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 $k_{cat}$ and $K_m$ 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 fluoreoscently 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 phosphate-buffered saline; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Pn, plasmin.
Properties of Plasminogen(S741C-Fluorescein)

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

<table>
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<th>No.</th>
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<tr>
<td>1</td>
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<td>Amplification</td>
</tr>
<tr>
<td>2</td>
<td>5′-GGCCCTCGGCGGCAGCA-3′ (1820–1843)</td>
<td>S741C</td>
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<td>3</td>
<td>5′-GGCCCGCTTGCGGCGGCAGCA-3′ (1820–1843)</td>
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<td>6</td>
<td>5′-GGCCCGCTTGCGGCGGCAGCA-3′ (1820–1843)</td>
<td>S741C</td>
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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 Ball-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 Ser741 (AGT) to Cys (TGT), whereas amplification of the 78 base pairs 3′-end of the plasminogen cDNA, achieved by oligonucleotides 1 and 2, the fragment used to be substituted with 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 (dihydrofolate reductase) was digested with SmaI, 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 and 5 μM valyl-phenylalaninyl-lysyl chloromethyl ketone for 1 h. The absence of traces of plasmin was verified by incubation 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 [Glu1]Plg(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 [Lys1]-Plg(S741C-fluorescein) forms to a fibrin clot was measured as follows. To a series of microcentrifuge tubes containing 2 μL of 50 mM CaCl2 equilibrated at 37 °C, were added 98 μL of HBST (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.02% Tween 80) and 1 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 fluorescent assay. Typical conditions were as described above but fibrin was kept constant at 1 μM and concentrations of [Lys1]-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 [Lys1]-Plg(S741C) at 37 °C 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. Typical conditions were as described above but fibrin was kept constant at 1 μM and concentrations of [Lys1]-Plg(S741C-fluorescein) ranged from 0.5 to 4 μM.

Fluorescent Plasminogen Cleavage by t-PA—Experiments to measure the kinetics of t-PA-catalyzed cleavage of [Glu1]- or [Lys1]-Plg(S741C) at 37 °C 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. Typical conditions were as described above but fibrin was kept constant at 1 μM and concentrations of [Lys1]-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\(^{+}\) were performed in 96-well fluorescence plates (Dynamech) 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 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 CaCl\(_2\) 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.

\[
dPn/dt = (P_0/L) \cdot dL/dt / (1 - r) \tag{Eq. 1}
\]

\(dPn/dt\) is rate of plasmin formation, \(P_0\) is initial [Glu] or [Lys]-Plg(S741C-fluorescein) concentration, \(L_0\) is initial fluorescence intensity, and \(r\) represents the ratio of the fluorescence intensities of the fluorescent plasmin and plasmin 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 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 (10\(^{6}\) 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\(^{268}\) and Thr\(^{445}\)) and 65% plasminogen-II (glycosylated at Thr\(^{445}\) 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\(^{1}\) form of this variant.
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 μM [Glu₁]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 (Δλₑ = 495, λₑ = 535). Data were corrected for dilution and fit by nonlinear regression to the equation for a single-site/ligand interaction, \[ \Delta \text{Intensity} = \Delta \text{max} \cdot [\text{6-AHA}] / (K_d + [\text{6-AHA}]). \] \( \Delta \text{max} \) is the maximum change at saturation, and \( K_d \) is the dissociation constant. The analysis provided values of \( \Delta \text{max} = 21.4 \pm 0.1 \) and \( K_d = 2.5 \pm 0.1 \) mm.

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 \( K_d \) of 2.52 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 \( K_f \) of 30 μM, whereas that of [Lys₇₈]Plg(S741C-fluorescein) was stronger (\( K_f = 1.2 \) 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 (\( K_f = 38 \) μM and 0.32, respectively) (28).

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 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 absence of such lysine analogs (26, 31). A similar effect of 6-AHA on the overall 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 [Glu₁]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 [Glu₁] plasmin molecule has a relaxed conformation similar to [Lys₇₈] 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 change of plasminogen activation by urokinase in solution has been well documented and resulted in the proposal of a tight, activation-resistant conformation of [Glu₁]plasminogen in the absence of such lysine analogs (26, 31). A similar effect of 6-AHA on activation rates is shown for [Glu₁]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 time courses of fluorescence intensity obtained when solutions of [Glu]$^1$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 CaCl$_2$ (10 μM, final), thrombin (6.0 nM, final), and t-PA (25 nM, final) are indicated in Fig. 7A. Upon initialization of the reaction, 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 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 $K_m$ of $k_{cat}$ and $K_m$.
solution lacking t-PA. Other controls without [Glu1]Plg(S741C-fluorescein). The experiment included 3.0 μM [Glu1]Plg(S741C-fluorescein) at a single input concentration of fibrinogen and thus compare them to values obtained by production level 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 the active site with a thioester.
Properties of Plasminogen(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 1.0% 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 125I-labeled fibrin films due to generated plasmin, kcat and Km values of 0.1 s⁻¹, 0.16 µM and 0.2 s⁻¹, 0.02 µM were obtained for [Glu¹]- and [Lys⁷⁸]plasminogen, respectively (2). Our results on the activation in an intact fully polymerized 3 µM fibrin clot by single-chain t-PA yield 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. The values for [Glu¹]Plg(S741C-Fluorescein) are similar to those reported on soluble fibrin monomer and native [Glu¹]plasminogen, where initial rates in the early phase of plasminogen activation were deduced from extrapolation to time zero (e.g. before plasmin feedback): 0.17 s⁻¹ and 1 µM (33).

It has been suggested that the conversion of single-chain t-PA to two-chain t-PA, [Glu¹]plasminogen to [Lys⁷⁸]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 Km values for plasminogen activation, being 0.52 µM ([Glu¹]plasminogen, intact fibrin, single chain t-PA; this study) to minimally 0.02 µM ([Lys⁷⁸]plasminogen, two chain t-PA, plasmin-modified fibrin; Ref. 2). At physiological levels of fibrin (9 µM) and plasminogen (1.5 µM), 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 Km 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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REFERENCES