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 $k_{cat}$ and $K_m$ values of (0.08 s$^{-1}$, 0.52 μM) and (0.092 s$^{-1}$, 0.098 μM) for [Glu$^1$]- and [Lys$^{78}$]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. 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we describe the expression of a variant of recombinant human plasminogen, in which the plasmin active site serine has been replaced by cysteine: Plg(S741C).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**

*A Novel Approach to Study Zymogen Activation Without Generation of Active Protease*®

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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 Bsu3II 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 change the codon for Ser448 (AGT) to Cys (TGT), whereas amplification of the 760 base pairs 3′-end of the plasminogen cDNA was achieved by oligonucleotides 1 and 4. The correct insertion and identity of 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 obtained by transfection of BHK-21 cells, from which (bovine) plasminogen was removed by passage over lysine-Sepharose. Baby hamster kidney (BHK-21) cells were transfected with [Glu1]Plg(S741C) cDNA. The correct insertion and identity of [Glu1]Plg(S741C) cDNA was evidenced by sequencing, using oligonucleotides 1, 3, and 4.

Properties of Plasminogen(S741C-Fluorescein) table I: Oligonucleotides used in this study (mutated nucleotides are in lowercase)

| No. | Sequence (bp) | Use |
|-----|--------------|-----|
| 1   | 5′-GGGAGGGTTGGTGCCAC-A | Amplification |
| 2   | 5′-TTTCTTCTTACTGGCC-3′ | Amplification |
| 3   | 5′-TGTGGACGAGGTAC-3′ | Amplification |
| 4   | 5′-AAGGTTCATGCAGACTTTTTTTCAGC-3′ | MTI sequencing |
| 5   | 5′-TATATAAAGGAGGGACGTT-3′ (pNUT) | pA sequencing |
| 6   | 5′-CCGGGCTTCCCTGCTGCTT-3′ (pNUT) | |

Fluorescent Plasminogen Cleavage by u-PA—Experiments to measure the kinetics of u-PA-catalyzed cleavage of [Glu1]- or [Lys78]Plg(S741C)-fluorescein (Atm5) were performed as described (21). A 1-mL DEAE-fast flow column (Pharmacia, Uppsala, Sweden), equilibrated with PBS, 10 mM HEPES-NaOH, pH 7.4, and the labeled protein eluted with HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.02% Tween 80 and stored in aliquots at –70°C. All chromatographic steps were performed in the dark, and control runs were performed with “wild-type” plasminogen to determine the absence of nespecific labeling. The amount of fluorescent incorporation was determined spectrophotometrically using an extinction coefficient at 495 nm for fluorescein of 84,000 M−1 cm−1 (Molecular Probes). The concentration of the labeled protein was determined from absorbance at 280 nm, after correction for the contribution of fluorescein (A280 = 0.19±A495). Typical incorporation levels were at 0.9 mol/mol. [Glu1]Plg(S741C-fluorescein) was converted to [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-aminohecanic acid. After 90 min, plasmin was removed by binding to 1 ml of aprotininagarose. The flow-through, containing the [Lys78]Plg(S741C-fluorescein), was treated with 10 µM v-lyphenylalaninyllyllylchomethyle ketone for 1 h. The absence of traces of plasmin was verified by incubating aliquots of the protein at 37°C with 1 mM S2251 for 2 h, in which period no increase of the absorbance at 405 nm was observed. Samples of Plg(S741C-fluorescein) (Glu1 and Lys78 form) were subjected to urea/ acetic acid 7.5% PAGE (23) at 210 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]Plg(S741C-fluorescein) forms to a fibrin clot was measured as follows. To a series of microcentrifuge tubes containing 2 µl of 50 µg/ml plasminogen in HBST, 0.5 mM CaCl2, equilibrated at 37°C, were added 98 µl of HBST with a fixed concentration of Plg(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 Plg(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 identical 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]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 [Lys78]Plg(S741C-fluorescein) ranged from 0.5 to 4 µM.
ure the kinetics of t-PA-catalyzed cleavage of [Glu1]- or [Lys78]Plg(S741C-fluorescein) at Arg283-Val342 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. 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 [Glu1]- or [Lys78]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 nM 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.

\[ \frac{dP_n}{dt} = \frac{P_c}{L_c} \cdot \frac{dP_n}{dt} / (1 - r) \quad (\text{Eq. 1}) \]

dPn/dt is rate of plasmin formation, Pc is initial [Glu1]- or [Lys78]Plg(S741C-fluorescein) concentration, Lc 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 of the well contents after solubilization with dilute acetic acid (0.2 M) and lyophilization. The respective r values for Glu1 and Lys78 forms of Plg(S741C-fluorescein) are 0.5 and 0.4.

**RESULTS**

**Production and Characterization of [Glu1]Plg(S741C)—Recombinant [Glu1]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). [Glu1]Plg(S741C) was purified from the BHK conditioned medium to apparent purity (S2251) corresponding to 0.0125% of the input concentration limit of 0.5 nM plasmin. Incubations of 5 µM [Glu1]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 Arg283-Val342, were performed in a similar fashion as described (19) and gave an amidolytic activity (S2251) corresponding to 0.0125% of the input [Glu1]Plg(S741C), when compared to the native plasminogen-streptokinase complex in an identical experiment. Finally, 5 µM [Glu1]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 [Glu1]Plg(S741C) when “activated” does not possess sufficient intrinsic amidolytic activity to perturb the experiments that are described in this paper.

**Fluorescent Labeling of [Glu1]Plg(S741C) and Characterization of [Glu1]Plg(S741C-fluorescein)—Fluorescent labeling of the introduced “active site” cysteine to generate [Glu1]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 [Glu1]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.
embodies binding to a single binding site with (21.4% saturation) and can be described best by a model that intrinsic fluorescence change (Fig. 2), the intensity decreases of the reporter group (Fig. 3). Contrary to the results on the was analyzed by quantifying the change in fluorescence intensity, the addition of urokinase and subjected to SDS-PAGE. The

The interaction of [Glu1]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 $K_d = 2.52 \pm 0.52$ mm. The Hill coefficient for the inferred binding suggested minimal cooperativity ($h = 1.1$). The decrease in fluorescence intensity of [Lys78]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 [Glu1]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 [Glu1]- as compared to [Lys78]plasminogen. To determine whether the Glu1 and Lys78 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 [Glu1]Plg(S741C-fluorescein) was weak with an estimated $K_d$ of 30 $\mu$m, whereas that of [Lys78]Plg(S741C-fluorescein) was stronger ($K_d = 1.2 \mu$m, $n = 1.8$ 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 ($K_d = 38 \mu$m and 0.32, respectively) (29).

The Activation Cleavage of Plg(S741C-fluorescein) by u-PA in Solution—[Glu1]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

![FIG. 3. Change in extrinsic fluorescence intensity of [Glu1]Plg(S741C-fluorescein) upon binding of 6-AHA. A 1.6-ml solution of 0.1 $\mu$m [Glu1]Plg(S741C-fluorescein) in 0.02 M HEPES, 0.15 M NaCl, 0.01% Tween 80, 10 mM CaCl$_2$ at 22 °C was titrated with 2.0-$\mu$l aliquots of 0.25 M 6-AHA and extrinsic fluorescence was measured ($\lambda_{ex} = 495$, $\lambda_{em} = 535$). Data were corrected for dilution and fit by nonlinear regression to the equation for a single site/ligand interaction, $\Delta I = \Delta I_{max}(6$-AHA)/($K_d + \text{[6-AHA]}$). $\Delta I_{max}$ is the maximum change in fluorescence intensity of saturating ligand and $K_d$ is the dissociation constant. The analysis provided values of $\Delta I_{max} = 21.4 \pm 0.1$% and $K_d = 2.5 \pm 0.1$ mm.](image)

![FIG. 4. Binding of [Glu1]- and [Lys78]Plg(S741C-fluorescein) to intact fibrin. The binding of either [Glu1]Plg(S741C-fluorescein) (●) or [Lys78]Plg(S741C-fluorescein) (○) at a fixed concentration (50 nM) to intact fibrin clots at various concentrations of fibrin was determined as described under "Experimental Procedures." The lines represent the results of nonlinear regression analysis of the data to $B = B_{max}P/F/K_{d,n} + F$, where $B$ is plasminogen bound to fibrin, $K_d$ is the dissociation constant for this binding, $n$ represents the number of plasminogen binding sites/fibrin monomer and $F$ is fibrin concentration. This analysis yields values for [Lys78]Plg(S741C-fluorescein) of $B_{max} = 0.75$ and $K_{d,n} = 2.20 \pm 0.05$ mm. The near linear relationship for [Glu1]Plg(S741C-fluorescein) precludes independent determination of these constants. When assuming $B_{max} = 0.75$ and $n = 1$, a $K_d$ of 30 $\mu$m is estimated. The inset shows the results, in Scatchard plot format, of a separate experiment in which the binding of [Lys78]Plg(S741C-fluorescein) at various concentrations to fibrin clots of fixed concentration (1 $\mu$m) was measured to obtain the separate values for $K_d$ and $n$. This analysis yielded a $K_d$ of 1.2 $\mu$m and $n = 1.8$ mol of Plg/mol of fibrin.](image)
The initial rate of fluorescence intensity obtained when solutions of [Glu1]Plg(S741C-fluorescein) (0.2 μM, final) and fibrinogen (0.05 μM or 3.0 μM, final) were added to the wells of microtiter plates, and the reactions were initiated with a solution of CaCl2 (10 mM, 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 can 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 [Glu1]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 [Lys78]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 kcat and K_m.
Values for the t-PA-catalyzed cleavage of [Glu1]Plg(S741C-fluorescein) and [Lys78]Plg(S741C-fluorescein) at a single input concentration 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 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 $I = I_0 - \Delta I_{\text{max}} (1 - \exp(-kt))$, where $I$ is the intensity, $\Delta I_{\text{max}}$ is the maximal decrease in intensity, $k$ is the first order rate constant, and $t$ is the time. In both instances the $\Delta I_{\text{max}}$ 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.

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 chloromethyl 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 thiol 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 active protease was formed, none of the positive feedback loops of fibrinolysis are all plasmamin-catalyzed, might result in substantial acceleration of plasmin-catalyzed, might result in substantial acceleration of plasmin, and that generate no plasmin activity upon cleavage by plasminogen activators.

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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