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Purification of a Form of Protease Nexin 1 That Binds Heparin with a Low Affinity†

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ABSTRACT: A form of protease nexin 1 (PN-1) that binds heparin with a low affinity (L-PN-1) was purified and studied since altered interactions with glycosaminoglycans could affect its inhibition of certain serine proteases. Purification of L-PN-1 and PN-1 was achieved by fractionating serum-free conditioned culture medium from human fibroblasts over dextran sulfate–Sepharose followed by immunoaffinity fractionation over a PN-1 monoclonal antibody–Sepharose column. The first step separated L-PN-1 from PN-1, and the second step resulted in apparently homogeneous L-PN-1 and PN-1. Comparisons of the two proteins showed that they could not be distinguished by the following properties: (a) molecular weight; (b) proteases complexed; (c) molecular weights of protease–L-PN-1 and protease–PN-1 complexes; (d) CNBr peptide maps; and (e) immunological cross-reactivity. Studies on activities that depend on the heparin binding domain revealed that heparin equally accelerated the rate of formation of ^{125}I-thrombin–L-PN-1 and ^{125}I-thrombin–PN-1 complexes even when the ratio of heparin to L-PN-1 or PN-1 was varied from 0.01 to 100. A functional difference, however, between L-PN-1 and PN-1 was observed in studies on the ability of the fibroblast surface to accelerate their reactions. Fixed fibroblasts accelerated the formation of ^{125}I-thrombin–L-PN-1 complexes 2-fold, whereas they accelerated the formation of ^{125}I-thrombin–PN-1 complexes 5-fold. The availability of purified L-PN-1 will permit studies on its functional relationship to PN-1.

Protease nexin 1 (PN-1)[1] is a 45-kDa serine protease inhibitor that is synthesized and secreted by cultured human fibroblasts (Baker et al., 1980, 1986; Scott et al., 1985) and several other cultured nonvascular cells (Eaton & Baker, 1983). PN-1 rapidly complexes thrombin, urokinase, plasmin, and trypsin (Scott et al., 1985). Previous studies showed that PN-1 binds to heparin with a high affinity (Baker et al., 1980; Scott & Baker, 1983; Scott et al., 1985) and that in the presence of heparin the rate of thrombin inactivation by PN-1 is markedly accelerated (Baker et al., 1980; Scott et al., 1985). This affinity for heparin has been employed in the procedures to purify PN-1 (Scott & Baker, 1983; Scott et al., 1985; Farrell et al., 1986). During these purifications, a form of PN-1 was noted which has a relatively low affinity for heparin. We have designated this form L-PN-1. Little is known about L-PN-1 because it has not been purified.

There are several important activities of PN-1 that likely depend on its heparin binding site and which could be modified in L-PN-1. For example, the surface of fibroblasts accelerates the formation of complexes between PN-1 and thrombin; this is due to glycosaminoglycans in the extracellular matrix (Farrell & Cunningham, 1986, 1987). Also, PN-1 is localized on the fibroblast extracellular matrix (D. H. Farrell et al., unpublished results). The molecules responsible for this association are not known but could involve heparin-like gly-

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[1] Abbreviations: PN-1, protease nexin 1; L-PN-1, protease nexin 1 that binds heparin with low affinity; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; kDa, kilodalton(s); Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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cosaminoglycans. Finally, heparin blocks the binding of ^{125}I-thrombin–PN-1 complexes to fibroblasts (Baker et al., 1982). These considerations prompted the present studies on L-PN-1.

Materials and Methods

Materials. Sepharose CL-6B, CNBr-activated Sepharose 4B, and dextran sulfate (M_r 500 000) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Heparin was from Calbiochem (San Diego, CA). CNBr was obtained from Aldrich (Milwaukee, WI). Cell culture supplies were from GIBCO (Grand Island, NY). Chloroglycoluril was from Pierce (Rockford, IL). Na^{125}I was purchased from New England Nuclear (Boston, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Purified thrombin (Fenton et al., 1977) was a gift of John W. Fenton, II (New York State Department of Health, Albany, NY), and was iodinated by the chloroglycoluril method (Glenn et al., 1980). Dextran sulfate–Sepharose (Van Nostrand & Cunningham, 1987) and heparin–Sepharose (Tollefsen et al., 1982) were prepared as described.

Cell Culture. Human fibroblasts were isolated from explants of neonatal foreskins and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum as previously described (Baker et al., 1980). Three-liter microcarrier cultures of the fibroblasts were prepared employing gelatin microcarrier beads as previously described (Van Nostrand & Cunningham, 1987).

Electrophoretic Techniques. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970); 7.5% total acrylamide gel were cast as 15 × 8 × 0.1 cm slabs. Gels were stained in methanol/acetic acid/water (5:1:5) containing 0.05% Coomassie Brilliant Blue R-250; they were destained in 5% ethanol/7% acetic acid. Slab gels containing radioactivity were dried and prepared for autoradiography as previously described (Baker et al., 1980).

Assay for PN-1-like Activity. To assay for PN-1 or L-PN-1, aliquots of the collected fractions were incubated with known quantities of ^{125}I-thrombin for 20 min at 37 °C. An equal volume of SDS–PAGE sample buffer was then added, and the mixtures were subjected to SDS–PAGE. After autoradiography, PN-1-like activity was monitored by the presence of an 80-kDa complex with ^{125}I-thrombin. To quantitate PN-1 activity, the autoradiograms were aligned with the dried gels, and the 80-kDa ^{125}I-thrombin–PN-1 or ^{125}I-thrombin–L-PN-1 complexes were located, excised, and measured in a γ counter. In Figures 1 and 3, this assay was used for a relative measure of PN-1-like activity to identify PN-1-containing or L-PN-1-containing fractions. A similar assay was also used to quantitate PN-1 and L-PN-1 activity as shown in Table I. However, in these measurements, pooled fractions from each step were compared to purified PN-1. One PN-1 unit is defined as the amount of ^{125}I-thrombin complexed by 10 pmol of standardized PN-1 as previously described (Farrell et al., 1986).

Collection of Serum-Free Conditioned Medium. The microcarrier beads were allowed to settle from the cultures, and the serum-containing medium was removed by aspiration. It was replaced with 2 L of DMEM buffered with 20 mM Hepes, pH 7.4, containing 100 units/mL penicillin and 100 μg/mL streptomycin. The microcarrier beads were again allowed to settle, the rinse medium was removed by aspiration and replaced with 2 L of DMEM buffered with 20 mM Hepes containing the antibiotics and 0.1% bovine serum albumin. The microcarrier culture was maintained in this medium for 24 h at 37 °C with stirring. This medium was then removed, and the cultures were incubated with fresh serum-free medium that contained bovine serum albumin for two subsequent 3-day periods. After each period, the "conditioned" medium was collected. Using two 3-L microcarrier cultures, we harvested 4 L of serum-free conditioned medium on each collection day. The cells were then returned to culture medium containing 5% bovine serum for 5–7 days after which they were cycled again for collection of serum-free conditioned medium. The serum-free conditioned medium was aspirated into a siliconized flask, filtered to remove particulates, and chilled to 4 °C. Phenylmethanesulfonyl fluoride, butylated hydroxytoluene, and sodium azide were added to final concentrations of 200 μM, 50 μM, and 0.04%, respectively. Conditioned medium was used within 24 h after collection. All subsequent chromatographic steps were conducted at 4 °C.

Dextran Sulfate–Sepharose Affinity Chromatography. Four liters of serum-free conditioned medium was applied to a column (2.5 × 40 cm) of dextran sulfate–Sepharose equilibrated with phosphate-buffered saline at a flow rate of 100 mL/h. After the column was loaded, it was washed with phosphate-buffered saline until the A_{280} returned to baseline. The adsorbed protein was eluted from the column with a 1.5-L linear gradient from 0.15 to 1.2 M NaCl in phosphate-buffered saline, and fractions of 20 mL were collected. Fractions containing L-PN-1 or PN-1 were identified by incubation with ^{125}I-thrombin and subsequent analysis by SDS–PAGE and autoradiography as described above.

Monoclonal Antibody–Sepharose Affinity Chromatography. A monoclonal antibody (mAbp18) that bound PN-1 was isolated, purified, and characterized as described in the preceding paper (Wagner et al., 1988). It was coupled to CNBr-activated Sepharose 4B as described by the manufacture. During the purification, mAbp18 did not detectably bind proteins other than PN-1 or L-PN-1. Pooled fractions containing L-PN-1 or PN-1 from dextran sulfate–Sepharose were individually applied to a column (0.5 × 10 cm) of mAbp18–Sepharose equilibrated with 20 mM potassium phosphate/1 M NaCl, pH 7.4, at a flow rate of 10 mL/h. After the column was loaded, it was washed with 5 column volumes of 20 mM potassium phosphate/1 M NaCl, pH 7.4, followed by 2 column volumes of 20 mM potassium/0.15 M NaCl, pH 7.4. The adsorbed L-PN-1 or PN-1 was eluted from the column with 0.2 M glycine hydrochloride/0.15 M NaCl, pH 3.0. One-milliliter fractions were collected in tubes containing 100 μL of 2 M Tris-HCl, pH 8.3, to neutralize the elution buffer. Concentrations of purified L-PN-1 or PN-1 were determined by their absorbance at 280 nm by using the specific absorption coefficient $A^{1\%}_{1cm}$ = 16.2 (Scott et al., 1985) or by the method of Bradford (1976).

CNBr Peptide Mapping. Ten micrograms of purified L-PN-1 or PN-1 was dialyzed for 24 h against distilled water at 4 °C; the samples were then frozen and lyophylized. Then, each protein was resuspended in 100 μL of 70% formic acid, and 2 μL of a stock solution of CNBr (2 mg/mL in acetonitrile) was added. The tubes containing the mixtures were saturated with nitrogen, capped, and left in the dark for 24 h at room temperature. Then, the solutions were evaporated under a stream of nitrogen, and the digested proteins were resuspended in 100 μL of distilled water, frozen, and lyophilized. Analysis of the peptide fragments was performed by using the peptide gel system of Swank and Munkres (1971). The lyophilized protein digests were resuspended in 40 μL of SDS-PAGE sample buffer containing 5% β-mercaptoethanol, boiled for 5 min, and electrophoresed on an 8 M urea/SDS

Table I: Purification of L-PN-1 and PN-1 from Serum-Free Culture Medium Conditioned by Human Fibroblasts

step	volume (mL)	protein (mg)	units	sp act. (units/mg)	% yield	x-fold purification
conditioned medium	4000	~4600[a]	4156	0.9	100	1
dextran sulfate–Sepharose (L-PN-1 pool)	200	13.7	802	59	19	65
dextran sulfate–Sepharose (PN-1 pool)	200	6.2	3031	489	74	543
mAbp18–Sepharose (L-PN-1)	5.5	0.345	492	1426	12	1584
mAbp18–Sepharose (PN-1)	5.5	1.150	2226	1936	54	2151

[a] Most of this protein is bovine serum albumin which was present in the medium added to the microcarrier cultures.

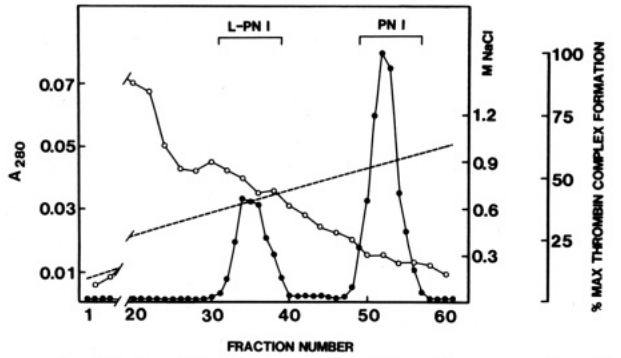

FIGURE 1: Elution of L-PN-1 and PN-1 from dextran sulfate–Sepharose. Four liters of serum-free conditioned medium was loaded on the column (2.5 × 40 cm) at 100 mL/h. The column was washed, and the adsorbed protein was eluted with a 1.5-L linear gradient from 0.15 to 1.2 M NaCl in phosphate-buffered saline. Fractions of 20 mL were collected. Aliquots of each fraction were quantitated for PN-1-like activity as described under Materials and Methods. Fractions 31–40 containing L-PN-1 activity were pooled, and fractions 48–57 containing PN-1 activity were pooled for subsequent immunoaffinity chromatography. (○) Absorbance at 280 nm; (●) PN I-like activity; (---) molar NaCl.

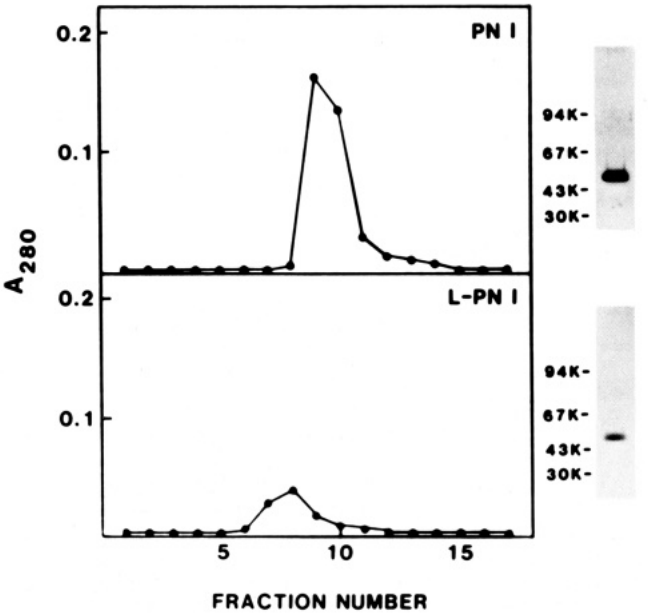

FIGURE 2: Elution of PN-1 and L-PN-1 from mAbp18–Sepharose. Pooled PN-I fractions or L-PN-I fractions from dextran sulfate–Sepharose were loaded on the mAbp18–Sepharose column (0.5 × 10 cm) which was preequilibrated with 20 mM potassium phosphate/1 M NaCl, pH 7.4. The column was washed, and the adsorbed PN-1 or L-PN-1 was eluted with 0.2 M glycine hydrochloride/0.15 M NaCl, pH 3.0. (●) Absorbance at 280 nm. Aliquots of eluted PN-1 or L-PN-1 were analyzed by nonreducing SDS–PAGE as described under Materials and Methods.

slab gel (15 × 8 × 0.1 cm) consisting of 13.3% total acrylamide. The resulting peptide profile was visualized by silver staining employing the method of Rubin et al. (1982).

Results

Purification of L-PN-1 and PN-1. We purified L-PN-1 and PN-1 from serum-free medium conditioned by human fibroblasts maintained in microcarrier cultures utilizing the procedures outlined under Materials and Methods. Table I summarizes the results of the purifications. Four liters of serum-free conditioned medium was applied to a dextran sulfate–Sepharose affinity column followed by elution of the adsorbed protein with a linear salt gradient. Figure 1 shows that this affinity column not only concentrated and enriched for PN-1 activity but also effectively separated the total activity into two different forms of PN-1. Aliquots of each fraction were incubated with ^{125}I-thrombin and analyzed by SDS–PAGE followed by autoradiography. PN-1-like activity was identified by the appearance of an 80-kDa ^{125}I-thrombin–PN-1 complex. As shown in Figure 1, L-PN-1 activity eluted from the dextran sulfate–Sepharose column at 0.60–0.65 M NaCl whereas PN-1 activity did not elute until the NaCl concentration reached 0.90 M. Importantly, 94% of the total PN-1-like activity in the conditioned medium was recovered after this purification step (Table I). Furthermore, fractionation over this column demonstrated that approximately 20% of the total PN-1 activity in conditioned medium was due to L-PN-1 (Table I).

Final purification of L-PN-1 and PN-1 was achieved by immunopurification with the mAbp18–Sepharose column. Pooled fractions containing either L-PN-1 or PN-1 were applied to the monoclonal antibody column followed by washing with 1 M NaCl. The adsorbed L-PN-1 or PN-1 was then eluted from the column with a glycine hydrochloride buffer at pH 3.0. Figure 2 shows the elution profiles of each form of PN-1 from the antibody affinity column; the corresponding Coomassie-stained gels show the apparent homogeneity of each purified protein. Approximately 70% of the total starting PN-1-like activity in the conditioned medium was recovered after the dextran sulfate–Sepharose and immunoaffinity steps (Table I). Also, L-PN-1 represented approximately 20% of the total recovered protein and activity (Table I).

Comparisons of L-PN-1 with PN-1. We first determined if purified L-PN-1 and PN-1 retained their respective affinities for heparin. The purified molecules were individually applied to a column of heparin–Sepharose. After the column was washed, the adsorbed L-PN-1 or PN-1 was eluted with a linear salt gradient. Aliquots of each fraction were tested for PN-1-like activity by formation of ^{125}I-thrombin–PN-1 complexes as described above. As shown in Figure 3, each purified protein eluted from the column as a single peak of activity and retained its relative affinity for heparin. L-PN-1 eluted from the column at approximately 0.3 M NaCl whereas PN-1 eluted at 0.5 M NaCl.

The following results suggest that L-PN-1 and PN-1 are very similar proteins and that minor differences must account for the different relative affinities for heparin. First, complexes of indistinguishable molecular weight were observed when purified L-PN-1 or PN-1 was incubated with ^{125}I-thrombin and analyzed by SDS–PAGE and autoradiography (Figure 4). In addition, L-PN-1 also formed complexes with urokinase, plasmin, and trypsin as previously described for PN-1 (Baker et al., 1980; Scott et al., 1985), and the protease–L-

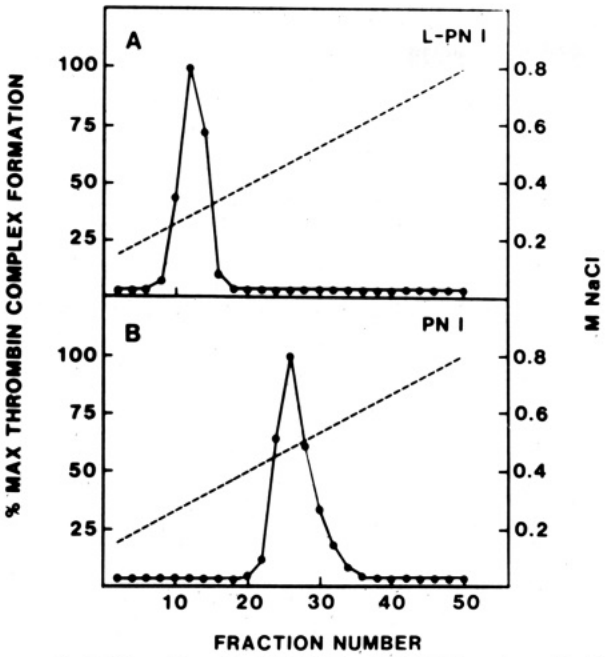

FIGURE 3: Elution of purified L-PN-1 and PN-1 from heparin–Sepharose. Purified L-PN-1 (200 μg) or PN-1 (200 μg) was individually bound to a column of heparin–Sepharose (1.0 × 12 cm) which was preequilibrated with 20 mM potassium phosphate/0.15 M NaCl, pH 7.4. The column was washed, and the adsorbed L-PN-1 or PN-1 was eluted with a 100-mL linear gradient from 0.15 to 0.8 M NaCl buffered with 20 mM potassium phosphate, pH 7.4. Fractions of 2 mL were collected at a flow rate of 7.5 mL/h. Aliquots of every other fraction were quantitated for PN-1-like activity as described under Materials and Methods. (●) PN I-like activity; (---) molar NaCl.

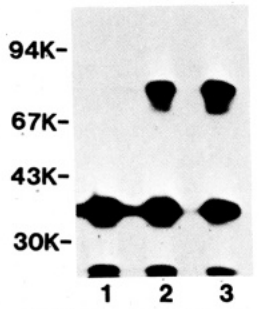

FIGURE 4: Formation of ^{125}I-thrombin–L-PN-1 or ^{125}I-thrombin–PN-1 complexes. ^{125}I-thrombin (100 ng) was incubated alone, with purified L-PN-1 (500 ng), or with purified PN-1 (500 ng) in 400 μL of 20 mM potassiuum phosphate/0.15 M NaCl, pH 7.4, for 15 min at 37 °C. An equal volume of SDS–PAGE sample buffer was added, and the samples were analyzed by nonreducing SDS–PAGE followed by autoradiography as described under Materials and Methods. Lane 1, ^{125}I-thrombin; lane 2, ^{125}I-thrombin + L-PN-1; lane 3, ^{125}I-thrombin + PN-1.

PN-1 complexes were indistinguishable in molecular weight from the corresponding protease–PN-1 complexes (data not shown). CNBr peptide analysis failed to reveal significant reproducible differences between L-PN-1 and PN-1 (Figure 5).

Effect of Heparin on the Functions of L-PN-1 and PN-1. Previous studies showed that ^{125}I-thrombin–PN-1 complexes bound to cells (Low et al., 1981; Scott & Baker, 1983) and that the cellular binding of these complexes was blocked by heparin (Baker et al., 1982; Scott & Baker, 1983). We determined if ^{125}I-thrombin–L-PN-1 complexes displayed similar interactions. To do this, increasing concentrations of purified L-PN-1 or PN-1 were preincubated with ^{125}I-thrombin, and the mixtures were then incubated with duplicate serum-free cultures of human fibroblasts in the presence or absence of heparin. Aliquots of the cell-bound radioactivity were analyzed

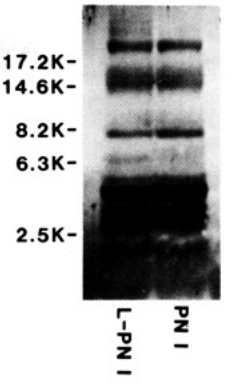

FIGURE 5: CNBr peptide mapping of purified L-PN-1 and PN-1. Ten micrograms of purified L-PN-I or PN-1 was digested with CNBr, and the resulting peptides were analyzed on the SDS–PAGE system of Swank and Munkres (1971) as described under Materials and Methods. The gel was silver-stained to visualize the peptides. Lane 1, L-PN-1; lane 2, PN-1.

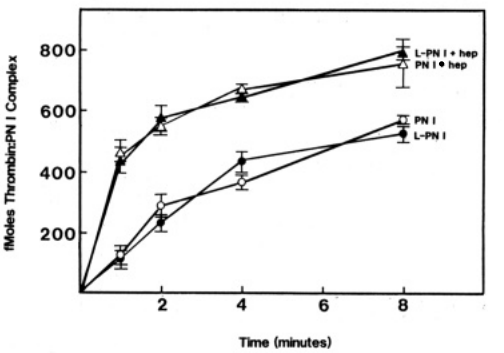

FIGURE 6: Heparin acceleration of ^{125}I-thrombin–L-PN-1 and ^{125}I-thrombin–PN-1 complex formation. ^{125}I-Thrombin (27 nM) was incubated with duplicate samples of purified L-PN-1 (270 nM) or PN-1 (270 nM) in the presence or absence of 0.4 unit/mL heparin for the designated times at 37 °C. The incubations were terminated by addition of SDS–PAGE sample buffer; aliquots of each sample were analyzed by nonreducing SDS–PAGE followed by autoradiography as described under Materials and Methods. The autoradiograms were aligned with the dried gels; the ^{125}I-labeled complexes were located, excised, and measured in a γ counter. (●) ^{125}I-Thrombin + L-PN-1; (○) ^{125}I-thrombin + PN-1; (▲) ^{125}I-thrombin + L-PN-1 + heparin; (△) ^{125}I-thrombin + PN-1 + heparin.

by SDS–PAGE and autoradiography. ^{125}I-Thrombin–L-PN-1 complexes as well as ^{125}I-thrombin–PN-1 complexes showed dose-dependent binding to the cells. In addition, both ^{125}I-thrombin–L-PN-1 complexes and ^{125}I-thrombin–PN-1 complexes exhibited the same heparin-sensitive binding (data not presented).

Heparin markedly accelerates the rate of inactivation of thrombin by PN-1 (Baker et al., 1980; Scott et al., 1985). Accordingly, we determined if heparin similarly accelerates the corresponding reaction with L-PN-1. The results in Figure 7 show that that excess heparin similarly accelerated the rates of complex formation between ^{125}I-thrombin and L-PN-1 or PN-1. We also varied the molar ratio of heparin to L-PN-1 or PN-1 from 0.01 to 100 and still found that it equally accelerated the rates of formation of ^{125}I-thrombin–L-PN-1 or ^{125}I-thrombin–PN-1 complexes (data not presented).

We recently reported that the surface of fixed human fibroblasts accelerates the reaction between ^{125}I-thrombin and PN-1 and that this is mostly due to heparin sulfate in the extracellular matrix (Farrell & Cunningham, 1986, 1987). Although the above experiments did not reveal a differential effect of heparin on the rate of formation of complexes between ^{125}I-thrombin and L-PN-1 or PN-1, we tested the possibility that the fibroblast surface might differentially accelerate the

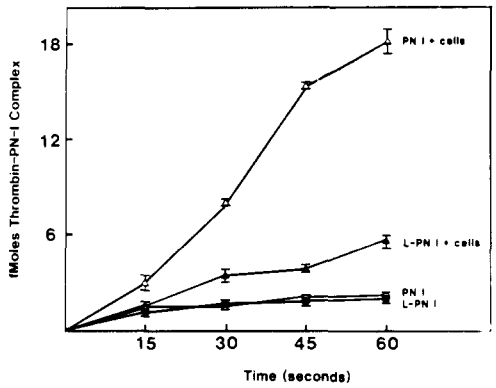

FIGURE 7: Acceleration of ^{125}I-thrombin–L-PN-1 and ^{125}I-thrombin–PN-1 complex formation by human fibroblasts. Confluent cultures of fibroblasts were serum-starved for 2 days, rinsed 5 times with phosphate-buffered saline, and fixed with 2% paraformaldehyde for 15 min at room temperature. The fixed cultures were then rinsed 3 times with phosphate-buffered saline. ^{125}I-Thrombin (2.7 nM) and purified L-PN-1 (27 nM) or PN-1 (27 nM) were added to duplicate fixed cultures and incubated for the designated times at 37 °C. Control incubations were conducted in culture dishes with no cells. In another control, ^{125}I-thrombin was incubated with fixed cells in the absence of added PN-1 or L-PN-1; the radioactivity in ^{125}I-thrombin–PN-1 or ^{125}I-thrombin–L-PN-1 complexes was negligible. The incubations were terminated by addition of SDS–PAGE sample buffer; aliquots of each sample were analyzed by nonreducing SDS–PAGE followed by autoradiography as described under Materials and Methods. The autoradiograms were aligned with the dried gels; the ^{125}I-labeled complexes were located, excised, and measured in a γ counter. (●) ^{125}I-Thrombin + L-PN-1; (○) ^{125}I-thrombin + PN-1; (▲) ^{125}I-thrombin + L-PN-1 + fixed cells; (△) ^{125}I-thrombin + PN-1 + fixed cells.

two reactions. The results in Figure 7 demonstrate that in the absence of cells both L-PN-1 and PN-1 complexed ^{125}I-thrombin at identical rates (3.3 ± 1.4 and 3.3 ± 1.3 fmol of complex/min, respectively). However, in the presence of fixed fibroblasts, the rate of complex formation with L-PN-1 was stimulated approximately 2-fold (5.7 ± 0.9 fmol of complex/min), whereas the rate with PN-1 was increased nearly 5-fold (16.8 ± 3.2 fmol of complex/min).

Discussion

During previous studies on PN-1, a related form was noted, now designated L-PN-1, which bound to heparin with a lower affinity than PN-1 (Scott & Baker, 1983; Scott et al., 1985; Farrell et al., 1986). Because of its higher affinity for heparin and greater concentration in conditioned cell culture medium, PN-1 has been easier to purify. On the other hand, because L-PN-1 has a lower affinity for heparin, the use of heparin–Sepharose or dextan sulfate–Sepharose as an affinity purification step is less effective because of coelution of many unwanted proteins.

It was possible to purify L-PN-1 to apparent homogeneity in the present studies because the dextran sulfate–Sepharose step completely separated it from PN-1 (Figure 1). This was necessary, because monoclonal antibody mAbp18 did not distinguish between PN-1 and L-PN-1. After the dextran sulfate–Sepharose step, it was possible to purify L-PN-1 and PN-1 to apparent homogeneity using mAbp18–Sepharose in an immunoaffinity step. This highly specific step was necessary for this purification and should be useful in purifying L-PN-1 or PN-1 from other sources as well.

Examination of purified L-PN-1 and PN-1 demonstrated that both proteins retained their respective relative affinities for heparin. Aside from this difference, several structural and functional comparisons between L-PN-1 and PN-1 revealed no significant differences. Both molecules were recognized equally by the anti-PN-1 antibodies tested (data not presented). The molecular weights of L-PN-1 and PN-1 as well as their complexes with proteases could not be distinguished by SDS–PAGE. In addition, CNBr peptide mapping studies showed no significant differences between the two proteins (Figure 5). Both L-PN-1 and PN-1 complexed the same proteases. ^{125}I-Thrombin–L-PN-1 complexes and ^{125}I-thrombin–PN-1 complexes both bound to fibroblasts, and their binding was blocked in the presence of heparin. These results clearly showed that L-PN-1 possessed the properties of PN-1 that enable it to regulate certain serine proteases in the immediate extracellular environment.

It was important to examine the possibility that L-PN-1 and PN-1 might be regulated differently by heparin-like molecules at the cell surface. Indeed, these studies showed that the fibroblast surface accelerated the formation of ^{125}I-thrombin–PN-1 complexes 5-fold, whereas it accelerated the formation of ^{125}I-thrombin–L-PN-1 complexes only 2-fold. In previous studies, we showed that this cell surface acceleration was mostly due to heparan sulfate (Farrell & Cunningham, 1986, 1987). Even though heparan sulfate and heparin are structurally very similar, we found that heparin equally accelerated the formation of complexes between thrombin and PN-1 or L-PN-1, even under conditions where the molar ratio of heparin to PN-1 or L-PN-1 was varied from 0.01 to 100. It is noteworthy that the inactivation of thrombin by antithrombin III is accelerated by heparin (Rosenberg & Damus, 1973) but that this reaction is not detectably accelerated by human fibroblasts (Cunningham & Farrell, 1986; McGuire & Tollefsen, 1987). Clearly, much remains to be learned about the cell surface molecules that accelerate the reactions between certain proteases and their inhibitors.

In recent studies, we found that PN-1 is present at the cell surface bound to the extracellular matrix (D. H. Farrell et al., unpublished results). The PN-1 could be extracted in an active form by 1.0 M NaCl. However, it was not detectably removed from the matrix by 0.5 M NaCl, indicating that it remained bound under physiological salt concentrations (D. H. Farrell et al., unpublished results). These studies did not distinguish between PN-1 and L-PN-1. This will be important in future studies, the heparin binding domain of PN-1 could be involved in its association with the matrix. Now that L-PN-1 has been purified, it will be possible to examine this and related questions.

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Mammalian High Molecular Weight and Monomeric Forms of Valyl-tRNA Synthetase†

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ABSTRACT: Valyl-tRNA synthetase from rat liver sediments at 15.5 S with a Stokes radius of 90 Å, corresponding to a native molecular weight of 585 000. Purification of valyl-tRNA synthetase to homogeneity by a combination of conventional and affinity column chromatography yields a fully active monomeric form of valyl-tRNA synthetase with a sedimentation coefficient of 7.7 S and a Stokes radius of 45 Å. The subunit molecular weight of the monomeric valyl-tRNA synthetase is 140 000, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. In the presence of 400 mM KCl, the purified monomeric valyl-tRNA synthetase associates to a high molecular weight form. The high molecular weight valyl-tRNA synthetase in the homogenate can be readily converted to the monomeric form by controlled trypsinization. The kinetic parameters of the two forms are nearly identical. The results suggest that the high molecular weight valyl-tRNA synthetase is a homotypic tetramer and converts to the monomeric valyl-tRNA synthetase after the cleavage of a small peptide.

Mammalian aminoacyl-tRNA synthetases exhibit distinctly different structural organization from their prokaryotic or low eukaryotic counterparts. At least eight of the synthetases (and likely prolyl-tRNA synthetase) apparently associate as a multienzyme complex (Cirakoglu et al., 1985; Deutscher, 1984). The rest of synthetases are free soluble enzymes, similar to those in lower organisms, with the exception of valyl-tRNA synthetase (Yang et al., 1985). Mammalian valyl-tRNA synthetase has not been well studied, due to the instability of the enzyme activity and lability of the structural integrity. In the crude extract, mammalian valyl-tRNA synthetase exhibits properties similar to the multienzyme complex of aminoacyl-tRNA synthetases [e.g., see Ussery et al. (1977)]. However, valyl-tRNA synthetase does not coelute with the synthetase complex (Kellermann et al., 1982). Valyl-tRNA synthetase is the only synthetase for branched amino acids which is not associated with the synthetase complex. Several viral RNAs contain tRNA-like structure which can be charged by valyl-tRNA synthetase (Briand et al., 1976; Florentz & Giege, 1986). Valyl-tRNA synthetase has been purified from *Escherichia coli* (Yaniv & Gros, 1969), *Bacillus stearothermophilus* (Samuelsson & Lundvik, 1978), yeast (Kern et al., 1975), *Euglena gracilis* (Imbault et al., 1979), and yellow lupin seed (Jakubowski & Pawelkiewicz, 1975). Mitochondrial and cytoplasmic valyl-tRNA synthetases may be the same (Suyama & Hamada, 1978). Plant chloroplast (Colas et al., 1982) valyl-tRNA synthetase, however, appears to be different from the cytoplasmic form. Genes coding for valyl-tRNA synthetase have been isolated from *E. coli* (Skogman & Nilsson, 1984) and *Bacillus stearothermophilus* (Brand & Fersht, 1986) and yeast (Jordana et al., 1987). Recent studies of yeast valyl-tRNA synthetase in a concentrated extract suggest that a high molecular weight form of valyl-tRNA synthetase is thio-dependent, arsenite-sensitive (Black, 1983) and in oscillatory interconversion with a soluble form (Black, 1986). Valyl-tRNA synthetase has not been highly purified from any mammalian sources.

In this paper, we report the purification of valyl-tRNA synthetase from rat liver. The purified valyl-tRNA synthetase is a monomeric form of valyl-tRNA synthetase with a subunit molecular weight of 140 000 which is greater than all previously reported valyl-tRNA synthetases from various sources. The structure of the high molecular weight form of valyl-tRNA synthetase is indirectly analyzed and is consistent with being a tetramer of valyl-tRNA synthetase.

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