Production and characterization of recombinant human plasminogen (S741C-fluorescein): a novel approach to study zymogen activation without generation of active protease
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A variant of recombinant plasminogen with the plasmin active site serine (S741) replaced by cysteine was produced and labeled with fluorescein at this residue to provide the derivative Plg(S741C-fluorescein). Studies of cleavage, conformation, and fibrin-binding properties of the derivative showed it to be a good model substrate to study plasminogen activation. Both in solution and in a fully polymerized fibrin clot, cleavage of the single chain zymogen to the two-chain “plasmin” molecule was accompanied by a 50% quench of fluorescence intensity. This change allows facile, continuous monitoring of the kinetics of cleavage. Measurements of cleavage by single chain t-PA within intact, fully polymerized 3 μm fibrin yielded apparent kcat and Km values of (0.08 s−1, 0.52 μM) and (0.092 s−1, 0.098 μM) for [Glu1]- and [Lys78]Plg(S741C-fluorescein), respectively. These values are similar to those obtained by others with plasma plasminogen. The approach used here might generally be useful in simplifying the analysis of zymogen activation kinetics in cases where the product (protease) has a great influence on its own formation via positive or negative feedback loops.

The fibrinolytic system leads to the formation of plasmin, which converts fibrin, the major protein constituent of blood clots, to soluble products. Activation of its inactive precursor plasminogen by tissue-type plasminogen activator (t-PA) occurs efficiently only when fibrin, but not fibrinogen, is present (1). Thus, fibrin is both a substrate for plasmin and a cofactor for plasmin formation (2). This makes an analysis of the t-PA-mediated activation process complex due to several proposed feedback loops. First, the generation of carboxyl-terminal lysines after limited proteolysis of the fibrin cofactor results in the generation of new, high affinity binding sites for plasminogen (3–5). Second, plasmin readily converts single chain t-PA to the more active two-chain form. Third, plasmin converts native [Glu1]plasminogen to the truncated form [Lys78] plasminogen, which is a superior substrate for t-PA (2, 6). The latter process is stimulated up to 200-fold by partly digested but not intact fibrin (7). A detailed kinetic analysis of the fibrinolytic process therefore necessitates the performance of steady state measurements during the different stages of fibrin degradation. Since this generates active plasmin, however, the structure of the cofactor fibrin, the substrate plasminogen and the enzyme t-PA will be subject to continuous change. Ideally, one would like to perform activation studies without the generation of active plasmin. All systems used so far, however, were based on measuring the generation of plasmin.

A clue to solving this problem came from the work of Drs. P. Bock and J. Shore, and co-workers, who constructed a plasminogen variant that was fluorescently labeled. They treated plasminogen with streptokinase in the presence of a chloromethyl ketone and thereby introduced a fluorescent label into plasminogen, such that subsequent cleavage could be monitored without the concurrent generation of active plasmin (8, 9). Studies with other proteases, inactivated by fluorescent labeling at the active site, also indicated that the properties of these labels thus positioned are sensitive probes to monitor changes in protease conformation (9–11). In this paper we describe the expression of a variant of recombinant human plasminogen, in which the plasmin active site serine has been replaced by cysteine: Plg(S741C). After labeling with a cysteine-specific fluorescent probe, we could quantify the rate of cleavage of this zymogen, Plg(S741C-fluorescein), without generating active plasmin.

**EXPERIMENTAL PROCEDURES**

**Materials—**The full-length plasminogen cDNA was a generous gift of Dr. L.-O. Heden (KabiGen AB, Stockholm, Sweden). The cysteine-specific fluorescent probe 5-iodoacetamidofluorescein (5-IAF) was obtained from Molecular Probes Inc. (Eugene, Oregon). Dulbecco’s modified Eagle’s medium:nutrient mixture F-12 (1:1), Opti-MEM I, and newborn bovine serum were from Life Technologies Inc. Methotrexate sodium injection (David Bull Laboratories, Mulgrave, Victoria, Australia) was purchased at the local hospital pharmacy. Chromogenic substrate D-Val-Leu-Lys-nitroanilide (S2251) and dansyl-Glu-Gly-Arg-chloromethyl ketone were obtained from Chromogenix (Molndal, Sweden) and Helena laboratories (Mississauga, Ontario, Canada), respectively. All DNA modifying enzymes were obtained from either Life Technologies, Inc. or Promega and were used according to the manufacturers’ instructions. Sequenase 2.0 was obtained from U. S. Biochemical Corp.

**Proteins—**Activase (t-PA) was generously provided by Dr. G. Vehar (Genentech, San Francisco, California). High molecular weight urokinase (u-PA) (>60,000 units/mg) was from Calbiochem; aprotinin was from Trasylol (Bayer, Leverkusen, Germany). Human α-thrombin was produced as described (12), and human fibrinogen (98% clottable) was prepared from fresh-frozen, citrated plasma according to published procedures (13, 14). Human [Glu1]plasminogen was isolated from fresh plasma.
Properties of Plasminogen(S741C-Fluorescein)

Oligonucleotides used in this study (mutated nucleotides are in lowercase)

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence position (bp)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′-GTGGGGGGGGTGTGCCCAC-3′ (1820–1843)</td>
<td>Amplification</td>
</tr>
<tr>
<td>2</td>
<td>5′-CTCCCAAGGAGTACCTTGCTG-3′ (2362–2331)</td>
<td>S741C</td>
</tr>
<tr>
<td>3</td>
<td>5′-AGTGGACCGGTTACCGGTTGC-3′ (2340–2371)</td>
<td>S741C</td>
</tr>
<tr>
<td>4</td>
<td>5′-AAGCGGATGTTAATACCCCTGACG-3′ (2581–2550)</td>
<td>Amplification</td>
</tr>
<tr>
<td>5</td>
<td>5′-ACTATAAGAGGCGGACCTG-3′ (pNUT)</td>
<td>MTI sequencing</td>
</tr>
<tr>
<td>6</td>
<td>5′-CCTCCGTTCTCCGCTGCG-3′ (pNUT)</td>
<td>pA sequencing</td>
</tr>
</tbody>
</table>

Frozen plasma on lysine-Sepharose (Pharmacia Biotech Inc., Uppsala, Sweden) as described (15). Lys-plasminogen and plasmin were produced as described (6). Concentrations of the proteins were determined by absorbance at 280 nm using the following specific absorbances for 1% protein solutions: fibrinogen = 16.0 (16), plasminogen = 16.2 (17).

Construction of Plasminogen(S741C)—The full-length plasminogen cDNA (18) was inserted as a Bsi/SphI fragment into the multiple cloning site (mp-18) of pATA-18 as described (19). Site-directed mutagenesis was achieved by the polymerase chain reaction overlap-extension technique (20). We employed the two partly complementary oligonucleotides 2 and 3 (Table I) to create the codon for Ser741 (AGT) to Cys (TGT), whereas amplification of the 76 base pairs 3′-end of the plasminogen cDNA was achieved by oligonucleotides 1 and 4 (Table I). The mutated fragment was used to substitute the “wild-type” EcoRV-SphI fragment of plasminogen in pATA-18. The absence of undesired mutations was verified by DNA sequencing of the entire fragment, using oligonucleotides 1, 3, and 4.

Construction of Stable Cell Lines—Stable cell lines expressing [Glu1]Plg(S741C) were constructed essentially as described for native Pig (19). In brief, cDNA for human (Glu1)[Glu1]Plg(S741C) was removed from the pATA-18 plasmid by digestion with HindIII and blunt ends were generated with T4-DNA polymerase. The expression vector pNUT (21) was digested with SmalI treated with alkaline phosphatase, and ligated to the [Glu1][Glu1]Plg(S741C) cDNA. The correct insertion and identity of [Glu1][Glu1]Plg(S741C) cDNA was evidenced by sequencing, using oligonucleotides 5 and 6. BHK cells were cultured in Dulbecco’s modified Eagle’s medium/F-12, supplemented with 5% newborn bovine serum from which (bovine) plasminogen was removed by passage over lysine-Sepharose. Baby hamster kidney (BHK-21) cells were transfected with the pNUT-[Glu1]Plg(S741C) plasmid according to the calcium phosphate precipitate method (22). Sixteen hours after transfection, the growth medium was supplemented with 0.44 mM methotrexate to select for pNUT (dihydrofolate reductase). Two weeks after transfection, individual clones were screened for [Glu1][Glu1]Plg(S741C) production by a sandwich enzyme-linked immunosorbent assay for human plasminogen (Affinity Biologicals, Ancaster, Ontario, Canada). Typical production levels were 10 μg/10⁷ cells/day.

Production, Purification, and Fluorescein Labeling of [Glu1][Glu1]Plg(S741C)—Cell lines were grown in 500-cm² triple flasks (Nunc) for large scale production. At confluence, the culture medium was replaced by serum-free Opti-MEM I, supplemented with 1 mM CaCl₂, equilibrated at 37 °C, were added 98 μl of HBST with a fixed concentration of Pig(S741C-Fluorescein) and various concentrations of fibrinogen. Clotting of the fibrinogen was complete within 1 min, after which the incubation was continued for 10 min at 37 °C. Subsequently, the fibrin clot was pelleted by centrifugation for 1 min at 10,000 × g and the supernatant was removed immediately. The amount of non-fibrin-bound Pig(S741C-Fluorescein) was determined by quantitation of fluorescence intensity of the supernatant, as follows. Aliquots of 25 and 75 μl of each supernatant were added to 75 and 25 μl of HBST in 96-well fluorescence plates, and fluorescence intensity was measured at excitation and emission wavelengths of 495 and 535, respectively, employing a 530-nm emission cut-off filter. Fluorescence intensities were converted to concentration of plasminogen in the supernatant by comparison to the standard of an otherwise identically treated sample that did not contain fibrin. The latter was identical to the intensity of untreated plasminogen, directly diluted from the stock solution. Fluorescence intensities obtained in this way were linear with respect to plasminogen concentration as established by measuring, under identical conditions, the fluorescence intensities in wells that contained serial dilutions of fluorescent plasminogen. In an alternative experiment, we determined the binding of various concentrations of [Glu1][Glu1]Plg(S741C-Fluorescein) to 1 μm fibrin clots. Experimental conditions were as described above, but fibrin was kept constant at 1 μM and concentrations of [Glu1][Glu1]Plg(S741C-Fluorescein) ranged from 0.5 to 4 μM.

Fluorescent Plasminogen Cleavage by u-PA—Experiments to measure the kinetics of u-PA-catalyzed cleavage of [Glu1] or [Lys78]Plg(S741C-Fluorescein) at Arg⁴⁸⁶—Val⁴⁸⁸ were performed in 96-well fluorescence plates (Dynatech) at 20 °C, using a Perkin Elmer LS50B Luminescence Spectrometer equipped with a fluorescence plate reader. Fluorescence intensities were measured at excitation and emission wavelengths of 495 and 535, respectively, employing a 530-nm emission cut-off filter. Fluorescent plasminogen solutions: fibrinogen (16.0 (16), plasminogen, and plasmin were produced as described (6)). Concentrations of the proteins were determined by absorbance at 280 nm, after correction for the contribution of fluoroscein (A₂₈₀ = 0.194A₂₈₀). Typical incorporation levels were at 0.9 mol/mol. [Glu1][Glu1]Plg(S741C-Fluorescein) was converted to [Lys78][Lys78][Lys78][Lys78][Lys78][Lys78][Lys78]Plg(S741C-Fluorescein) by adding 5 μl of 188 μM plasmin to 2.5 ml of 15 μM plasminogen in HBST supplemented with 5 mM 6-aminohexanoic acid. After 90 min, plasmin was removed by binding to 1 ml of aprotinin agarose. The flow-through, containing the [Lys78][Lys78][Lys78][Lys78][Lys78][Lys78][Lys78]Plg(S741C-Fluorescein), was treated with 10 mM valyphenylalaninyllysyl chloromethyl ketone for 1 h. The absence of traces of plasmin was verified by incubating aliquots of the protein at 37 °C with 1 mM S2231 for 2 h, in which period no increase of the absorbance at 405 nm was observed. Samples of Pig(S741C-Fluorescein) (Glu1 and Lys78 form) were subjected to urea/acetic acid 7.5% PAGE (23) at 120 V in a minigel system and mobilities were compared to native plasminogen. Gels were stained with Coomassie Brilliant Blue and destained.

Fibrin-binding Assay—Binding of [Glu1]- or [Lys78][Lys78][Lys78][Lys78][Lys78][Lys78][Lys78]Plg(S741C-Fluorescein) forms to a fibrin clot was measured as follows. To a series of microcentrifuge tubes containing 2 μl of 30 mM a-thrombin in HBST, 0.5 mM CaCl₂, equilibrated at 37 °C, were added 98 μl of HBST with a fixed concentration of Pig(S741C-Fluorescein) and various concentrations of fibrinogen. Clotting of the fibrinogen was complete within 1 min, after which the incubation was continued for 10 min at 37 °C. Subsequently, the fibrin clot was pelleted by centrifugation for 1 min at 10,000 × g and the supernatant was removed immediately. The amount of non-fibrin-bound Pig(S741C-Fluorescein) was determined by quantitation of fluorescence intensity of the supernatant, as follows. Alogues of 25 and 75 μl of each supernatant were added to 75 and 25 μl of HBST in 96-well fluorescence plates, and fluorescence intensity was measured at excitation and emission wavelengths of 495 and 535, respectively, employing a 530-nm emission cut-off filter. Fluorescence intensities were converted to concentration of plasminogen in the supernatant by comparison to the standard of an otherwise identically treated sample that did not contain fibrin. The latter was identical to the intensity of untreated plasminogen, directly diluted from the stock solution. Fluorescence intensities obtained in this way were linear with respect to plasminogen concentration as established by measuring, under identical conditions, the fluorescence intensities in wells that contained serial dilutions of fluorescent plasminogen. In an alternative experiment, we determined the binding of various concentrations of [Lys78][Lys78][Lys78][Lys78][Lys78][Lys78][Lys78]Plg(S741C-Fluorescein) to 1 μM fibrin clots. Experimental conditions were as described above, but fibrin was kept constant at 1 μM and concentrations of [Glu1][Glu1]Plg(S741C-Fluorescein) ranged from 0.5 to 4 μM.
ure the kinetics of t-PA-catalyzed cleavage of [Glu]- or [Lys]-Plg-(S741C-fluorescein) at Arg-Val and were performed in 96-well fluorescence plates (DynaTech) at 20 °C, using a Perkin Elmer LS50B Luminescence Spectrophotometer equipped with a fluorescence plate reader. Fluorescence intensities were measured at excitation and emission wavelengths of 495 and 535 nm, respectively, employing a 530-nm emission cut-off filter. Wells were pre-equilibrated with HBST for 1 h to prevent absorbance of proteins to the plastic. Subsequently, wells were loaded with 90 μl of HBST containing various concentrations of [Glu]- or [Lys]-Plg(S741C-fluorescein) and 3.2 μM fibrinogen, and were equilibrated at 20 °C. The stability of the fluorescence intensity was verified for 5 min. The reaction was initiated by adding 10 μl of HBST, 100 mM CaCl₂ containing 60 mM human α-thrombin and t-PA (1–10 nM final concentration). Data were collected every 60 s and stored as print files for each individual well using a data acquisition program written by Dr. W. K. Stevens in our laboratory. Initial rates of fluorescence decrease were determined by linear regression analysis and converted to rates of plasminogen activation according to Equation 1.

\[
\frac{dP_n}{dt} = (P/I)_0 \cdot \frac{dI}{dt} (1 - r) \quad (\text{Eq. 1})
\]

\(dP_n/dt\) is rate of plasmin formation, \(P_0\) is initial [Glu]- or [Lys]-Plg(S741C-fluorescein) concentration, \(I_0\) is initial fluorescence intensity, and \(r\) represents the ratio of the fluorescence intensities of the fluorescent plasmin and plasminogen analogues. The \(r\) values were determined in separate but equivalent reactions, in which the reactions were allowed to go to completion, and total cleavage was confirmed by SDS-PAGE analysis of the well contents after solubilization with dilute acetic acid (0.2 M) and lyophilization. The respective \(r\) values for Glu and Lys forms of Plg(S741C-fluorescein) are 0.5 and 0.4.

RESULTS

Production and Characterization of [Glu]-Plg(S741C)—Recombinant [Glu]-Plg(S741C) was expressed in stably transformed BHK cells at production levels of 10 μg of plasminogen/ml of serum-free medium (10⁶ cells/day). [Glu]-Plg(S741C) was purified from the BHK conditioned medium to apparent homogeneity by conventional affinity chromatography on lysine-Sepharose. Analysis of the protein on acid urea gels (Fig. 1) showed the absence of detectable amounts of degraded products. Laser densitometry of Coomassie-stained acid/urea gels showed that the recombinant plasminogen displays a similar ratio of the two glycoforms: 35% plasminogen-I (glycosylated at Asn-288 and Thr-345) and 65% plasminogen-II (glycosylated at Thr-345) only as observed for plasma plasminogen (24) and recombinant wild-type plasminogen (19).

The production levels in BHK cells and yields after purification on lysine-Sepharose of [Glu]-Plg(S741C) were identical to those for wild-type recombinant plasminogen (19). This suggests that no gross alterations in the structure result from the introduction of a new, free cysteine replacing serine 741. To further substantiate the validity of this plasminogen variant as a model, we analyzed by intrinsic fluorescence one of the most striking properties of [Glu]-plasminogen, namely its tight, activation-resistant conformation (25, 26). The increase of this intrinsic fluorescence (Fig. 2) has a sigmoidal relationship with increasing concentrations of the lysine analog 6-AHA as shown previously for plasma derived plasminogen (27) and “wild-type” recombinant plasminogen (19), indicating the tight (activation-resistant) conformation of the Glu form of this variant.

**Fig. 1.** Analysis of wild-type and recombinant plasminogen by acid/urea PAGE. Lanes 1 and 8 represent wild-type [Glu]-plasminogen, lanes 2 and 3 are [Glu]-Plg(S741C); lanes 4 and 5 are [Glu]-Plg(S741C-fluorescein), lane 6 is [Lys]-Plg(S741C-fluorescein), and lane 7 represents plasma [Lys]-plasminogen.

**Fig. 2.** Change in intrinsic fluorescent properties of [Glu]-Plg(S741C) upon binding of 6-aminohexanoic acid. Changes in intrinsic fluorescence of [Glu]-Plg(S741C) upon binding of 6-AHA were quantified as described for recombinant and plasma plasminogen (19). Excitation and emission wavelengths were at 290 and 540 nm, respectively. The increase in fluorescence shows a sigmoidal relation to 6-AHA concentration. The corresponding Hill plot is shown in the inset as reported (19). The Hill coefficient was 2.0, and the transition midpoint was 0.52 mM.

*Plg(S741C) Lacks Detectable Proteolytic Activity—*The absence of amidolytic and proteolytic activity in the variant plasminogen molecule (S741C) was substantiated by “activation” with the three plasminogen activators u-PA, t-PA, and streptokinase as follows. [Glu]-Plg(S741C) at 5 μM was incubated with 150 units/ml u-PA in the presence of the chromogenic substrate S2251 (1.0 mM), and the absorbance at 405 nm was followed in time for 20 h. The progress curve did not vary from similar experiments without plasminogen, whereas identical experiments with native plasminogen indicated a lower detection limit of 0.5 mM plasmin. Incubations of 5 μM [Glu]-Plg(S741C) with 5 or 50 units/ml streptokinase, which generates an active site in plasminogen by 1:1 complex formation rather than cleavage at Arg-Val, were performed in a similar fashion as described (19), and gave an amidolytic activity (S2251) corresponding to 0.0125% of the input [Glu]-Plg(S741C), when compared to the native plasminogen-streptokinase complex in an identical experiment. Finally, 5 μM [Glu]-Plg(S741C) was included in a clot lysis experiment with 3 μM fibrin and 5 nM t-PA, as described previously (19, 28). The turbidities of these clots were stable for 16 h, whereas in control experiments run simultaneously at little as 0.1 nM native plasminogen can be detected by a decrease in turbidity due to lysis of the fibrin. Based on these assays we conclude that [Glu]-Plg(S741C) when “activated” does not possess sufficient intrinsic amidolytic activity to perturb the experiments that are described in this paper.

**Fluorescent Labeling of [Glu]-Plg(S741C) and Characterization of [Glu]-Plg(S741C)-fluorescein**—Fluorescent labeling of the introduced “active site” cysteine to generate [Glu]-Plg-(S741C)-fluorescein could be accomplished nearly quantitatively (0.85 ± 0.1 mol of fluorescein/mol of plasminogen, n = 10) as described in detail under “Experimental Procedures.” Upon full conversion of [Glu]-Plg(S741C)-fluorescein to the two-chain “plasmin” form by u-PA, the fluorescein label was bound exclusively to the protease domain of plasminogen, as deduced from SDS-PAGE analysis (see below). Recombinant “wild-type” plasminogen did not incorporate detectable levels of fluorescein when produced and subjected to labeling under identical conditions.
Properties of Plasminogen(S741C-Fluorescein)

![Graph](image)

**Fig. 3.** Change in extrinsic fluorescence intensity of [Glu\(^1\)]Plg(S741C-Fluorescein) upon binding of 6-AHA. A 1.6-ml solution of 0.1 μM [Glu\(^1\)]Plg(S741C-Fluorescein) in 0.02 M HEPES, 0.15 M NaCl, 0.01% Tween 80, 10 mM CaCl2 at 22 °C was titrated with 2.0-μl aliquots of 0.25 mM 6-AHA and extrinsic fluorescence was measured (λ\text{ex} = 495, λ\text{em} = 535). Data were corrected for dilution and fit by nonlinear regression to the equation for a single-site/ligand interaction, ∆I = ∆I\text{max}([6-AHA]/Kd + [6-AHA]). ∆I\text{max} is the maximum change at saturation, and Kd is the dissociation constant. The analysis provided values of ∆I\text{max} = 21.4 ± 0.1% and Kd = 2.5 ± 0.1 mM.

The interaction of [Glu\(^1\)]Plg(S741C-Fluorescein) with 6-AHA was analyzed by quantifying the change in fluorescence intensity of the reporter group (Fig. 3). Contrary to the results on the intrinsic fluorescence change (Fig. 2), the intensity decreases (21.4% at saturation) and can be described best by a model that embodies binding to a single binding site with Kd of 2.52 mM. The Hill coefficient for the inferred binding suggested minimal cooperativity (h = 1.1). The decrease in fluorescence intensity of [Lys\(^78\)]Plg(S741C-Fluorescein) was < 1.5% at 10 mM 6-AHA. Intrinsic (Trp) fluorescence cannot be studied for these molecules due to interference by the fluorescein label, precluding a direct comparison with plasma plasminogen or [Glu\(^1\)]Plg(S741C). Comparison of the results of Figs. 2 and 3, however, suggests that the interaction between 6-AHA and plasminogen measured by intrinsic fluorescence, with a Hill coefficient of 2.0 and a transition midpoint at 0.52 mM 6-AHA, is different from the interaction measured by extrinsic fluorescence.

The existence of an activation-resistant conformation has been used to rationalize the weak fibrin binding of [Glu\(^1\)]- as compared to [Lys\(^78\)]plasminogen. To determine whether the Glu\(^1\) and Lys\(^78\) forms of the fluorescent plasminogen exhibit these differences in affinity for fibrin, we measured their binding to fully polymerized clots (Fig. 4). Fibrin binding of [Glu\(^1\)]Plg(S741C-Fluorescein) was weak with an estimated Kd of 30 μM, whereas that of [Lys\(^78\)]Plg(S741C-Fluorescein) was stronger (Kd = 1.2 μM, n = 1.8 sites/fibrin). These trends are identical to those of the "wild-type" recombinant plasminogen species (19), and the values of the binding parameters are similar to those reported for the plasma plasminogen forms (Kd = 38 μM and 0.32, respectively) (29).

The **Activation Cleavage of Plg(S741C-Fluorescein) by u-PA in Solution**—[Glu\(^1\)]Plg(S741C-Fluorescein) was treated with 6-AHA, and the initial decrease in fluorescence was measured. Then urokinase was added and the progressive decrease over time was monitored continually. An example is shown in Fig. 5A. Functional homogeneity of the fluorescent plasminogen is suggested by the coincidence of data and the line obtained by linear regression to the equation for first order decay. In this experiment, samples were withdrawn at regular intervals after the addition of urokinase and subjected to SDS-PAGE. The fluorescent bands were photographed (Fig. 5A, inset), and the gel was stained with Coomassie Blue, destained, and scanned with a densitometer. As the inset of Fig. 5A indicates, the progressive decline in intensity correlates with cleavage and, as expected, the fluorescence is associated exclusively with either the zymogen or the light chain, with no visible fluorescence in the heavy chain. Although the data are not shown, densitometry indicated that the decline in fluorescence after the addition of urokinase was linear in the extent of cleavage of [Glu\(^1\)]Plg(S741C-Fluorescein), and linear regression of intensity values to the extent of the reaction predicted an overall drop in intensity (including that due to 6-AHA) of 54% at 100% completion of the reaction.

Notably, the maximal decrease in fluorescence intensity of [Glu\(^1\)]Plg(S741C-Fluorescein) upon activation at different 6-AHA concentrations was inversely proportional to the initial change upon adding 6-AHA (data not shown), making the combined effects of 6-AHA and u-PA constant at about a 50% decrease. From this observation we suggest that the [Glu\(^1\)] plasmin molecule has a relaxed conformation similar to [Lys\(^78\)] plasminogen, and therefore no effect of 6-AHA on the overall intensity change is observed. This is in agreement with results on the properties described for active site blocked Glu-plasmin, which are similar to Lys-plasminogen, with respect to, for example, affinity for fibrin (30). The fluorescence-6-AHA on the rise of plasminogen activation by urokinase in solution has been well documented and resulted in the proposal of a tight, activation-resistant conformation of [Glu\(^1\)]plasminogen in the absence of such lysine analogs (26, 31). A similar effect of 6-AHA on activation rates is shown for [Glu\(^1\)]Plg(S741C-Fluorescein) in Fig. 5B. The decrease at higher concentrations of 6-AHA has been shown to result from the direct inhibitory effect of lysine analogs on the activity of u-PA (31). The maxi-
Properties of Plasminogen(S741C-Fluorescein)

The time courses of fluorescence intensity obtained when solutions of [Glu\(^1\)]Plg(S741C-Fluorescein) (0.2 \(\mu\)M, final) and fibrinogen (0.05 \(\mu\)M or 3.0 \(\mu\)M, final) were added to the wells of microtiter plates, and the reactions were initiated with a solution of CaCl\(_2\) (10 \(\mu\)M, final), thrombin (6.0 nM, final), and t-PA (25 nM, final) are indicated in Fig. 7A. Upon initialization of the reactions, an initial decrease in intensity (\(-10\%\)) occurred because of dilution. Although a small additional change (4.8%) followed the polymerization of fibrin at the high input level of fibrinogen, as evidenced by the difference in control (minus t-PA) signals of Fig. 7A, the magnitude of the subsequent decreases when the reactions approached completion were virtually identical at both the high and low fibrin concentrations. The relative lack of influence of fibrin polymerization on the signal, potentially due to, for example, light scattering could most likely be attributed to the plate reader format whereby both the excitation and emission optics are above the sample and the sample well is quite reflective. The lines of Fig. 7A are the regression lines obtained by fitting the data to the equation for first order decay, a procedure that is justified because of the relatively low input concentration of [Glu\(^1\)]Plg(S741C-Fluorescein). The good fit of the data to the equation implies functional homogeneity. The regression analysis indicated a 50% decrease in intensity upon completion of the reaction. The data from similar experiments with [Lys\(^{78}\)]Plg(S741C-Fluorescein) did not fit as well to the first order decay equation (due to low \(K_m\)), but monitoring for extended periods indicated a 40% decline in intensity at completion of the reaction (data not shown), at both low and high input concentrations of fibrinogen. In the absence of the fluorescein-labeled protein, the signal was negligible (7.0% or less than that with the fluorescent protein over the range of protein concentrations studied). This blank value, however, was subtracted from all relevant data.

In order to measure initial rates, activator concentrations were decreased so that the approximately linear portion of the reaction could be measured. An example is shown in Fig. 7B. In this case the magnitude of signal change encompassed by the exhibited data is about 10% of the total signal, which corresponds to about 20% consumption of the substrate. Over this range the rate was essentially constant. In typical measurements of initial rates, data such as those of Fig. 7B were subjected to linear regression to determine the slope. This approach was employed to obtain the apparent \(k_{cat}\) and \(K_m\)
A four solutions (90 μl) containing [Glu¹][Plg(S741C-fluorescein)] (0.2 μM) and fibrinogen at concentrations of either 50 nM (●, ▲) or 3.0 μM (○, △) were added to the wells of a microtiter plate. Initial values of intensities then were recorded for 150 s. The reactions were started by adding aliquots (10 μl) of a solution consisting of CaCl₂ (100 mM), thrombin (60 nM), and t-PA (250 nM) to two of the wells (●, ○). The other two controls (▲, △) received this solution lacking t-PA. Other controls without [Glu¹][Plg(S741C-fluorescein)], including one with HBST only, all yielded an intensity value of 7.5, and this has been subtracted from the data indicated. The initial decrease upon addition of the 10-μl aliquot to start the reactions is consistent with dilution. As the subsequent data indicate, fibrin polymerization only marginally affected the initial (control) signal, which was 94.9 at 50 nM fibrin (●) and 90.4 at 3.0 μM fibrin (△). The lines through the data obtained with t-PA (●, ○) are regression lines that resulted upon fitting the experimental points by nonlinear regression to the equation

$$I = I_o - \Delta I_{max} \left(1 - \exp\left(-k t\right)\right),$$

where $I$ is the intensity, $\Delta I_{max}$ is the maximal decrease in intensity, $k$ is the first order rate constant, and $t$ is the time. In both instances the $\Delta I_{max}$ was 50% of the initial (control) intensity. $B$, an example of the use of linear regression to determine the initial rate of cleavage of 1.5 μM [Glu¹][Plg(S741C-fluorescein)]. The experiment included 3.0 μM fibrin and 3.3 nM t-PA. The total consumption of substrate over the interval was 20% of the initial amount.

values for the t-PA-catalyzed cleavage of [Glu¹]- and [Lys⁷⁸][Plg(S741C-fluorescein)] at a single input concentration of fibrinogen and thus compare them to values obtained by others with native plasminogen utilizing soluble substitutes for fibrin or fibrin films. Initial rates of cleavage versus nominal concentrations of the substrate fit well to a rectangular hyperbola, indicating Michaelis-Menten-like kinetics (Fig. 8).

DISCUSSION

We describe the production of a variant of plasminogen in which the serine of the plasmin catalytic triad has been replaced by cysteine. This enabled the introduction of a fluorescein label at the position of this residue in plasminogen to produce [Glu¹][Plg(S741C-fluorescein)]. Characterization of the activating cleavage, conformation, and fibrin-binding properties of this variant showed it to be a good model substrate to study plasminogen activation. Both in solution and in a fully polymerized fibrin clot, the activation of the single chain zymogen to the two-chain “plasmin” molecule was accompanied by a substantial change in the micro-environment of this probe upon occurrence of the activation cleavage. Results for active proteases, which had been labeled via protein-chemical approaches, had already indicated the sensitivity of this position within the protease domain (9–11). We show that this approach yields a variant of the zymogen, at high levels of expression, which differs from the native zymogen only by a serine to cysteine substitution. In the case of plasminogen, we did not find indications of malfolding as a result of this change, since production levels in BHK cells as well as other properties of this variant were identical to those obtained for “wild-type” plasminogen (19).

Recently Bock and co-workers (9) described the production of a fluorescent plasminogen derivative similar to that reported here. They treated plasma plasminogen with streptokinase and covalently modified the active site with a thioester chlormethyl ketone. The thioester was subsequently hydrolyzed with hydroxylamine, and the thiol group was covalently modified with an anilinonaphthylsulfonate moiety. The fluorescent derivative was then separated from streptokinase. The active site-modified fluorescent derivative yielded readily measured spectral changes upon interaction with lysine analogues, with streptokinase, and upon cleavage to the plasmin derivative. The kinetics of cleavage by urokinase of the derivative and unmodified plasma plasminogen were very similar, indicating...
that the derivative is a good surrogate for unmodified plasminogen whereby the properties of plasminogen can be monitored. The cleaved form of the derivative yielded plasmin activity of about 1.0% of native plasmin. The presently described derivative Plg(S741C-fluorescein) has many of the properties of the derivative described by Bock et al. (9). Its fluorescence properties are sensitive to lysine analogues and cleavage by plasminogen activators. In addition, it appears to be a good derivative for analyzing the interactions and reactions of plasminogen. Unlike the derivative prepared with streptokinase, however, the presently described derivative exhibits no detectable plasmin activity when cleaved by plasminogen activators, whether or not cysteine 741 is modified with a fluorescent probe. This lack of activity is undoubtedly the result of the cysteine for serine substitution at position 741. Thus, Plg(S741C) appears ideally suited as a tool to produce plasminogen derivatives that can be labeled with specific probes and that generate no plasmin activity upon cleavage by plasminogen activators.

Plg(S741C-fluorescein) enabled us for the first time to do steady state determinations of activation rates in a fully polymerized fibrin clot without solubilization of the clot. Since no active protease was formed, none of the positive feedback loops of fibrinolysis have taken place. The stimulation factors (rate fibrin absent/rate fibrin present) were several hundredfold for both forms of plasminogen. In a very detailed study in which plasminogen activation rates were deduced from rates of release of isotope from \( {^{125}}I \)-labeled fibrin films due to generated plasmin, \( k_c \) and \( K_m \) values of 0.1 s\(^{-1}\), 0.16 \( \mu \)M and 0.2 s\(^{-1}\), 0.02 \( \mu \)M were obtained for [Glu\(^1\)]- and [Lys\(^{78}\)]plasminogen, respectively (2). Our results on the activation in an intact fully polymerized 3 \( \mu \)M fibrin clot by single-chain t-PA yield apparent \( k_c \) and \( K_m \) values of 0.08 s\(^{-1}\), 0.52 \( \mu \)M and 0.092 s\(^{-1}\), 0.098 \( \mu \)M for [Glu\(^1\)]- and [Lys\(^{78}\)]Plg(S741C-fluorescein), respectively. The values for [Glu\(^1\)]Plg(S741C-fluorescein) are similar to those reported on soluble fibrin monomer and native [Glu\(^1\)]plasminogen, where initial rates in the early phase of plasminogen activation were deduced from extrapolation to zero time (e.g. before plasmin feedback): 0.17 s\(^{-1}\) and 1 \( \mu \)M (33). It has been suggested that the conversion of single-chain t-PA to two-chain t-PA, [Glu\(^1\)]plasminogen to [Lys\(^{78}\)]plasminogen, and intact fibrin to proteolytically modified fibrin, which are all plasmin-catalyzed, might result in substantial acceleration of the rate of plasminogen activation. The kinetic parameters presented in the present study, however, are very similar to those obtained in assays in which all of the mentioned feedback loops do occur. Hence, our results indicate that the effects of the proposed feedback loops in a polymerized fibrin clot might be limited under non-pathological conditions. Differences are restricted to decreased \( K_m \) values for plasminogen activation, being 0.52 \( \mu \)M ([Glu\(^1\)]plasminogen, intact fibrin, single chain t-PA; this study) to minimally 0.02 \( \mu \)M ([Lys\(^{78}\)]plasminogen, two chain t-PA, plasin-modified fibrin; Ref. 2). At physiological levels of fibrin (9 \( \mu \)g) and plasminogen (1.5 \( \mu \)g), this will result in a 25% increase in rate of plasminogen activation.

Adverse conditions like clot retraction, however, might lead to plasminogen depletion, and therefore the decrease in \( K_m \) could have a more dramatic effect.

In conclusion, the approach to label a zymogen at serine of the corresponding active site of the enzyme via mutation to cysteine and incorporation of a fluorescent, cysteine-specific probe might have wide applicability, since it enables steady state activation studies through a readily measured signal. In addition, it simplifies the interpretation of kinetics in cases where the product (protease) has a great influence on its own formation via positive or negative feedback loops.

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