Covalent Molecular Weight ~92 000 Hybrid Plasminogen Activator Derived from Human Plasmin Amino-Terminal and Urokinase Carboxyl-Terminal Domains†

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ABSTRACT: The preparation of a new class of covalent hybrid plasminogen activators containing the fibrin-binding domains of human plasmin(ogen) and the catalytic active center of human urokinase will be described. Hybridization of the sulfhydryl form of the NH_2-terminal plasmin-derived heavy (A) chain (Pln_A) with the sulfhydryl form of the COOH-terminal urokinase-derived active heavy (B) chain (u-PA_B) was carried out; a covalent Pln_A–u-PA_B hybrid plasminogen activator was prepared. The sulfhydryl form of Pln_A ($Pln_A(SH)_2$) was isolated from reduced Lys-2-plasmin by L-lysine-substituted Sepharose column chromatography. For the isolation of the sulfhydryl form of u-PA_B (u-PA_B(SH)), high molecular weight urokinase was adsorbed onto a benzamidine–Sepharose column and reduced with 100 mM 2-mercaptoethanol on the column. The urokinase NH_2-terminal light (A) chain was washed off the column, and the u-PA_B(SH) chain was eluted from the column. The specific activity of the isolated u-PA_B(SH) chain was determined to be 242 000 IU/mg of protein. The $Pln_A(SH)_2$ and u-PA_B(SH) chains were mixed at a molar ratio of $Pln_A(SH)_2$ to u-PA_B(SH) of 3:2; the reducing agents were then removed by gel filtration. The hybridization (reoxidation) reaction was allowed to proceed for 48 h at 4 °C. The covalent hybrid activator, in 40% yield, was purified from the reaction mixture to homogeneity, by a sequential affinity chromatography method with L-lysine-substituted Sepharose followed by anti-low molecular weight urokinase IgG–Sepharose, and then gel filtration through Sephadex G-150. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis, one molecular species of the covalent Pln_A–u-PA_B hybrid activator was found, M_r ~92 000, indicating that it contains 1 mol of each chain. Reduction of the covalent hybrid activator gave the two parent chains, Pln_A and u-PA_B. The specific activity of the covalent hybrid activator was determined to be 71 500 IU/mg of protein in an amidolytic assay with a specific tripeptide chromogenic substrate, whereas its specific fibrinolytic activity was determined to be 279 000 IU/mg of protein, in a fibrin clot lysis assay, a 4-fold increase in the presence of a fibrin clot. The Glu-plasminogen activator activity of the covalent hybrid enzyme was significantly stimulated in the presence of soluble fibrin, about 5-fold, whereas the activator activity of high molecular weight urokinase was stimulated by soluble fibrin only about 0.5-fold. The hybrid activator was also more strongly adsorbed to a fibrin clot, about 25%, than was high molecular weight urokinase, about 3%. These findings indicate that this new covalent hybrid plasminogen activator, M_r ~92 000, is a more potent plasminogen activator in the presence of fibrin than is high molecular weight urokinase, M_r ~54 000.

Human plasminogen (Plg)[1] is the zymogen of the proteolytic (fibrinolytic) enzyme plasmin (Pln). Native human plasminogen (Glu_1-Plg), M_r ~90 000, consists of a single-chain molecule of 790 amino acid residues (Sottrup-Jensen et al., 1978). Glu_1-Plg is a mixture of two variant forms, Glu_1-1-Plg and Glu_1-2-Plg, which differ in their isoelectric points and states of glycosylation (Summaria et al., 1976; Hayes & Castellino, 1978a–c). Activation of Glu_1-Plg results in the loss of 76 amino acids from the NH_2-terminus to give Lys_{77}-Pln, a two-chain molecule linked by two disulfide bonds (Sottrup-Jensen et al., 1978). The NH_2-terminal Lys_{77} heavy (A) chain (Pln_A) and the COOH-terminal Val_{561} light (B) chain (Pln_B), containing the active center, have been isolated after reduction of the two interchain disulfide bonds of Pln, and a recombinant Lys-Pln was prepared from the sulfhydryl forms of both chains (Summaria et al., 1979). Pln_A contains five regions of sequence homology called kringle domains (Sottrup-Jensen et al., 1978), which have specific binding regions for fibrin (Lucas et al., 1983; Vali & Patthy, 1984), α_2-antiplasmin (Ichinose et al., 1984), and ω-aminocarboxylic acids (Markus et al., 1979).

Human high molecular weight urokinase (HMW-u-PA), a Plg activator, M_r ~54 000, is synthesized in the kidney and excreted into the urine. The active enzyme is a two-chain molecule linked by a single disulfide bond (Günzler et al., 1982a). The NH_2-terminal (A) chain, u-PA_A, has a single kringle domain that shows extensive homology with the plasminogen kringles (Günzler et al., 1982b), whereas the active center of the enzymes is located in the COOH-terminal chain, u-PA_B (Steffens et al., 1982). Recently, we reported

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[1] Abbreviations: Plg, plasminogen; Pln, plasmin; Pln_A, plasmin NH_2-terminal heavy (A) chain; Pln_B, plasmin COOH-terminal light (B) chain; Glu_1, NH_2-terminal glutamic acid; Lys_{77}, NH_2-terminal lysine; HMW-u-PA, high molecular weight urokinase; LMW-u-PA, low molecular weight urokinase; u-PA_A, high molecular weight urokinase NH_2-terminal A chain; u-PA_B, high molecular weight urokinase COOH-terminal B chain; t-PA, tissue plasminogen activator; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; (SH), one interchain sulfhydryl; $(SH)_2$, two interchain sulfhydryls; IU, international units; pyro-Glu-Gly-Arg-pNA, L-pyroglutamylglycyl-L-arginyl-*p*-nitroanilide; H-D-Val-Leu-Lys-pNA, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide; PBS, phosphate-buffered saline; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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on the isolation of a functionally active u-PA_B chain, M_r ~32000, from minimally reduced and alkylated HMW-u-PA (Sumi & Robbins, 1983).

The objective of the studies reported in this paper is to determine if molecules with high fibrin-binding capacity, like the Pln_A chain with its five kringle domains, can be hybridized with the active center domain, or uPA_B chain, of HMW-u-PA to form a plasminogen activator with higher fibrin-binding capacity than the parent HMW-u-PA and with higher catalytic efficiency in the presence of fibrin. Fibrin binding is a specific property of tissue plasminogen activator (t-PA), which has two kringle domains differentiating this activator from HMW-u-PA which has one kringle domain with relatively low fibrin-binding capacity (Pennica et al., 1983). Also, soluble fibrin enhances the Glu-Plg activator of t-PA but not u-PA, another method for differentiating t-PA from u-PA (Hoylaerts et al., 1982). The significance of the studies described in this paper is in the development of methodology for the preparation of new types of plasminogen activators, covalent hybrids, with properties that will change the properties of native plasminogen activators, e.g., u-PA and t-PA, particularly their fibrin-binding capacities, and their enzymatic activities in the presence of fibrin. Their reactions with specific plasma inhibitors may also change with the possibility of obtaining higher catalytic efficiencies in plasma. Covalent hybrid activators with the fibrin-binding domains of other proteins, e.g., fibronectin, are other possibilities.

In this paper, we will report studies on the isolation of the sulfhydryl forms of Pln_A [$Pln_A(SH)_2$] from Lys_{77}-2-Pln and u-PA_B(SH) from HMW-u-PA. Also, studies were carried out on the hybridization of the $Pln_A(SH)_2$ chain with the u-PA_B(SH) chain to obtain a new covalent Pln_A-u-PA_B hybrid Plg activator, and its isolation and characterization will be described. The covalent hybrid activator contains both the fibrin-binding sites of Pln (Plg) and the active center of u-PA. Studies on the effect of fibrin on the activator activity of the covalent hybrid and the binding of the covalent hybrid activator to fibrin clots, compared to u-PA, will be described.

Experimental Procedures

Materials. The u-PA chromogenic substrates pyro-Glu-Gly-Arg-pNA (S-2444) and the Pln chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251) were purchased from Kabi Diagnostica, Sweden. Dansyl chloride and dansyl amino acid standards (Sigma), 2-mercaptoethanol (Aldrich), dithioerythritol (Sigma), agarose (Litex, type HSA) leupeptin (Peptide Institute, Inc.), and Trasylol (M_r ~6500) (Bayer), 6000 kallikrein inhibitor units/mg of protein, were purchased. Sephadex G-25, G-75, G-100, and G-150, Sephacryl S-200, and Sepharose CL-4B were purchased from Pharmacia. Polyamide sheets were obtained from Chang Chin Trading Co. The molecular weight marker proteins for SDS–PAGE and gel filtration were purchased from Pharmacia and Sigma. All other reagents and chemicals were of the highest grade available.

Preparation of Human Glu_1-2-Plg. Human Glu_1-Plg was prepared from human plasma fraction III by an affinity chromatography method with L-lysine-substituted Sepharose (Summaria et al., 1972). Trasylol was added to the fraction III extract (2.3 nM) and to all solutions (0.23 nM) used in the chromatography procedures. Glu_1-2-Plg was obtained from this preparation by a previously published method (Summaria et al., 1976) with minor modifications. Glu_1-Plg (100 mg) was dissolved in 5 mL of 0.1 M phosphate buffer, pH 7.4, containing 0.23 nM Trasylol and applied to a column of L-lysine-substituted Sepharose (1.5 × 28 cm) at 25 °C, previously equilibrated with the same buffer. The column was washed with 40 mL of the same buffer, and the Plg was eluted with a linear gradient consisting of 200 mL of the same buffer as the starting solvent and 200 mL of 7 mM ε-aminocaproic acid in the same buffer, as the limit solvent. Fractions were collected at a flow rate of 50 mL/h at 25 °C. The yield of Glu_1-2-Plg (peak 2) of the total Plg applied to the column was 60–70%. Glu_1-2-Plg was precipitated out of solution by adding 0.35 g of $(NH_4)_2SO_4$/mL at 25 °C, and the precipitate was removed by centrifugation and dissolved in 0.05 M Tris-HCl, 0.02 M lysine, and 0.1 M NaCl, pH 9.0, buffer, containing 0.23 nM Trasylol. The Glu_1-2-Plg solution was applied to a column of Sephacryl S-200 (2 × 60 cm) previously equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 20 mL/h at 4 °C. The main peak fractions were pooled, and the Glu_1-2-Plg was again precipitated out of solution by the addition of 0.35 g of $(NH_4)_2SO_4$/mL at 4 °C as described. The Plg precipitate was dissolved in 25% glycerol, 0.05 M Tris-HCl, 0.02 M lysine, and 0.1 M NaCl, pH 9.0, buffer, at a protein concentration of 15 mg/mL. The Glu_1-2-Plg was found to be homogeneous in SDS–PAGE, and the specific proteolytic activity was determined to be 32 IU/mg of protein (casein assay).

Preparation of HMW-u-PA. Human urinary HMW-u-PA was purified from a partially purified preparation by benzamidine–Sepharose chromatography (Holmberg et al., 1976) followed by gel filtration through Sephadex G-100 (2 × 100 cm) in 0.1 M ammonium acetate buffer, pH 4.5. In the gel filtration step, HMW-u-PA was separated from LMW-u-PA. The HMW-u-PA preparation was found to be homogeneous in SDS–PAGE; its specific activity was determined to be 128000 IU/mg of protein (amidolytic assay). The LMW-u-PA preparation was also found to be homogeneous in SDS–PAGE; its specific activity was determined to be 240000 IU/mg of protein (amidolytic assay). Both preparations were lyophilized and stored at −70 °C.

Preparation of Plg-Free Thrombin. Bovine thrombin was made Plg free by passing the solution through an L-lysine-substituted Sepharose column. The thrombin was eluted in the unadsorbed fraction.

Preparation of Anti-LMW-u-PA IgG–Sepharose and Double Immunodiffusion. Rabbit antibodies to human LMW-u-PA were prepared by immunizing rabbits (New Zealand white female, 6 weeks old) by intracutaneous injection with 0.15 mg of the LMW-u-PA in complete Freund's adjuvant; a double booster injection of the same amount of protein was given at 3-week intervals. After another 10-day period, blood was drawn and incubated at 37 °C for 3 h and 4 °C for 18 h, and the serum was separated by centrifugation at 2500 rpm. The IgG was separated from the serum by both ammonium sulfate fractionation and DEAE-cellulose column chromatography (Fahey, 1967). The anti-LMW-u-PA IgG was coupled to cyanogen bromide activated Sepharose CL-4B (Cuatrecasas, 1970) (15 mg of IgG bound/mL of Sepharose).

Double immunodiffusion analysis was carried out by the Ouchterlony method in 1% agarose in 0.1 M Tris–phosphate buffer, pH 8.5, at 4 °C for 48 h. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 25% methanol/10% acetic acid, destained with 90% methanol/10% acetic acid, and dried. Rabbit anti-LMW-u-PA IgG (5 mg/mL) and rabbit anti-Lys_{77}-Plg IgG (5 mg/mL) were used.

Preparation of $Pln_A(SH)_2$ (Lys_{77}–Arg_{560}). Glu_1-2-Plg (25 mg) was converted to Lys_{77}-2-Pln by activation with HMW-u-PA in 25% glycerol, 0.05 M Tris-HCl, 0.02 M lysine, and 0.1 M NaCl, pH 9.0, at a molar ratio of Plg to u-PA of 1000:1

at 25 °C for 16 h (Robbins et al., 1967); the Plg concentration was 15 mg/mL. Reduction of Lys_{77}-2-Pln and the preparation of Lys_{77}-2-Pln_A [$Pln_A(SH)_2$] were carried out according to a method previously described (Summaria et al., 1979), with minor modifications. The Lys_{77}-2-Pln was reduced in a 25% glycerol, 0.05 M Tris-HCl, 0.02 M lysine, and 0.1 M NaCl, pH 9.0, buffer, containing 50 mM dithioerythritol, 40 mM leupeptin, and 5 mM EDTA, at 0 °C for 3 h under nitrogen, at a protein concentration of 10 mg/mL. The reduced sample was applied to a column of L-lysine-substituted Sepharose (1.5 × 5.8 cm), previously equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 3 mM dithioerythritol, 0.2 mM leupeptin, 5 mM EDTA, 0.23 nM Trasylol. The column was washed with 1–2 volumes of the same buffer and then washed with 1–2 volumes of the same buffer without leupeptin in order to remove Pln_B. The $Pln_A(SH)_2$ chain was eluted with 0.1 M phosphate buffer, pH 7.4, containing 0.2 M ϵ-aminocaproic acid, 3 mM dithioerythritol, 5 mM EDTA, and 0.23 nM Trasylol. A linear ϵ-aminocaproic acid gradient, the same as used for the separation of the Plg forms, can also be used to elute $Pln_A(SH)_2$. This alternative method is useful if the $Pln_A(SH)_2$ chain is partially degraded; the degraded chains in the preparation are eluted before the major native chain. Pln_A (Lys_{77}–Arg_{560}) has a molecular weight of ~60 000, with an $E^{1\%}_{1cm}$ (280 nm) of 17.0 (Summaria et al., 1979; Barlow et al., 1984).

Preparation of u-PA_B(SH) (Ile_{159}–Leu_{411}). HMW-u-PA (12.7 mg, 1.626×10^6 IU) was dissolved in 3 mL of sodium acetate buffer, pH 6.8, and adsorbed on a benzamidine–Sepharose column (1.5 × 5.8 cm), previously equilibrated with the same buffer, and the column was washed with the same buffer. The column was then equilibrated with 50 mL of 0.1 M sodium acetate buffer, pH 6.8, containing 100 mM 2-mercaptoethanol and 5 mM EDTA. The reduction of the interchain disulfide bond connecting the u-PA_A and u-PA_B chains of HMW-u-PA was carried out in the column at 25 °C for 16 h. The column was then washed with 150 mL of the 2-mercaptoethanol–EDTA buffer to remove unadsorbed u-PA_A. The column was further washed with 100 mL of 0.1 M sodium acetate buffer, pH 6.8, containing 3 mM dithioerythritol and 5 mM EDTA. The adsorbed u-PA_B, on the column, was eluted with 0.1 M acetic acid containing 3 mM dithioerythritol and 5 mM EDTA. u-PA_B (Ile_{159}–Leu_{411}) has a molecular weight of ~32 000 (Sumi & Robbins, 1983) with an E_{1cm} (280 nm) of 13.2 (White et al., 1966).

Hybridization of $Pln_A(SH)_2$ with u-PA_B(SH). $Pln_A(SH)_2$ (9.7 mg in 5 mL) and u-PA_B(SH) (3.6 mg, 871 000 IU, in 5 mL) were mixed at 0 °C (ratio of $Pln_A(SH)_2$ to u-PA_B(SH) of 3 mol:2 mol). The mixture was immediately passed through a Sephadex G-25 column (3.2 × 45 cm), which was previously equilibrated with 0.1 M NH_4HCO_3 containing 0.23 nM Trasylol at 4 °C, and eluted with the same solution, in order to remove the reducing agents and other salts. The protein fractions were pooled (24 mL; total A_{280} of 21.24; total activity of 866 000 IU), and then 6 mL of leupeptin solution (10 mg/mL in 0.1 M NH_4HCO_3) was added to the pooled fraction. The hybridization (reoxidation) reaction was then allowed to proceed at 4 °C for 48 h, with continuous stirring. Maximum hybridization was obtained under these conditions.

Purification of Covalent Pln_A–u-PA_B Hybrid Activator. A small amount of precipitate that formed during the hybridization reaction was removed by centrifugation at 2000 rpm for 20 min at 4 °C. The supernatant was applied to a column of L-lysine-substituted Sepharose (1.5 × 5.8 cm), previously equilibrated with 0.1 M NH_4HCO_3. The column was washed with 150 mL of the same solution to remove unhybridized u-PA_B(SH). The covalent hybrid and the unhybridized $Pln_A(SH)_2$, which were adsorbed, were eluted with 0.1 M NH_4HCO_3 containing 20 mM ϵ-aminocaproic acid; the eluted fractions were pooled.

One-third of the pooled–eluted fraction (5 mL; total A_{280} of 5.81; total activity of 117 000 IU) was applied to a column of anti-LMW-u-PA IgG–Sepharose (1.5 × 5.8 cm), previously equilibrated with 0.1 M NH_4HCO_3. The column was washed with 50 mL of 0.1 M NH_4HCO_3 to remove non-u-PA proteins, $Pln_A(SH)_2$, and possible Pln_A dimer. The hybrid activator was eluted with 0.1 M Tris–phosphate buffer, pH 8.0, containing 8 M urea and 2 M NaCl. The immunoadsorbent eluate was immediately dialyzed against 0.05 M NH_4HCO_3 at 4 °C for 48 h. The antibody affinity chromatography step was repeated 2 times. The dialyzed samples from three separate immunoadsorbent affinity column chromatographies were combined (50 mL; total A_{280} of 4.89; total activity of 240,000 IU) and applied to a benzamide–Sepharose column (1.5 × 2.9 cm), previously equilibrated with 0.1 M phosphate buffer, pH 7.4, to concentrate the protein. The hybrid activator was adsorbed and then eluted with 0.1 M acetic acid. The eluate was immediately neutralized to pH 7.0 with 2 M NH_4HCO_3. The solution was concentrated with Centricon 30 (Amicon) to a volume of 1 mL. This preparation was gel-filtered through Sephadex G-150 (1.5 × 90 cm), previously equilibrated with 0.1 M NH_4HCO_3, and eluted with 0.1 M NH_4HCO_3, at a flow rate of 18 mL/h at 25 °C. An $E^{1\%}_{1cm}$ (280 nm) of 15.6 was calculated for the covalent Pln_A-u-PA_B hybrid on the basis of 1 mol of each chain in the hybrid.

Determination of Activator Activity. u-PA amidolytic activity was determined with pyro-Glu-Gly-Arg-pNA by an end point method (Claeson et al., 1978) in a system, in a total volume of 0.9 mL, with 700 μL of 0.05 M Tris-HCl buffer, pH 8.8, containing 38 mM NaCl, 0.1% gelatin, 100 μL of activator, and 100 μL of 3 mM pyro-Glu-Gly-Arg-pNA. After incubation at 37 °C for 10 min, 0.1 mL of 50% acetic acid was added to stop the reaction. The absorbance was measured at 405 nm; a u-PA standard curve was used.

Fibrinolytic activity was measured in a standard fibrin clot lysis assay, in a system containing bovine Plg-rich fibrinogen at a final concentration of 2 mg/mL in 0.1 M phosphate buffer, pH 7.4, at 37 °C, and covalent hybrid activator at various concentrations. The incubation was started after addition of 10 μL of bovine thrombin (100 NIH units/mL in 0.1 M phosphate buffer, pH 7.4). Standard curves were constructed by plotting log of clot lysis time vs. log of activator activity, using a u-PA standard curve, 5–100 IU.

Glu-Plg activator activity was determined by an end-point method with H-D-Val-Leu-Lys-pNA (Wohl et al., 1979, 1980), with and without soluble fibrin (Ranby et al., 1982). Each activator (10 IU) was incubated at 37 °C in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1.25 μM Glu-Plg, and various amounts of soluble fibrin in a total volume of 0.8 mL. After 5 min, generated Pln activity was measured by adding 0.1 mL of 3 mM H-D-Val-Leu-Lys-pNA to the solution and incubating for 1 min at 37 °C; 0.1 mL of 50% acetic acid was added to stop the reaction. The absorbance was read at 405 nm. A Pln standard curve was prepared by using a WHO plasmin standard. Amidolytic activity was determined, with and without soluble fibrin, by the end-point method described earlier, in a system containing 0.05 M Tris-HCl, pH 8.8, 38 mM NaCl, 0.1% gelatin, hybrid activator or HMW-u-PA (40 IU), various amounts of soluble fibrin, and 100 μL of 3 mM pyro-Glu-Gly-Arg-pNA, in a total volume of 0.9 mL. After

incubation at 37 °C for 5 min, 0.1 mL of 50% acetic acid was added to stop the reaction. The absorbance was read at 405 nm.

Amidase parameters were determined with pyro-Glu-Gly-Arg-pNA in a 0.05 M Tris–0.10 M NaCl buffer, pH 9.0, at 37 °C as previously described (Wohl et al., 1979, 1980). The molar concentration of active sites was determined from amidase activity on pyro-Glu-Gly-Arg-pNA; E_0 was 2.67×10^{-8} M in the stock solution and 5.35×10^{-9} M in the assay.

Adsorption of Covalent Pln_A–u-PA Hybrid Activator and HMW-u-PA to Fibrin Clots. The adsorption of the covalent Pln_A–u-PA_B hybrid activator and HMW-u-PA to a fibrin clot was determined by mixing 0.5 mL of 1 mg/mL bovine Plg-free fibrinogen dissolved in PBS (0.01 M phosphate buffer, pH 7.4, containing 0.14 M NaCl), 5 μL of activator (12000 IU/mL), and 5 μL of bovine Plg-free thrombin (100 NIH units/mL). The mixtures were incubated at 37 °C for 30 min. The clots were then removed by centrifugation at 10000 rpm for 5 min at 25 °C. The supernatants were used for the measurement of enzyme activity (unadsorbed proteins). The clots were each washed with 1 mL of PBS 2 times. The washed clots were first extracted with 0.5 mL of 0.2M ε-aminocaproic acid in PBS for 30 min at 25 °C and then centrifuged at 10000 rpm for 5 min to obtain extract I. The clots were further extracted with 0.5 mL of 0.1 M phosphate buffer, pH 8.0, containing 8 M urea and 2 M NaCl at 25 °C for 30 min. After centrifugation at 10000 rpm for 5 min, extract II was obtained. Amidolytic activities of the clot supernatants, and the extracts, were measured with pyro-Glu-Gly-Arg-pNA as the substrate.

NH_2-Terminal Amino Acid Analysis. NH_2-Terminal amino acid analysis was carried out by the dansyl method (Gros & Labouesse, 1969). Before dansylation, samples were denatured by boiling in 0.1% SDS for 5 min and dialyzed against 0.1% SDS to remove low molecular weight materials. The dansylated protein was hydrolyzed in constant boiling 6 M HCl at 105 °C for 18 h in vacuo. After removal of the HCl, the residue was dissolved in ethyl acetate and applied to a polyamide sheet according to a previously described method (Woods & Wang, 1969).

SDS–PAGE. SDS–PAGE was carried out by using a previously described method (Laemmli, 1970), in the Hoefer apparatus (14 × 16 cm, 1.5 mm thick gel). Samples were treated in 0.25 M Tris-HCl buffer, pH 6.8, containing 1% SDS and 10% glycerol, with or without 1% 2-mercaptoethanol (final concentration). The samples were placed on a 5% stacking gel and a 10% running gel and run at a constant current of 70 mA at 4 °C for 90 min in a 0.05 M Tris–glycine buffer, pH 8.3, containing 0.1% SDS in the upper and lower chambers. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 dissolved in 45% methanol/10% acetic acid and destained with 25% methanol/7% acetic acid. The molecular weights and concentrations of each component were determined with a simultaneous run of marker proteins in the same gel; the gel scanned after drying.

RESULTS

Preparation of $Pln_A(SH)_2$. The elution profile of the reduced Lys_{77}-2-Pln preparation from L-lysine-substituted Sepharose is shown in Figure 1. The pass-through fraction contains $Pln_B(SH)_2$. The adsorbed–eluted fraction contains $Pln_A(SH)_2$; its yield was 54% as judged from the absorbance measurements at 280 nm. $Pln_A(SH)_2$ was found to be homogeneous is SDS–PAGE in the presence of 2-mercaptoethanol; an M_r ~60000 was estimated. The predominant NH_2-terminal amino acid of the $Pln_A(SH)_2$ preparation was determined to be Lys.

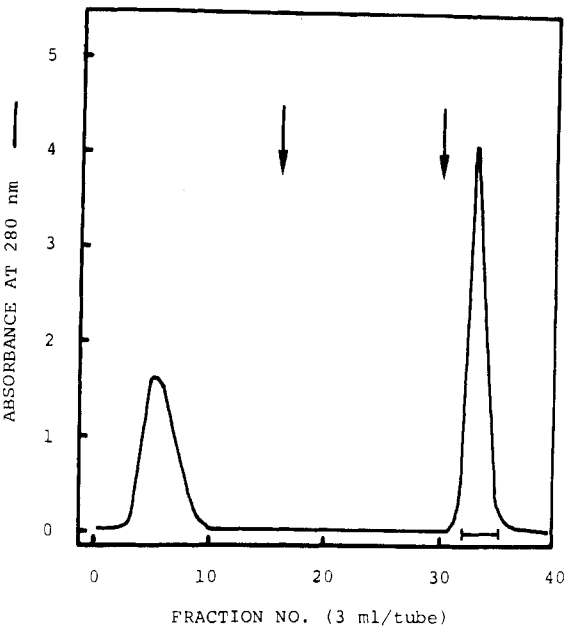

FIGURE 1: Isolation of $Pln_A(SH)_2$ with L-lysine-substituted Sepharose. Reduced Pln (25 mg) was applied to an L-lysine-substituted Sepharose column (1.5 × 5.8 cm), previously equilibrated with 0.1 M phosphate buffer containing 3 mM dithioerythritol, 5 mM EDTA, 0.2 mM leupeptin, and 0.23 nM Trasylol, pH 7.4, and the column was washed with the same buffer. The first arrow marks the point of the buffer change to the equilibration buffer without leupeptin. The second arrow marks the start of elution of $Pln_A(SH)_2$ with 0.1 M phosphate buffer containing 0.2 M ε-aminocaproic acid, 3 mM dithioerythritol, and 0.23 nM Trasylol, pH 7.4. (—) Absorbance at 280 nm.

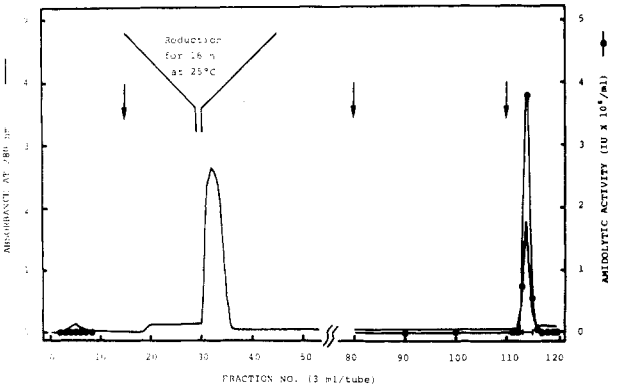

FIGURE 2: Isolation of u-PA_B(SH) with benzamidine–Sepharose. HMW-u-PA (12.7 mg) was applied to a benzamidine–Sepharose column 1.5 × 5.8 cm), previously equilibrated with 0.1 M sodium acetate buffer, pH 6.8, and the column was washed with the same buffer. The first arrow marks the point of buffer change to the equilibration buffer containing 100 mM 2-mercaptoethanol and 5 mM EDTA. Reduction of the HMW-u-PA was carried out in the column at 25 °C for 16 h. After reduction, the column was washed with the same 2-mercaptoethanol buffer. The second arrow marks the point of the buffer change to 3 mM dithioerythritol and 5 mM EDTA in 0.1 M sodium acetate buffer, pH 6.8. The third arrow indicates elution of u-PA_B(SH) with 0.1 M acetate acid containing 3 mM dithioerythritol and 5 mM EDTA. (—) Absorbance at 280 nm; (●) amidolytic activity.

Preparation of u-PA_B(SH). u-PA_B(SH) was isolated from HMW-u-PA by benzamidine–Sepharose affinity chromatography as shown in Figure 2. After reduction of the HMW-u-PA on the column with 100 mM 2-mercaptoethanol at 25 °C for 16 h, u-PA_A(SH) was removed from the column under the same reducing conditions. The column was washed with a 3 mM dithioerythritol buffer, and the u-PA_B(SH) was eluted from the column with 0.1 M acetic acid containing 3 mM dithioerythritol and 5 mM EDTA. The eluted fraction

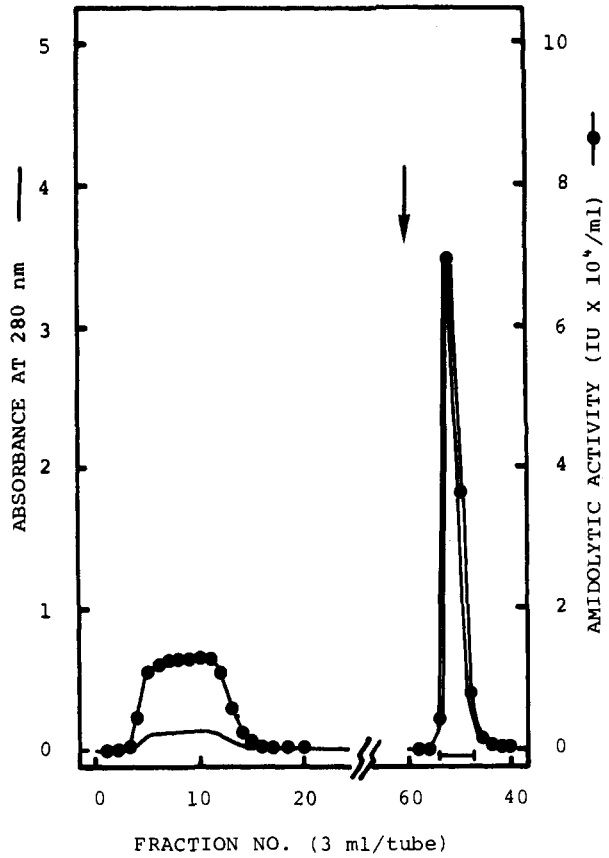

FIGURE 3: L-Lysine-substituted Sepharose column chromatography of hybridization mixture. The sample after hybridization was applied to an L-lysine-substituted Sepharose column (1.5 × 5.8 cm), previously equilibrated with 0.1 M NH_4HCO_3, and washed with the same solution. The arrow marks the start of elution of bound proteins with 20 mM ϵ-aminocaproic acid in 0.1 M NH_4HCO_3. Fractions under the bar were pooled. (—) Absorbance at 280 nm; (●) amidolytic activity.

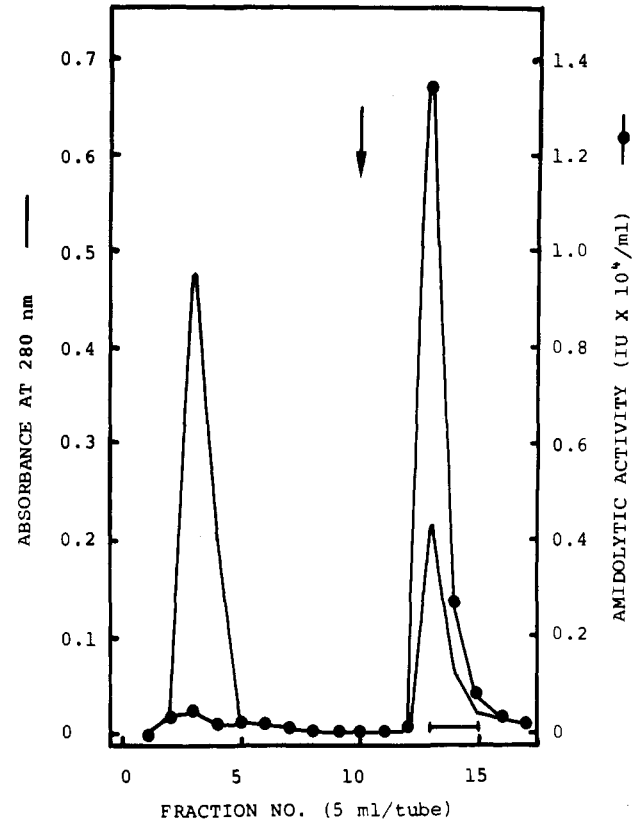

FIGURE 4: Anti-LMW-u-PA IgG–Sepharose column chromatography of the adsorbed–eluted fraction from L-lysine-substituted Sepharose. The adsorbed–eluted fraction from L-lysine-substituted Sepharose (see Figure 3) was applied to an anti-LMW-u-PA IgG–Sepharose column (1.5 × 5.8 cm), previously equilibrated with 0.1 M NH_4HCO_3, and washed with the same solution. The arrow marks the start of elution of the hybrid activator with 0.1 M Tris–phosphate buffer, pH 8.0, containing 8 M urea/2 M NaCl. Fractions under the bar were pooled. (—) Absorbance at 280 nm; (●) amidolytic activity.

(fractions 113–115) contained 6 mg of protein with an activity of 1.452×10^6 IU (specific activity 242000 IU/mg of protein). The yields of protein and activity were 47.2% and 89.3%, respectively. u-PA_B(SH) was determined to be homogeneous in SDS–PAGE in the presence of 2-mercaptoethanol; an M_r ~33000 was estimated. The predominant NH_2-terminal amino acid of u-PA_B(SH) was determined to be Ile. u-PA_B(SH) can also be prepared from LMW-u-PA by the same procedure.

Hybridization and Purification of Covalent Pln_A–UK_B Hybrid Activator. The $Pln_A(SH)_2$ chain was hybridized with the u-PA_B(SH) chain at a molar ratio of $Pln_A(SH)_2$ to u-PA_B(SH) of 3:2, in 0.1 M NH_4NCO_3 containing 0.23 nM of Trasylol and 2 mg/mL of leupeptin at 4 °C for 48 h. There was no apparent loss in enzyme activity or of protein during the hybridization reaction.

After hybridization (reoxidation), the reaction mixture was passed through a L-lysine-substituted Sepharose column in order to adsorb the covalent hybrid, with the unhybridized u-PA_B(SH) passing through the column (Figure 3). About 60% of the activity applied to the column was found in the unadsorbed fraction. The specific activity of the unadsorbed protein was about 170000 IU/mg of protein, which is almost the same specific activity as the parent u-PA_B(SH) which was 242000 IU/mg of protein, indicating that the unadsorbed protein is mainly unreacted u-PA_B(SH). About 80% of the applied protein was eluted with 20 mM ϵ-aminocaproic acid. The adsorbed–eluted fraction contained about 40% of the activity applied to the column and also contained some unhybridized $Pln_A(SH)_2$ and possible Pln_A–Pln_A hybrids. Therefore, the percent yield of activity in the adsorbed–eluted fraction (40%) is a reflection of the percent hybridization. The specific activity of the adsorbed–eluted fraction is about 50% of the specific activity of the original hybridization mixture at zero time.

In the next step, the adsorbed–eluted fraction from the L-lysine-substituted Sepharose column was chromatographed on an anti-LMW-u-PA IgG–Sepharose column to remove the unreacted Pln_A and possible Pln_A dimer. A typical elution profile is shown in Figure 4. About 60% of applied protein was found in the unadsorbed fraction, which had almost no enzyme activity. About 70% of the activity and 30% of the protein applied to the column was recovered by elution with Tris–phosphate buffer, pH 8.0, containing 8 M urea and 2 M NaCl. The specific activity of the fraction increased about 2.5-fold, due to the removal of contaminating Pln_A. Analysis of the eluted fraction by SDS–PAGE showed three components, with M_r ~130000–150000 (4%), M_r ~92000 (91%), and M_r ~70000 (5%).

After dialysis and concentration of the immunoadsorbent adsorbed–eluted fraction, it was gel-filtered through Sephadex G-150 (Figure 5). A main peak of enzyme activity was eluted at a position of about M_r ~90000. The main peak (fractions 38–42) was pooled and concentrated with Centricon 30; it had a specific activity of 12000 IU/mL. As shown in the figure, about 10% of the applied activity was eluted at a position corresponding to M_r ~30000. This protein appears to be u-PA_B(SH) which could result from the dissociation of a

Table I: Purification of Covalent Hybrid Activator

step	A_{280}	activity (IU)	specific activity (IU/A_{280})	activity yield (%)
(1) hybridization mixture through Sephadex G-25	21.24	866 000	40 800	(100.00)
(2) L-lysine-substituted Sepharose	17.43	350 000	20 100	40.4
(3) anti-LMW-u-PA IgG–Sepharose[a]	4.89	240 000	49 100	27.7
(4) Sephadex G-150[b]	1.05	48 000	45 700[c]	5.5

[a] Three times. [b] Selected peak fractions. [c] 71 300 IU/mg of protein.

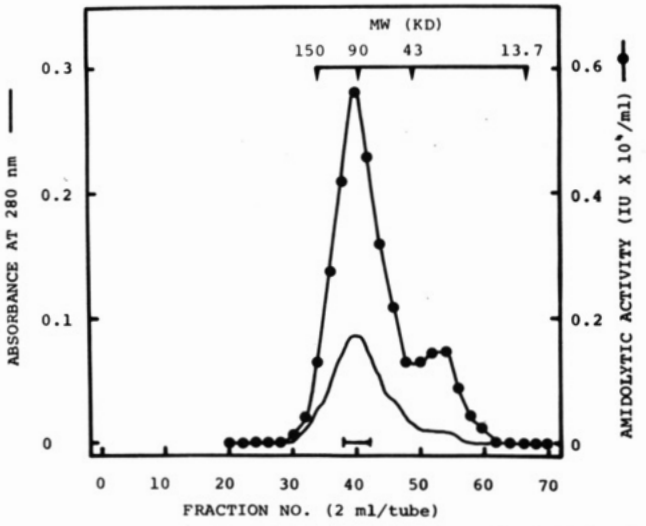

FIGURE 5: Gel filtration of the immunoadsorbent adsorbed–eluted fraction through Sephadex G-150. The adsorbed–eluted fraction from the immunoadsorbent column (see Figure 4), after concentration, was gel-filtered on a column of Sephadex G-150 (1.5 × 90 cm) with 0.1 M NH_4HCO_3. The numbers show the molecular weight of marker proteins [from left to right: rabbit IgG (150 000), Glu_1-Plg (90 000), ovalbumin (43 000), and ribonuclease A (13 700)]. (—) Adsorbance at 280 nm; (●) amidolytic activity.

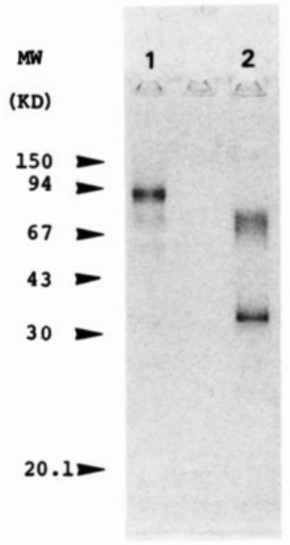

FIGURE 6: SDS–PAGE of the purified covalent hybrid activator. Lane 1, purified covalent Pln_A–UK_B hybrid activator (2 μg); lane 2, purified covalent hybrid activator (3 μg) reduced with 1% 2-mercaptoethanol. The numbers show the molecular weight of marker proteins [from top to bottom: rabbit IgG (150 000), phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), and soybean trypsin inhibitor (20 100)]. Electrophoresis was carried out on a 5% stacking gel and a 10% separating gel.

possible noncovalent hybrid activator during the final purification steps.

A summary of the purification method is given in Table I. The activity yields at each purification step were about 40%, 28%, and 6% (selected peak fractions), respectively.

SDS–PAGE. The purified covalent Pln_A–u-PA_B hybrid activator was analyzed by SDS–PAGE (Figure 6). A molecular weight of ~92 000 was estimated for the covalent hybrid activator which shows that it contains 1 mol of each parent chain. The theoretical molecular weight of the hybrid is ~92 000 (Pln_A chain–M_r ~60 000 plus u-PA_B chain–M_r ~32 000). After complete reduction of the covalent hybrid activator with 2-mercaptoethanol, it was separated into two components that had electrophoretic mobilities similar to those of the reduced parent Pln_A and u-PA_B chains, respectively.

Double Immunodiffusion. Double immunodiffusion analysis of the covalent Pln_A–u-PA_B hybrid activator using both anti-LMW-u-PA IgG and anti-Plg IgG is shown in Figure 7. This analysis shows that the hybrid does indeed contain both Pln and u-PA domains.

Specific Activity of Covalent Pln_A–u-PA_B Hybrid Activator. The specific activity of the purified covalent Pln_A–u-PA_B hybrid activator was measured by the amidolytic assay with pyro-Glu-Gly-Arg-pNA and by the fibrin clot lysis assay. In the amidolytic assay, the specific activity of the hybrid activator was found to be 71 300 IU/mg of protein, whereas in the fibrin clot lysis assay, against the same HMW-u-PA standard, its specific activity was found to be 279 000 IU/mg of protein. This indicates that the covalent Pln_A–u-PA_B hybrid activator has about a 4-fold higher fibrinolytic activity than amidolytic activity. In the amidolytic assay, the specific activity of HMW-u-PA, of M_r ~54 000, is 128 000 IU/mg of protein, whereas the specific activity of LMW-u-PA, of M_r ~31 000, is 240 000 IU/mg of protein. These data infer an intact active center in the covalent hybrid activator.

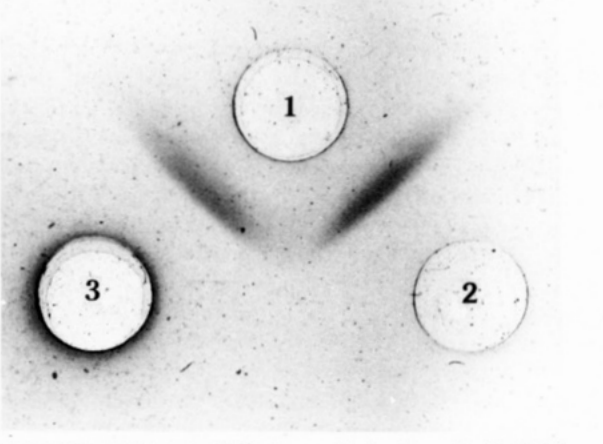

FIGURE 7: Double immunodiffusion analysis of covalent hybrid activator. Well 1 contained 10 μL of covalent hybrid activator (0.08 mg/mL), well 2 contained 10 μL of anti-Plg IgG (5.0 mg/mL), and well 3 contained 10 μL of anti-LMW-u-PA IgG (5.0 mg/mL).

Amidase kinetic parameters of the covalent hybrid activator were determined. The apparent Michaelis constant (K_m) was 110 μM, and the catalytic rate constant (k_{cat}) was 21.1 s^{-1}. They were similar to those kinetic parameters obtained in our laboratory with HMW-u-PA and LMW-u-PA (Sumi & Robbins, 1983; Wohl et al., 1979, 1980) and the functionally active u-PA_B chain (Sumi & Robbins, 1983). These data

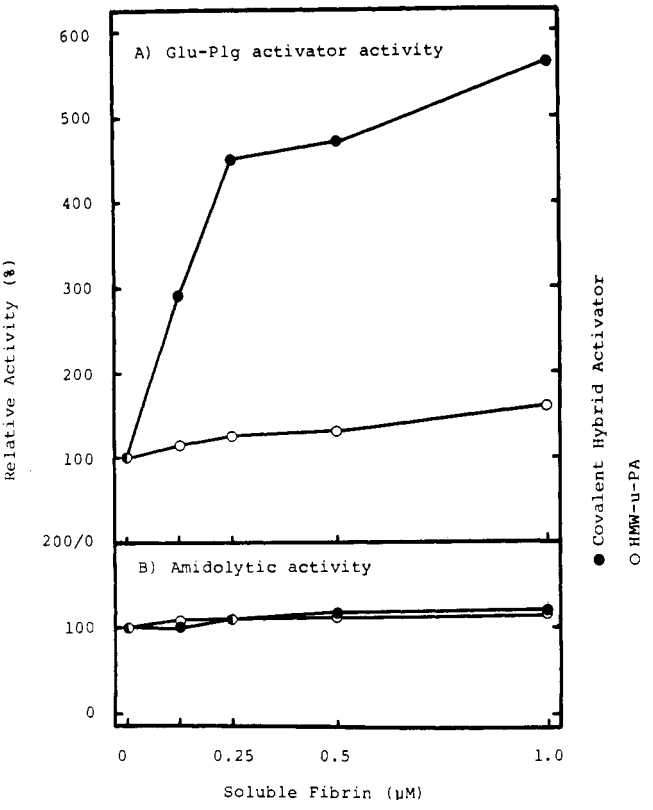

FIGURE 8: Effect of soluble fibrin on Glu-Plg activator activity and amidolytic activity of covalent hybrid activator. (A) Glu-Plg activator activity; (B) amidolytic activity with pyro-Glu-Gly-Arg-pNA as the substrate. (●) purified hybrid activator; (○) HMW-u-PA. The activities in the presence of soluble fibrin are expressed as percents of the activities in the absence of soluble fibrin.

showed that the covalent hybrid activator has the same catalytic properties as native HMW-u-PA.

Effects of Soluble Fibrin on Glu-Plg Activator Activity and Amidolytic Activity of the Covalent Pln_A–u-PA_B Hybrid Activator. Soluble fibrin has a significant effect on the Glu-Plg activator activity of the purified covalent Pln_A–u-PA_B hybrid activator (Figure 8A). Soluble fibrin increases the Glu-Plg activator activity of the hybrid activator significantly, but it has little effect on the Glu-Plg activator activity of HMW-u-PA. The activity of the hybrid activator was 4.7-fold and 5.7-fold higher at soluble fibrin concentrations of 0.5 and 1.0 μM, respectively, than that in the absence of soluble fibrin. The activity of u-PA at similar soluble fibrin concentrations increases about 0.5-fold. Soluble fibrin has no effect on the amidolytic activity of the covalent hybrid activator or on the amidolytic activity of HMW-u-PA (Figure 8B).

Adsorption of the Covalent Pln_A–u-PA_B Hybrid Activator and HMW-u-PA to Fibrin Clots. The results of the adsorption experiments of the purified covalent Pln_A–u–PA_B hybrid activator and HMW-u-PA are summarized in Table II. The hybrid activator was bound more strongly than the HMW-u-PA to fibrin clots. A significant amount of the hybrid activator was adsorbed to the fibrin, while the adsorption of the HMW-u-PA to the clot was negligible. About 25% of the activity present in the clotting mixture was recovered from the washed clot by extraction first with 0.2 M ε-aminocaproic acid (14.4%) and then with 8 M urea/2 M NaCl (10.5%).

Table II: Adsorption of Covalent Hybrid Activator and HMW-u-PA to Fibrin Clots

	% activity[a]	
	covalent hybrid activator	HMW-u-PA
unbound	67.1 ± 3.8	100.2 ± 2.3
clot extract		
extract I[b]	14.4 ± 3.5	1.0 ± 0.5
extract II[c]	10.5 ± 3.0	2.2 ± 0
total extract	24.9 ± 1.7	3.2 ± 0.5

[a] Results were expressed as mean ± SD (n = 3). [b] Clots were extracted with 0.1 M ε-aminocaproic acid. [c] Clots were extracted with 8 M urea/2 M NaCl.

Discussion

It has been established that native direct-type plasminogen activators like urokinase (u-PA) and tissue plasminogen activator (t-PA) differ in their ability to activate human Plg in the presence of fibrin (Hoylaerts et al., 1982). The enzymatic activity of t-PA is greatly enhanced in the presence of fibrin whereas soluble fibrin has a slight effect on u-PA activation of Plg. It appears that this effect is probably due to the preferential binding of t-PA to fibrin clots, perhaps a reflection of the two kringle domains in the NH_2-terminal region of t-PA (Pennica et al., 1983). It is possible that the single-chain zymogen of u-PA (pro-u-PA), which has a single kringle domain in the NH_2-terminal region (Günzler et al., 1982b), may also have some fibrin-binding properties (Takada et al., 1984), but apparently not as great as the two kringle domains of t-PA. It has not been proven definitively that the kringle domains in either t-PA or u-PA have fibrin-binding properties. Plg (Pln) has specific fibrin-binding domains in the NH_2-terminal region; the major fibrin-binding domain may be kringle 2 (Van Zonneveld et al., 1985).

The studies reported in this paper were carried out to determine if molecules with high fibrin-binding capacity, like the Pln_A chain, derived from the NH_2-terminus of Plg, containing kringles 1–5, can be combined with the catalytic active center of u-PA. We had previously reported studies on the isolation of the sulfhydryl forms of the Pln_A and Pln_B chains, after reduction of the two interchain disulfide bonds, and a recombinant covalent Pln was prepared by hybridization of the two chains (Summaria et al., 1979). Also, we had reported on the isolation of a functionally active u-PA_B chain from HMW-u-PA (Sumi & Robbins, 1983); this chain was alkylated after minimal reduction of HMW-u-PA in which the single interchain disulfide bond was reduced. Although the technology was available in our laboratory for preparing mixed covalent hybrids from the $Pln_A(SH)_2$ and u-PA_B(SH) chains, the outcome was not predictable.

The two interchain disulfide bonds connecting the NH_2-terminal Pln_A and COOH-terminal Pln_B chains are Cys_{557}–Cys_{565} and Cys_{547}–Cys_{665} (Sottrup-Jensen et al., 1978) which are close to the Arg_{560}–Val peptide bond cleaved in Plg by activators to form Pln (Robbins et al., 1967). The Pln_A chain, Lys_{77}–Arg_{560}, contains five kringle domains where each domain is a triple loop, three disulfide bridge region. The two-chain u-PA molecule has a single interchain disulfide bond, Cys_{148}–Cys_{279}, connecting the NH_2-terminal u-PA_A and COOH-terminal u-PA_B chains (Bachman & Kruithof, 1984). The single-chain inactive zymogen of u-PA (pro-u-PA) is cleaved by a serine protease, perhaps Pln, at Lys_{158}–Ile to form the two-chain active u-PA (Günzler et al., 1982b; Bachman & Kruithof, 1984); a second bond cleavage also occurs at Lys_{135}–Lys resulting in the low molecular weight u-PA form. Perhaps the Lys_{135}–Lys peptide bond cleavage occurs before the Lys_{158}–Ile bond is cleaved, since a u-PA_B chain was isolated from HMW-u-PA with NH_2-terminal lysine (Sumi & Robbins, 1983). The two-chain t-PA also has a single interchain disulfide bond, Cys_{267}–Cys_{398}, connecting the NH_2-terminal t-PA_A and COOH-terminal t-PA_B chains (Pennica et al., 1983). The t-PA NH_2-terminal chain has a molecular weight

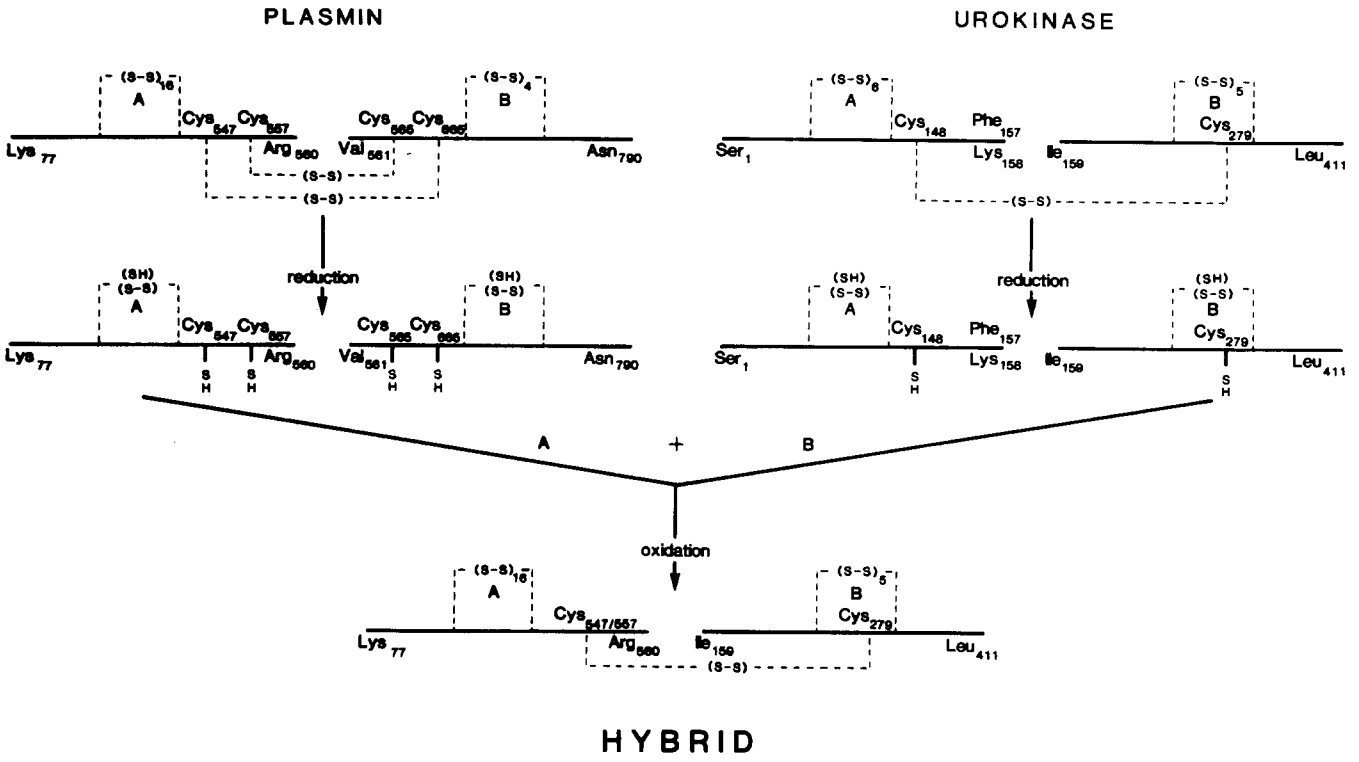

FIGURE 9: Schematic presentation of the structures of the parent molecules, the sulfhydryl forms of the chains, and the covalent hybrid.

of ~36 000, and the COOH-terminal chain containing the active center has a molecular weight of ~32 000. There is a high degree of homology between the u-PA and t-PA active center regions, as well as in the kringle regions (Bachman & Kruithof, 1984).

The new M_r ~92 000 covalent hybrid activator contains 1 mol each of Pln_A, M_r ~60 000, and u-PA_B, M_r ~32 000. Pln_A with two free interchain sulfhydryl groups, at Cys_{547} and Cys_{557}, probably reacted with the single interchain sulfhydryl group in u-PA_B, at Cys_{279}. After reoxidation, the covalent hybrid probably formed a disulfide bond between Pln_A-Cys_{547} and u-PA-Cys_{279} or between $Pln_A Cys_{557}$ and u-PA-Cys_{279}. It does not appear to be possible to have Pln_A-Cys_{547}/Cys_{557} reacting with two molecules of u-PA_B-Cys_{279}, due to steric hindrance, since the two Pln_A sulfhydryl groups are separated by nine amino acid residues. Since hybridization occurs in yields of about 40% (Table I), we believe that reoxidation in this system of monomers with free sulfhydryl groups to form covalent hubrids shows that successful disulfide pairing is possible. We have made the assumption that if a few of the intrachain disulfide bonds are cleaved in the two monomers during minimal reduction (Summaria et al., 1979; Sumi & Robbins, 1983), they are re-formed during reoxidation. It has been reported that kringle 4 loses its affinity for L-lysine-substituted Sepharose upon reductive cleavage of its disulfide bridges (Trexler & Patthy, 1983). However, aerobic incubation of the reduced kringle results in the rapid restoration of the disulfide bonds with concomitant recovery of lysine–Sepharose affinity. However, there is the possibility that a single interchain disulfide bond, Cys_{168}–Cys_{296} connecting kringles 2 and 3 may not re-form after cleavage of the disulfide bonds, and a covalent hybrid form with $Pln_A(SH)_2$ is possible. This could result in a Pln_A–$(u\text{-}PA_B)_2$ hybrid with M_r ~120 000. We have found small amounts of a higher molecular weight form (see Figure 5, in fractions 32–36). A schematic presentation of the structures of the parent molecules, the sulfhydryl forms of the chains, and the covalent hybrid is seen in Figure 9.

The purification method used to isolate the hybrid activator was designed to first separate the Pln_A–u-PA_B hybrid (and unhybridized Pln_A) from unhybridized u-PA_B(SH), on an L-lysine-substituted Sepharose affinity column (Figure 3). In the next step, an immunoaffinity method with anti-LMW-u-PA IgG–Sepharose was used to separate the Pln_A–u-PA_B hybrids from unhybridized $Pln_A(SH)_2$ (Figure 4). The gel filtration step with Sephadex G-150 was used to select out the homogeneous covalent hybrid containing 1 mol of each chain (Figure 5). Throughout the isolation procedures, there was some evidence of different covalent Pln_A–u-PA_B hybrid forms. In the first step, gradient elution of the hybrid from L-lysine-substituted Sepharose with ϵ-aminocaproic acid gave several ill-defined peaks with different specific activities. In the gel filtration step (Figure 5), early fractions selected before the peak fractions were found to contain small amounts of a higher molecular weight component, M_r ~120 000, perhaps a covalent Pln_A–$(u\text{-}PA_B)_2$ hybrid. They had 2-fold higher activities in the fibrin clot lysis assay over the highly purified covalent hybrid (unpublished data).

The new class of plasminogen activators described in this paper, the covalent Pln_A-u-PA_B hybrid, which contains the Plg(Pln) fibrin-binding domains and the u-PA_B activator active center, has properties that resemble t-PA, namely, high fibrin-binding affinity (Table II) and a 5-fold enhancement of Glu-Plg activation in the presence of soluble fibrin (Figure 8). Probably, t-PA_B can also supply the activator active center to form a covalent hybrid with $Pln_A(SH)_2$. A useful method has been described in this paper for preparing covalent hybrid activators from other types of fibrin-binding proteins, e.g., fibronectin, together with other plasminogen activator active centers.

Registry No. Plg, 9001-91-6.

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