A Virgin Enzyme Derived from Human Plasminogen. Specific Cleavage of the Arginyl-560–Valyl Peptide Bond in the Diisopropoxyphosphinyl Virgin Enzyme by Plasminogen Activators†

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ABSTRACT: An active site is formed in human Lys-plasminogen, a single chain monomer, in a stoichiometric Lys-plasminogen·streptokinase (Lys-Plg·SK) complex. The zymogen with an active site, Lys-Plg_a, or the virgin enzyme, and Lys-Plg, can be dissociated from the SK moiety at pH 2.2. Acid dissociation of a Lys-Plg_a·SK complex, with about 50% active sites, resulted in complete precipitation of the SK moiety with the bulk of the Lys-Plg_a and parent Lys-Plg recovered in the supernatant solution. This Lys-Plg_a preparation containing 54% active sites was further purified by an affinity chromatography method with a Trasylol–Sepharose column to separate Lys-Plg_a from the contaminating parent Lys-Plg. The final Lys-Plg_a preparation, a single chain monomer, contained 69% active sites and had a specific proteolytic activity of 23.6 IU/mg of protein; it had no SK and no human or bovine plasminogen activator activity. The only contaminant was the parent Lys-Plg, about 31%, which was completely activatable. The amidase parameters of this Lys-Plg_a preparation were determined on two synthetic chromogenic substrates. With H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide, a K_m of 417 μM and a k_{cat} of 8.44 s^{-1} were obtained; with Tos-glycyl-L-prolyl-L-lysyl-*p*-nitroanilide, a K_m of 307 μM and a k_{cat} of 12.83 s^{-1} were obtained. Lys-Plg_a is inhibited by leupeptin and by the human plasma α_2-plasmin inhibitor. The K_i for leupeptin with Lys-Plg_a was determined to be 8.2 μM, whereas the K_i for the plasma α_2-plasmin inhibitor with Lys-Plg_a was determined to be 0.75 μM for the reversible first step. DIP-Lys-Plg_a, when isolated from the DIP-Lys-Plg_a·SK complex by acid dissociation, could be cleaved at the intact Arg_{560}–Val peptide bond by either UK or the various SK·Plg activator enzyme species, except for SK, to give an inactive DIP-Pln. However, the DIP-Lys-Plg_a·SK complex could not be cleaved at the intact Arg_{560}–Val peptide bond in the Plg moiety by these activator enzymes, indicating that the SK moiety in the complex occupies the activator binding site which is in the vicinity of the Arg_{560}–Val peptide bond.

When human plasminogen and streptokinase react to form a stoichiometric complex, either plasminogen·streptokinase (Plg·SK)[1] or plasmin·streptokinase (Pln·SK) can be identified, depending upon the conditions of the reaction (De Renzo et al., 1963; Hummel et al., 1966; Buck et al., 1968; Summaria et al., 1968). In the equimolar human Plg·SK (with either Glu-Plg or Lys-Plg) complex, an active site develops in the Plg moiety in minutes prior to the formation of plasmin (Glu-Pln or Lys-Pln) (McClintock & Bell, 1971; Reddy & Markus, 1972, 1973). This enzyme has been shown to react with serine protease inhibitors, such as DFP, NPGB, and bovine pancreatic trypsin inhibitor or Trasylol. However, the enzyme apparently does not react with the naturally occurring plasma protease inhibitors, such as α_2-plasmin inhibitor, α_2-macroglobulin, α_1-antitrypsin, or antithrombin III (Summaria et al., 1968; McClintock & Bell, 1971; Reddy & Markus, 1972–1974; Summaria et al., 1977; Wohl et al., 1979; Cederholm-Williams et al., 1979). Kinetic parameters have been determined for both the Plg·SK and Pln·SK enzyme complexes (Buck & Boggiano, 1971; Reddy & Markus, 1974; Wohl et al., 1977–1980; Morris et al., 1981). Various methods have been used to dissociate the Plg·SK enzyme complex in an effort to study the individual moieties (De Renzo et al., 1967; Buck et al., 1968; Summaria et al., 1968, 1971; Brockway & Castellino, 1974), but a pure, structurally intact Plg moiety, with or without an active site, had not been previously isolated. We had previously reported on a preparation of Lys-Plg_a with 43% active sites, containing ~57% Plg, isolated directly from a fraction $III_{2,3}$ preparation (Wohl et al., 1977). A fibrinogen fragment E·Lys-Plg complex was recently isolated and reported to hydrolyze a synthetic chromogenic substrate specific for Pln and to possess 30% of the activity of Pln toward [^{125}I]fibrin (Adams et al., 1980).

In this report, we will describe a method for dissociating a Lys-Plg·SK complex containing ~50% active sites and isolating a Lys-Plg moiety with an active site (Lys-Plg_a). The amidase parameters of Lys-Plg_a were determined with two different synthetic chromogenic substrates, and the kinetic constants for its interaction with two plasmin inhibitors were determined. A DIP-Lys-Plg_a·SK complex was prepared, and DIP-Lys-Plg_a was isolated from this complex. Studies on the cleavage of the Arg_{560}–Val sensitive peptide bond essential for the formation of plasmin were carried out with DIP-Lys-Plg_a·SK, and DIP-Lys-Plg_a separated from the complex, by different UK and SK activator species.

Experimental Procedures

Plasminogen and Plasmin and SK Activator Preparations. Human Lys-Plg was prepared from plasma fractions $III_{2,3}$ by an affinity chromatography method with L-lysine-substituted

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[1] Abbreviations: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; IU, international units established by World Health Organization for streptokinase, urokinase, and plasmin; H-D-Val-Leu-Lys-pNa, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide; Tos-Gly-Pro-Lys-pNA, Tos-glycyl-L-prolyl-L-lysyl-*p*-nitroanilide; Cbz, benzyloxycarbonyl; Plg, plasminogen; Pln, plasmin; Glu-Plg, NH_2-terminal glutamyl plasminogen; Lys-Plg, NH_2-terminal lysyl plasminogen; Lys-Plg_a, NH_2-terminal lysyl plasminogen virgin enzyme with an active site; Lys-Pln, NH_2-terminal lysyl-77 and valyl-561 plasmin; SK, streptokinase; UK, urokinase; DFP, diisopropyl phosphorofluoridate; DIP, $(i\text{-}PrO)_2P(O)$– or diisopropoxyphosphinyl; MSF, methanesulfonyl fluoride.

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Sepharose (Robbins & Summaria, 1976). The specific proteolytic activity was 30 IU/mg of protein determined in a casein assay (Robbins & Summaria, 1976). The preparation was >98% activatable with catalytic amounts of either SK or UK. Lys-Pln, with a specific activity of 30 IU/mg of protein, was prepared from Lys-Plg by activation with catalytic amounts of UK in a standard 25% glycerol buffer system, at pH 9, by methods previously described (Robbins et al., 1967). The human Lys-Plg_a·SK complex and the Lys-Pln·SK complex were prepared by reacting 0.3 mL of SK at a concentration of 8.8 mg/mL in 0.1 M phosphate buffer, pH 7.0, with either 0.7 mL of Lys-Plg at a concentration of 7.2 mg/mL in 0.05 M Tris/0.02 M lysine/0.1 M NaCl buffer, pH 9.0, or 0.7 mL of Lys-Pln at a concentration of 7.2 mg/mL in 25% glycerol/0.04 M Tris/0.02 M lysine/0.08 M NaCl buffer, pH 9.0, at 0 °C, for 10 min. These activator complexes have a specific activity of ~75 000 SK equiv units/mg of protein (casein assay). The DIP-Lys-Plg_a·SK complex was prepared by reacting equimolar quantities of Lys-Plg and SK in the presence of 0.1 M DFP in the 25% glycerol buffer system, at 0 °C, for 24 h, with subsequent removal of the DFP by dialysis (no Lys-Plg·SK). The Pln-derived light (B) chain·SK activator complex was prepared from a reduced and alkylated Lys-Pln·SK complex by previously described methods; it had a specific activity of 158 000 SK equiv units/mg of protein (casein assay).

Materials. SK was a highly purified preparation with a specific activity of 100 000 IU/mg of protein (fibrin assay) and was a gift from Dr. R. Lundēn (AB Kabi). A high molecular weight UK preparation with a specific activity of 118 000 IU/mg of protein (fibrin assay) was a gift from Dr. N. Aizawa (Mochida Pharmaceutical Co.). Leupeptin (Ac-L-Leu-L-Leu-L-argininal) was a gift from Dr. T. Aoyagi (Institute of Microbial Chemistry, Japan). NPGB, DFP, Trasylol, dodecyl sulfate (sodium lauryl sulfate; BDH), and acrylamide gel reagents (Eastman and Ortec) were purchased. Trasylol–Sepharose was prepared by the method described by Cuatrecasas et al. (1968).

Methods. The steady-state kinetic methods used to determine the amidase parameters and the inhibitor dissociation constants were previously reported (Wohl et al., 1977, 1979, 1980). Inhibition by α_2-plasmin inhibitor was measured with normal plasma (Wohl et al., 1979). Human and bovine plasminogen activator activities were determined by methods previously described (Summaria & Robbins, 1976; Wohl et al., 1980). Proteolytic activity was determined in a casein assay (Robbins & Summaria, 1976). Active-site titrations were carried out by a previously described method (Chase & Shaw, 1969). Active-site titrations carried out at 1.5 °C showed bursts with either Pln, Plg_a, or preincubated Plg·SK complex. The percent of active site formed was calculated, after subtraction of nonspecific hydrolysis of NPGB by the buffer, by Plg, and by SK, separately. All of the samples used for kinetic and active-site titrations were adjusted to pH 7.4 or to pH 8.2 with NaOH. Acrylamide gel/dodecyl sulfate electrophoresis was carried out in the Ortec acrylamide gel system by methods previously described (Summaria et al., 1975). The gels were dried and scanned with a Beckman Model 110 microzone scanner both to identify and to quantitate each component.

Results and Discussion

Active-Site Formation at 1.5 °C. Equimolar amounts of human Lys-Plg and SK were mixed and incubated at 1.5 °C for 5 min in the presence of NPGB; approximately 50% of the potential active sites were obtained in this time. Equimolar quantities of Lys-Plg and SK were also reacted at 0 °C for 5 min in the absence of NPGB and then treated with 1×10^{-4} M NPGB. After reduction and alkylation, the sample when analyzed by acrylamide gel/dodecyl sulfate electrophoresis showed no plasmin. Plasmin is found in preparations incubated longer than 5 min at 0 °C. The results obtained were similar to those reported by McClintock & Bell (1971) and Reddy & Markus (1972), using different conditions for the reaction.

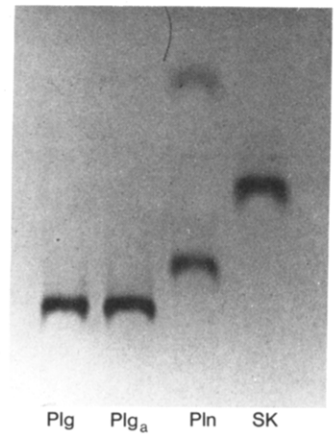

FIGURE 1: Acrylamide gel/dodecyl sulfate electrophoretogram of a Lys-Plg_a preparation. (1) Lys-Plg, reduced and alkylated; (2) Lys-Plg_a, reduced and alkylated; (3) Lys-Pln, reduced and alkylated; (4) SK, reduced and alkylated.

Isolation of Lys-Plg_a. Equimolar quantities of human Lys-Plg (M_r 82 400) and SK (M_r 44 000) were mixed at 0 °C, pH 8.2, at a Lys-Plg concentration of 5.0 mg/mL and an SK concentration of 2.65 mg/mL in a 1.0-mL volume and incubated at 0 °C for 5 min; 0.1 mL of 1 M HCl was then added to the preparation at 0 °C in order to adjust the mixture to pH 2.2. At this pH, the bulk of the complex dissociated with an immediate precipitation of all of the SK moiety and some undissociated complex, but the preparation was incubated for 2 h at 0 °C to ensure complete precipitation of SK. The precipitate was removed by centrifugation at 0 °C for 1 h at 16 000 rpm in an International Model PR-2 centrifuge, high speed rotor no. 296, and discarded. The supernatant solution containing Lys-Plg_a (76% recovery of starting Lys-Plg), with 54% active sites, had a specific activity of 13.6 IU/mg of protein. It was adjusted to pH 7.0 with 1 N NaOH at 0 °C. One milliliter of the preparation was passed through a 50-mL Trasylol–Sepharose column (2 × 10 cm) (containing 10 mg of bound Trasylol) at 4 °C; approximately 40% of the protein adsorbed to the column. The protein was eluted with 40 mL of 1 M KCl at pH 2.0; 28% of the applied protein was recovered. This Lys-Plg_a preparation (0.3 mg of protein/mL), with 69% active sites, had a specific activity of 23.6 IU/mg of protein. It was stored at pH 2.0 at −40 °C.

Characterization of Lys-Plg_a. The Lys-Plg_a preparation was analyzed, after reduction and alkylation, by acrylamide gel/dodecyl sulfate electrophoresis (Figure 1). The preparation showed a single homogeneous component with neither SK nor Pln components. Lys-Plg is a single chain monomer containing an NH_2-terminal Lys_{77} residue by Edman degradation (Robbins et al., 1972), with a molecular weight of 82 400 (Robbins et al., 1975). The Lys-Plg_a preparation appears to have the same molecular weight as Lys-Plg without any bond cleavages. Neither SK nor Lys-Pln contamination is seen in the acrylamide gel system, and the preparation did not have any Plg activator activity on either human or bovine Plg. The residual protein (31%) was parent Lys-Plg which was completely activatable; the total specific activity of the

Table I: Amidase Parameters of Lys-Plg_a

enzyme	substrate	amidase parameters[a] K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Lys-Plg_a	H-D-Val-Leu-Lys-pNA	417 ± 78	8.44 ± 0.91	0.020
Lys-Plg_a	Tos-Gly-Pro-Lys-pNA	307 ± 24	12.83 ± 0.47	0.042

[a] Values given are means ± standard error.

preparation was 29.7 IU/mg of protein which is the highest specific activity found for either native Lys-Plg or Lys-Pln.

The amidase parameters of the Lys-Plg_a preparation were determined by using two different synthetic chromogenic peptide substrates, and the data are summarized in Table I. Since the parent Lys-Plg had no activity on either protein or peptide substrates, the amidase parameters were compared to Plg-derived enzymes. For example, with H-D-Val-Leu-Lys-pNA, the K_m of Lys-Plg_a was similar to the K_m of Lys-Pln and two activator enzyme species, the Lys-Plg_a·SK complex and the Pln-derived light (B) chain·SK complex, but about 3-fold lower than that of the Pln-derived light (B) chain (Wohl et al., 1979, 1980). The k_{cat} of Lys-Plg_a on this substrate was 2-fold lower than the k_{cat} of the Pln-derived light (B) chain, 3-fold lower than the k_{cat} of Lys-Pln, and 5-fold lower than the k_{cat} of the activator species, the Lys-Plg_a·SK complex and the Pln-derived light (B) chain·SK complex. These data show that the active site in Lys-Plg_a is different from, and less efficient than, the active site in either Lys-Pln or the Lys-Plg·SK and Pln-derived light (B) chain·SK complexes, but more efficient than the active site in the Pln-derived light (B) chain. However, these data do show that Lys-Plg_a is a catalytically efficient enzyme on both the synthetic peptide and protein substrates. The k_{cat}/K_m determined for Lys-Plg_a is 6-fold lower than the k_{cat}/K_m determined for Lys-Pln; this difference was substantially less than the observed k_{cat}/K_m difference between trypsinogen and trypsin, or chymotrypsinogen and chymotrypsin (Lonsdale-Eccles et al., 1978). It was found that trypsinogen had a k_{cat}/K_m which was 49–186-fold lower than the k_{cat}/K_m of trypsin, and chymotrypsinogen had a k_{cat}/K_m which was 450–2240-fold lower than the k_{cat}/K_m of chymotrypsin, with three different substrates. Also, the k_{cat}/K_m ratio of Lys-Pln to Lys-Plg_a is 6.1 which is very similar to the ratio of the second-order rate constants of bovine factor X_a to factor X, which was reported to be 8.5 with DFP and 6.7 with MSF (Kerr et al., 1978).

The Lys-Plg_a isolated in this study cannot be compared on a kinetic basis with a recently isolated fibrinogen fragment E·Lys-Plg complex, since no kinetic data were reported (Adams et al., 1980). But, it was reported that the fibrinogen fragment E·Lys-Plg complex hydrolyzed a tripeptide substrate specific for Pln, contained 30% of the activity of Pln toward [^{125}I]fibrin, had no Plg activator activity, and was inhibited by bovine pancreatic trypsin inhibitor. It is possible that the active site in that complex is similar to the active site in Lys-Plg_a.

Lys-Plg_a was found to be inhibited by bovine pancreatic trypsin inhibitor or Trasylol, leupeptin, and the plasma α_2-plasmin inhibitor. The K_i of leupeptin with Lys-Plg_a was determined to be 8.2 μM. This value is identical with the K_i of 8.5 μM reported for leupeptin with Lys-Pln and the K_i of 9.5 μM reported for leupeptin with the Lys-Plg_a·SK complex but different from the K_i of 30.0 μM reported for the Lys-Pln·SK complex (Wohl et al., 1977). The K_i of the Lys-Plg_a–α_2-plasmin inhibitor reversible first step was determined to be 0.75 μM; a K_i of 0.19 nM for the Lys-Pln–α_2-plasmin inhibitor reversible step was previously reported (Wiman & Collen, 1978). The fact that the K_i for Lys-Plg_a is 4000-fold higher than the K_i for Lys-Pln indicates that the binding site in Lys-Plg_a for the α_2-plasmin inhibitor is grossly different from the binding site of Pln for the α_2-plasmin inhibitor. It is possible that the intact Arg_{560}–Val peptide bond near the active site prevents the interaction of Lys-Plg_a with the inhibitor.

Interaction of a DIP-Lys-Plg_a·SK Complex with Plasminogen Activators. A DIP-Lys-Plg_a·SK complex at a concentration of 2 mg/mL in the 25% glycerol buffer, pH 8.2, was incubated at 25 °C for 4 h with either high molecular weight UK, Lys-Plg_a·SK complex, Lys-Pln·SK complex, Pln-derived light (B) chain·SK complex, or SK, at a molar ratio of DIP-Lys-Plg_a·SK complex to activator of 50:1. The preparations were then reduced, alkylated, and analyzed by acrylamide gel/dodecyl sulfate electrophoresis for the presence of the DIP-Lys-Pln-derived heavy (A) and light (B) chains. The acrylamide gel scans showed that no Lys-Pln-derived heavy (A) and light (B) chains were formed from any of the DIP-Lys-Plg_a·SK preparations, indicating that none of the activators could convert the DIP-Lys-Plg_a·SK complex to the DIP-Lys-Pln·SK complex. A control Lys-Plg preparation treated with DFP was quantitatively converted to Lys-Pln by all of the activator species.

The DIP-Lys-Plg_a·SK complex was dissociated at pH 2.2, and the DIP-Lys-Plg_a moiety was isolated by the method described above used to isolate Lys-Plg_a (first step only). After the DIP-Lys-Plg_a moiety was dialyzed in the pH 8.2 25% glycerol buffer, it was incubated at a concentration of 2 mg/mL for 4 h at 25 °C with the same five plasminogen activator preparations used above and at the same molar ratios. With the exception of SK, each activator quantitatively converted DIP-Lys-Plg_a to DIP-Lys-Pln, as determined from scans of the acrylamide gel/dodecyl sulfate electrophoretograms after reduction and alkylation of the preparations. SK, as expected, did not convert DIP-Lys-Plg_a to DIP-Lys-Pln since it did not form an active activator species with DIP-Lys-Plg_a. The inability of these plasminogen activators to convert the DIP-Lys-Plg_a·SK complex to the DIP-Lys-Pln·SK complex indicates that all of the Plg activator enzymes bind to the same binding site on Plg, which must be in the vicinity of the Arg_{560}–Val peptide bond, and if that binding site is occupied by SK, no activator can cleave the Arg_{560}–Val peptide bond in the Plg moiety. The conversion of the DIP-Lys-Plg_a moiety to DIP-Lys-Pln by these activators showed that the DIP moiety in the active site did not prevent Plg activators from binding to DIP-Lys-Plg_a and cleaving the Arg_{560}–Val peptide bond. Kinetic data have shown that all activators probably bind to Plg somewhere in the vicinity of the Arg_{560}–Val peptide bond (Wohl et al., 1980). It has been previously reported that SK binds only to the Pln-derived light (B) chain, the COOH-terminal portion of the plasmin and plasminogen molecules (Summaria & Robbins, 1976). These experiments also show that when SK binds to Plg, it does so in the vicinity of the Arg_{560}–Val peptide bond, probably COOH terminal to it, and prevents the cleavage of that bond by any other Plg activator enzyme. Since it was not possible to convert the DIP-Lys-Plg_a·SK complex to the DIP-Lys-Pln·SK complex with the activator enzymes, the mechanism by which the Lys-Plg·SK complex converts to the Lys-Pln·SK complex must be primarily by an intramolecular, rather than by an intermolecular, process. The concept of intramolecular activation has been previously proposed (Bajaj & Castellino, 1976), and our results provide further evidence to substantiate such a mechanism.

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