced below 150 mm, and this aggregated material was functionally inactive. The possible dependence of PAI-1 activity on high ionic strength also requires investigation.
Further experiments are needed to precisely define biological roles for PAI-1 in various physiological and pathophysiological states. The ability to produce and purify this recombinant form of PAI-1, which shares many properties in common with natural active PAI-1, is an important step in defining such roles for PAI-1.

Acknowledgments—We would like to thank Walt Manger and Gene Corman for the NH2-terminal sequencing, Shubada Kaemekar and Alex Boon for technical assistance, Dr. Richard Yates for fermentation analysis, and Claire Stecher for preparation of the manuscript.

REFERENCES


36. Hekman, C. M., and Loskutoff, D. J. (1986) Fibrinolysis 1, Suppl. 46a
Purification and characterization of recombinant plasminogen activator inhibitor-1 from Escherichia coli.
T M Reilly, R Seetharam, J L Duke, G L Davis, S K Pierce, H L Walton, D Kingsley and W P Sisk


Access the most updated version of this article at http://www.jbc.org/content/265/16/9570

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

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

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/16/9570.full.html#ref-list-1