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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°C% quench of fluorescein 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 M 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 plasin, 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)1 occurs efficiently only when fibrin, but not fibrinogen, is present (1). Thus, fibrin is both a substrate for plasin 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). Dako’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 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 manufactures’ instructions. Sequenase 2.0 was obtained from U. S. Biochemical Corp.

Proteases—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

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§ The abbreviations used are: t-PA, (recombinant) tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; Plg(S741C), recombinant human plasminogen with a serine 741 to cysteine mutation; Plg(S741C- fluorescein), Plg(S741C) covalently labeled at cysteine 741 with fluorescein; [Glu1]- and [Lys78]Plg(S741C- fluorescein), Glu1- or Lys78 forms of Plg(S741C- fluorescein); PAGE, polyacrylamide gel electrophoresis; 5-IAF, 5-iodoacetamidofluorescein; 6-AHA, 6-aminohexanoic acid; BHK, baby hamster kidney; PBS, phosphate-buffered saline; dansyl, 5-dimethylaminonaphthalene-1-sulfonfonyl; Pn, plasmin.
Properties of Plasminogen(S741C-Fluorescein)

Table I

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence position (bp)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′-GTGGGGGGGGTGTGCCCCAC-3′ (1820-1843)</td>
<td>Amplification</td>
</tr>
<tr>
<td>2</td>
<td>5′-CCTCCGACCGGACCTCGTGCTGCCC-3′ (2362-2331)</td>
<td>S741C</td>
</tr>
<tr>
<td>3</td>
<td>5′-AGTGGCAGGAGGACGTTCTCTGCTGAC-3′ (2340-2371)</td>
<td>S741C</td>
</tr>
<tr>
<td>4</td>
<td>5′-AACCTTGTACGCTAAAATCTCTCTGCCAG-3′ (2581-2550)</td>
<td>Amplification</td>
</tr>
<tr>
<td>5</td>
<td>5′-ACTAATAAGAGGCGGACGTCGCTC-3′ (pNUT)</td>
<td>MT1 sequencing</td>
</tr>
<tr>
<td>6</td>
<td>5′-CCCCAGGCTCTTCTCGGCCTC-3′ (pNUT)</td>
<td>pA sequencing</td>
</tr>
</tbody>
</table>

Properties of Plasminogen(S741C-Fluorescein) were studied in this study (mutated nucleotides are in lowercase).

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 Bst/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 change the codon for Ser44 (AGT) to Cys (TGT), whereas amplification of the 760 base pair 3′-end of the plasminogen cDNA, achieved by oligonucleotides 1 and 4, 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]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]Plg(S741C) cDNA. The correct insertion and identity of [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-Plg(S741C) plasmid according to the calcium phosphate precipitation 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]Plg(S741C) production by a sandwich enzyme-linked immunosorbent assay for human plasminogen (Affinity Biologicals, Ancaster, Ontario, Canada). Typical production levels were 10 µg/10⁶ cells/day.

Production, Purification, and Fluorescein Labeling of [Glu1]Plg(S741C)—Cell lines were grown in 500-cm² triple flasks (Nunc) for large scale production. At confluence, the growth medium was replaced by serum-free Opti-MEM I, supplemented with 50 µM ZnCl₂. Conditioned media were collected every other day; supplemented with 1 µM dapsyl-Glu-Gly-Arg-chloromethyl ketone, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA; and loaded onto lysine-Sepharose. After washing the column with PBS (20 mM NaF, pH 7.4, 150 mM NaCl) until the A₂₈₀ of the effluent was <0.002, [Glu1]Plg(S741C) was eluted with PBS, 10 mM 6-aminohexanoic acid. Fractions containing <0.25 mg/ml [Glu1]Plg(S741C) based on A₂₈₀ were pooled. Labeling was performed directly on this pooled fraction (typically 10–15 ml, 6–10 µM [Glu1]Plg(S741C)) by adding 150 µl of 20 µM 5-IAF in N,N-dimethylformamide. The reaction was continued for 30 min at room temperature in the dark. Excess label was removed from this incubation mixture on a 1 ml DEAE-fast flow column (Pharmacia, Uppsala, Sweden), equilibrated and run in PBS, 0.05% Tween 80, resulting in elution of [Glu1]Plg(S741C) in the flow-through and binding of 5-IAF to the resin. Subsequently, the protein was concentrated and freed from remaining traces of 5-IAF and 6-aminohexanoic acid (6-AHA) by adding to a 5-ml DEAE-fast flow column after a 1:5 dilution with 20 mM Tris- HCl, pH 8.0. The column was washed with 20 ml 100 mM HEPES- NaOH, pH 7.4, and the eluted protein eluted with HBST (20 mM 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 nonspecific labeling. The amount of fluorescein incorporation was determined spectrophotometrically using an extinction coefficient of 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 ([Lys78]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-aminohexanoic 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 µl vaflyphenylalaninyllysyllchloromethyl ketone for 1 h. The absence of traces of plasmin was verified by incubating aliquots of the protein at 37 °C with 1 ml 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) forms 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.

Fluorescent Plasminogen Cleavage by u-PA—Experiments were done to measure the kinetics of u-PA-catalyzed cleavage of [Glu1]- or [Lys78]-Plg(S741C- fluorescein) at Arg⁴⁵⁶-Val⁴⁶⁵ in serum-free Opti-MEM I at 37 °C. Fluorescent plasminogen (1 µM) was added to wells with HBST and equilibrated until a stable fluorescence signal was obtained. Then, the plate was transferred to a 96-well fluorescence plate (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. Fluorescence intensities were converted to concentration of plasminogen in the supernatant by comparison to the supernatant of an otherwise identical 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]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.

Fluorescent Plasminogen Cleavage by t-PA—Experiments were done to measure the kinetics of t-PA-catalyzed cleavage of [Glu1]- or [Lys78]Plg(S741C- fluorescein) at Arg⁴⁵⁶-Val⁴⁶⁵ were performed in 96-well fluorescence plates (DyneTech) 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 (1 µM) was added to wells with HBST and equilibrated until a stable fluorescence signal was obtained. Then, the plate was transferred to a 96-well fluorescence plate (DyneTech) 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. Fluorescence intensities were converted to concentration of plasminogen in the supernatant by comparison to the supernatant of an otherwise identical 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 fluorescent plasminogen to fibrin clots. Experimental conditions were as described above, but fibrin was kept constant at 1 µM and concentrations of fluorescent plasminogen ranged from 0.5 to 4 µM.
ure the kinetics of t-PA-catalyzed cleavage of [Glu]- or [Lys]-plasminogen, lanes 2 and 3 are [Glu]-plasminogen (S741C-fluorescein) at Arg561-Val562 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 [Glu]- or [Lys]-plasminogen (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.

ϕn/dt = (ϕn/Lo) ∗ (dϕn/dt)/(1 − r) (Eq. 1)

Δϕn/dt is rate of plasmin formation, ϕn is initial [Glu]- or [Lys]-plasminogen (S741C-fluorescein) concentration, L0 is initial fluorescence intensity, and r represents the ratio of the fluorescence intensities of the fluorophores before and after cleavage. 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 Lys76 forms of [Glu]-plasminogen (S741C-fluorescein) were 0.5 and 0.4.

RESULTS

Production and Characterization of [Glu]-Plasminogen (S741C)—Recombinant [Glu]-plasminogen (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]-plasminogen (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 of the well contents after solubilization with dilute acetic acid 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 Asn288 and Thr345) and 65% plasminogen-II (glycosylated at Thr345 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]-plasminogen (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 Glu1 form of this variant.

Fluorescent Labeling of [Glu]-Plasminogen (S741C) and Characterization of [Glu]-plasminogen (S741C-Fluorescein)—Fluorescent labeling of the introduced “active site” cysteine to generate [Glu]-plasminogen (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]-plasminogen (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 density of the reporter group (Fig. 3). Contrary to the results on the was analyzed by quantifying the change in fluorescence intensity, with a Hill coefficient of 2.0 to fully polymerized clots (Fig. 4). Fibrin binding of these differences in affinity for fibrin, we measured their bind-

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 \( K_D = 2.52 \text{ mM} \). 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 \( K_D = 30 \text{ mM} \), whereas that of [Lys^78]Plg(S741C-fluorescein) was stronger \( (K_D = 1.2 \text{ mM}, n = 1.8 \text{ sites/fibrin}) \). 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 \( (K_D = 38 \text{ mM} \text{ and } 0.32, \text{ respectively}) \) (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 is 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) at 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 of 6-AHA on the site 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-
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 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 [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 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 \(K_m\).
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FIG. 7. Activation of [Glu1]Plg(S741C-fluorescein) by t-PA in a polymerized fibrin clot. A, four solutions (90 μl) containing [Glu1]Plg(S741C-fluorescein) (0.2 μM) and fibrinogen at concentrations of either 50 mM (●, ▼) 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 mM) 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 mM 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 $k = k_{cat}P/(K_m + P)$, where $k$ is the t-PA turnover number (s$^{-1}$) and $P$ is nominal Glu1 or Lys82[Plg741C-fluorescein] concentration.

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 values for the t-PA-catalyzed cleavage of [Glu1]- and Lys82[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).

FIG. 8. Initial rates of activation of [Glu1]- and Lys82[Plg(S741C-fluorescein)] by t-PA in a fully polymerized fibrin clot. Rates of activation were measured as in Fig. 7B at the indicated [Glu1]- or Lys82[Plg(S741C-fluorescein)] concentrations in a 3 μM polymerized fibrin clot and 3.75 nM t-PA and are expressed as turnover number (mol of plasmin generated/mol of t-PA/s). Experimental details and data analysis are described under “Experimental Procedures.” The drawn line represents the results of nonlinear regression to the equation $k = k_{cat}P/(K_m + P)$, where $k$ is the t-PA turnover number (s$^{-1}$) and $P$ is nominal Glu1 or Lys82[Plg(S741C-fluorescein)] concentration. The analyses yielded apparent $k_{cat}$ and $K_m$ values of 0.08 s$^{-1}$ and 0.52 μM for [Glu1]Plg(S741C-fluorescein) (○) and 0.092 s$^{-1}$ and 0.998 μM for Lys82[Plg(S741C-fluorescein)] (●).
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% 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 cysteine and incorporation of a fluorescent, cysteine-specific probe would 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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