Evidence that the conformation of unliganded human plasminogen is maintained via an intramolecular interaction between the lysine-binding site of kringle 5 and the N-terminal peptide

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Human Glu-plasminogen adopts at least three conformations that provide a means for regulating the specificity of its activation in vivo. It has been proposed previously that the closed (α) conformation of human Glu-plasminogen is maintained through the physical interaction of the kringle 5 domain and a lysine residue within the N-terminal peptide (NTP). To examine this hypothesis, site-directed mutagenesis was used to generate variant proteins containing substitutions either for aspartic acid residues within the anionic centre of the kringle 5 domain or for conserved lysine residues within the NTP. Size-exclusion HPLC and rates of plasminogen activation by urokinase-type plasminogen activator were used to determine the conformational states of these variants. Variants with substitutions within the kringle 5 lysine-binding site demonstrated extended conformations, as did variants with alanine substitutions for Lys89 and Lys92. In contrast, molecules in which NTP residues Lys90 or Lys95 were replaced were shown to adopt closed conformations. We conclude that the lysine-binding site of kringle 5 is involved in maintaining the closed conformation of human Glu-plasminogen via an interaction with the NTP, probably through Lys90 and/or Lys95. These conclusions advance the current model for the initial stages of fibrinolysis during which fibrin is thought to compete with the NTP for the kringle 5 lysine-binding site.

INTRODUCTION

Glu-plasminogen (Glu-Plg) is a single-chain 92 kDa glycoprotein [1] containing 791 amino acid residues [2]. It consists of seven domains: an N-terminal peptide (NTP), five kringle domains and a serine protease domain [3]. Activation of Plg on the fibrin surface, generating the trypsin-like fibrinolytic enzyme plasmin, is the initiating step of fibrinolysis at the onset of blood clot degradation. The rate of Glu-Plg activation by activator enzymes such as tissue-type or urokinase-type Plg activators (t-PA or u-PA respectively), is dependent on its conformational state [4]. Human Glu-Plg adopts at least three conformational states [5], mediated by intramolecular interactions involving the binding of intrachain lysine residues to the lysine-binding sites within at least two of the five kringle domains. Saturating concentrations of lysine analogues such as 6-aminohexanoic acid (6-AHA) induce conformational transitions in the molecule from the closed (α) conformation to more extended β and γ conformations [4,5]. Transitions in Plg conformation can be induced by interactions with biological macromolecules in vivo, particularly intact and degraded fibrin [4–6], which promote the accessibility of the Arg561-Val565 scissile bond to Plg activator enzymes [7–10].

The native closed α conformation is thought to adopt a right-handed ‘spiral’ conformation [11–13] with close proximity between the N- and C-terminal domains. Proteolytic removal of the NTP, yielding Lys-Plg, results in an extended conformation designated β, suggesting the involvement of the NTP in maintaining the closed α conformation [14]. This conformational transition can also be induced by the addition of benzamidine, a ligand with a known specificity for kringle 5 [15,16], strongly suggesting that kringle 5 is the NTP-binding domain [5].

Two additional lysine-binding sites, those within kringles 1 and 4, have also been proposed to possess NTP-binding functions [17–19]. Indeed, full-length Glu-Plg variants containing substitutions within these two lysine-binding sites possess high activation rates by low-molecular-mass urokinase-type Plg activator [20] and high sedimentation velocities [21] indicative of fully-extended molecules. These apparent discrepancies in the literature can be resolved in part by a recent suggestion that kringle 4 is not involved in binding to the NTP but mediates a second (β to γ) conformational change [5]. In addition, a role for kringle 1 in binding the NTP is not substantiated by ligand binding studies [9,22].

Studies of molecular conformation and proenzyme activation, although insightful, do not provide an understanding of binding events at the resolution of individual amino acids. However, a combination of such studies with site-directed mutagenesis allows a determination of the contribution of particular amino acids to molecular function. Here, site-directed mutagenesis has been used (1) to examine the hypothesis that the lysine-binding site of...
kringle 5 binds the NTP and (2) to determine the identity of the specific NTP residue(s) involved in this interaction. Glu-Plg variants with amino acid substitutions within the anionic centre of the kringle 5 lysine-binding site were constructed, as were NTP variants with alanine substitutions for each conserved lysine residue. The conformation of each variant protein expressed in Chinese hamster ovary cells was assessed from the results of two complementary methods. Size-exclusion (SE) HPLC provided a biophysical measurement of molecular size; measurement of u-PA-mediated Glu-Plg activation in the presence and in the absence of 6-AHA indicated the degree of accessibility of the u-PA active site to the Glu-Plg activation site in unliganded and liganded (fully extended) conformations.

**EXPERIMENTAL**

**Rationale for mutagenesis of kringle 5 and the NTP**

Known structures of ligand-bound kringle domains [23,24] show that the ε-amino groups of lysine residues or lysine analogues form ion-pair interactions with the carboxylates of Asp55 and Asp57 (consensus numbering as in [25]). For human Glu-Plg kringle 5, these residues correspond to Asp516 and Asp518, which are absolutely conserved for all known Plg sequences. Replacement of either aspartic residue within a kringle with an uncharged residue by mutagenesis is known to decrease binding function [26]. Consequently, two Glu-Plg kringle 5 variants were constructed, Plg(D518Q) and Plg(D516Q, D518Q), in which a single aspartic residue Asp518 or the pair of aspartic residues Asp516 and Asp518 were replaced with glutamine. Glutamine was chosen as it is uncharged and is similar to lysine in volume and side chain length. The selection of residues within the NTP was based on the occurrence of lysine residues that are conserved in known Plg sequences (namely human, mouse, Rhesus macaque, pig, cow and hedgehog). Of the five lysine residues that are absolutely conserved between these sequences, four (Lys59, Lys82, Lys101 and Lys102, but not Lys109) were considered as possible ligands of a kringle lysine-binding site. Lys57 was not chosen because it is absent from an NTP fragment (residues 1–68) that is known to bind Lys-Plg via a lysine-binding site [17]. Arginine residues were not considered to be potential ligands because kringle 5 has been shown to possess a low affinity for internal arginine analogues [14]. Four lysine residues were individually replaced with glutamine. Glutamine was added to the fractions to inhibit proteolysis.

**Mutagenesis**

A Plg cDNA coding for a Glu-Plg protein of length 791 residues [2] was cloned from a human liver cDNA library (JGT11 construct), obtained from Clontech (Palo Alto, CA, U.S.A.) by using a random hexanucleotide-labelled partial clone [28]. Site-directed mutagenesis was performed on the partial Plg gene fragment (EcorV to HindIII) cloned into M13mp18. Table 1 shows the variants with corresponding primers that were used for mutagenesis by the method of Kunkel et al. [29]. The DNA was cloned as an EcoRV to Sp61 fragment (0.85 kb), replacing the wild-type sequence. The full-length gene was cloned into a pGWIHG expression vector, putting the Plg gene under control of the hCMV.M1E promoter and the SV40 polyadenylation signal. For a double substitution variant Plg(D516Q,D518Q), the Plg gene fragment in M13mp18 was mutated as a partial HindIII to Sp61 segment (1.8 kb) with direct insertion into the expression vector containing the remaining wild-type segment. Successful mutagenesis of the selected sequences was confirmed by sequencing the DNA before transfection. In addition, a Plg with wild-type sequence was expressed by inserting the unmutagenized Plg gene into the expression vector; this gene product was used in control experiments.

**DNA transfection, selection of cell lines and protein production**

Plasmid DNA (40 μg linearized with Not1) was electroporated into a Chinese hamster ovary K1 cell line. Cell lines were selected in cell-growth medium containing 1.4 mM xanthine/15.6 μM mycophenolic acid/0.1 mM hypoxanthine/16 μM thymidine. Highest producing cell lines were harvested in Dulbecco’s modified Eagle’s medium-F12 (DMEM-F12)/2 mM glutamine/10 mM sodium bicarbonate/20 kallikrein inhibitor units (KIU)/ml apronin after an initial 24 h conditioning period. Procedures used were similar to those described previously [30]. Plg was harvested every 24 h and purified by lysine-Sepharose chromatography [31]. Plg was eluted from the column in 50 mM sodium phosphate buffer (pH 6.8)/2 mM glutamine/200 μm 6-AHA, and subsequently buffer-exchanged with 50 mM sodium phosphate, pH 6.8, with an Amicon Centricon 10 membrane concentrator (Amicon, Beverly, MA, U.S.A.) until the concentration of 6-AHA was less than 0.1 mM. Concentrations of eluted Plg were estimated by using absorbance measurements at 280 nm and an absorption coefficient of $\epsilon_{280} = 16.8$ [32]. A 5-fold molar excess of the plasmin inhibitor p-nitrophenyl p-guanidinobenzoate was added to the fractions to inhibit proteolysis.

**Table 1 Variants with corresponding primers used for mutagenesis by the method of Kunkel et al. [29]**

<table>
<thead>
<tr>
<th>Kringle 5 mutants</th>
<th>Mutagenesis primer and corresponding primary sequence</th>
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<tbody>
<tr>
<td>D518Q</td>
<td>$5' - GACCACTACTTGACATCGGTAC - 3'$&lt;br&gt; C-term G G V D Q P N N-term</td>
</tr>
<tr>
<td>D516Q,D518Q</td>
<td>$5' - GACCACTACTTGACATCGGTAC - 3'$&lt;br&gt; C-term G G V D Q P N N-term</td>
</tr>
<tr>
<td>NTP mutants</td>
<td></td>
</tr>
<tr>
<td>K20A</td>
<td>$5' - CTCCCAAGCTGCGCTTATGACAC - 3'$&lt;br&gt; C-term G L Q K A K T V N-term</td>
</tr>
<tr>
<td>K33A</td>
<td>$5' - GTCCCTCACTGCTGCAATTC - 3'$&lt;br&gt; C-term D E E C K A A A C T N-term</td>
</tr>
<tr>
<td>K50A</td>
<td>$5' - CATTTGTCACCTGCTGCATGATGTC - 3'$&lt;br&gt; C-term Q Q E K A S H Y Q N-term</td>
</tr>
<tr>
<td>K62A</td>
<td>$5' - GATTATGAGGGACGCCCCCTGAGC - 3'$&lt;br&gt; C-term I S S K A R N E A N-term</td>
</tr>
</tbody>
</table>
Proteins

Plasma-derived Plgs were obtained from the Plasma Fractionation Laboratory (Oxford, U.K.) and from Kabo Pharmaceuticals (Stockholm, Sweden). Two-chain u-PA was from Hitachi (Tokyo, Japan). Streptokinase was from Behringwerke AG (Marburg, Germany). Wild-type recombinant Lys-Plg was obtained from wild-type Glu-Plg dissolved in 50 mM phosphate buffer, pH 7.4, left at −20 °C for 1 month, allowing for gradual proteolysis, until a band of 94.5 % Lys-Plg was observed by acid/urea PAGE. The protein was subsequently resolved as one peak by SE-HPLC. Plg concentrations were determined spectrophotometrically using the following values of $A_{\text{ex}}$ for Glu-Plg: 16.8 [32]; Lys-Plg, 17.4 [33]; u-PA, 13.2 [34]; streptokinase, 9.5 [35].

Characterization of variant proteins

Variant and wild-type Plgs were characterized by acid/urea PAGE to determine the percentage of Glu- and Lys-type Plgs on the basis of their charge separation [36]. The predominance of Glu-type Plg was confirmed by N-terminal sequencing on an Applied Biosystems 470A/120A protein sequencer. The purity of proteins was assessed by SDS/PAGE [37,38] before experimentation and after SE-HPLC and urokinase activity studies to assess whether significant changes had occurred over the time course of experiments. Native-like folding of recombinant proteins was assessed by using an anti-Plg monoclonal ELISA [30] and four monoclonal antibodies; these recognized the first eight residues of the NTP or Plg domains K1-3, K4 or mini-plasminogen (American Diagnostica Plg monoclonal antibodies, numbers 3641, 3642, 3647 and 3644 respectively). As a further test of correct protein folding, zymogen activation of the Plg serine protease was examined with a streptokinase activity assay. Plg (10 µl) at a final concentration of 0.16 µM with 30 µl of 50 mM sodium phosphate buffer, pH 7.4, and 10 µl of streptokinase at 0.16 µM was mixed with 50 µl of the chromogenic substrate S-2251 (p-(p-nitroanilino)-N-benzoyl-L-leucyl-L-lysine, Sigma) at 0.6 mM. The absorbance of the mixture was measured at 405 nm at 1 min intervals as a measure of the amidolytic activity of the Plg–streptokinase complex generated. The assay was performed in duplicate for each variant and for wild-type Plg.

SE-HPLC

A Hewlett-Packard HP1090 SE-HPLC machine equipped with a diode array detector and fitted with a Tosa Haas TSK-G3000SWxl column (30 cm x 0.78 cm internal diam.) was used to characterize the conformations of variants and wild-type molecules [5]. Samples of 25 µl (concentrations 250 µg/ml or greater) were applied to the column equilibrated in 0.1 M sodium phosphate, pH 6.8, or the same buffer with 50 mM 6-AHA as indicated. Run times were 15 min with a flow rate of 1 ml/min at ambient temperatures. Experiments were performed with the single column over a period of 6 months, during which time the standard deviation of Glu-Plg control runs was 0.033 min (n = 4). The absorbance of the eluate was measured at 205 or 210 nm in the presence of 6-AHA. Peak elution times, $\tau_e$, were obtained with the Hewlett-Packard integrator. Changes in $\tau_e$ values in the absence and in the presence of ligand provided a measurement of the conformational changes induced by 50 mM 6-AHA. SE-HPLC results were obtained in duplicate by using the products of two consecutive but separate 24 h harvests of conditioned media.

Amidolytic assays in the absence and in the presence of 6-AHA

Addition of the ligand 6-AHA has previously been reported to increase the u-PA-mediated activation of Plg as a consequence of the Plg conformational change [4,39–41]. The measurement of plasmin activity in the absence and in the presence of 6-AHA provides an indication of the rate of Plg activation and thereby its conformational state. Concentrations of 6-AHA greater than 10 mM were found to inhibit the activity of u-PA as previously reported [4,39]. 6-AHA (5 mM) was found to result in maximum enhancement of u-PA-mediated Glu-Plg activation, so subsequent activation assays were performed in the presence or in the absence of this concentration of 6-AHA. Plg (10 µl) sample at a concentration of 0.65 µM was added to 90 µl of the chromogenic substrate S-2251 at a concentration of 1.78 mM in 50 mM sodium phosphate buffer (pH 7.4)/0.01 % (v/v) Tween 20. A concentration of 30 units/ml u-PA (100 µl) was added. Absorbance was measured at 405 nm at 1 min intervals. Experiments for each protein were performed in triplicate. Measurements of the rate of activation were recorded when the rates of hydrolysis reached linearity.

RESULTS

Characterization of recombinant proteins

This study of conformational changes in Plg required that recombinant proteins be folded correctly and were full-length Glu-Plg molecules. For each analysis of variant and wild-type Plgs a Glu-Plg proportion of greater than 93 % for all harvests was determined by acid/urea PAGE (results not shown). The N-terminal sequence of each of these molecules was also confirmed by N-sequencing of the first ten residues of each protein; these sequences were identical with that reported for plasma-derived Plg with glutamic acid at the N-terminus [1,2]. All variants and the wild-type produced one band on SDS/PAGE gels both

Table 2 SE-HPLC peak elution times ($\tau_e$) and X values for native and recombinant plasminogens

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\tau_e$ (min)</th>
<th>X</th>
</tr>
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<tbody>
<tr>
<td>p-Plg</td>
<td>8.87</td>
<td>8.26</td>
</tr>
<tr>
<td>r-Glu-Plg</td>
<td>8.90</td>
<td>8.36</td>
</tr>
<tr>
<td>G153S</td>
<td>8.47</td>
<td>8.22</td>
</tr>
<tr>
<td>Lys-Plg</td>
<td>8.77</td>
<td>8.56</td>
</tr>
<tr>
<td>r-Lys-Plg</td>
<td>8.91</td>
<td>8.37</td>
</tr>
<tr>
<td>Glu-Plg(D518Q)</td>
<td>8.93</td>
<td>8.36</td>
</tr>
<tr>
<td>r-Glu-Plg(D518Q)</td>
<td>8.42</td>
<td>8.22</td>
</tr>
<tr>
<td>r-Lys-Plg(D518Q)</td>
<td>8.46</td>
<td>8.27</td>
</tr>
<tr>
<td>Glu-Plg(K20A)</td>
<td>8.59</td>
<td>8.44</td>
</tr>
<tr>
<td>r-Glu-Plg(K20A)</td>
<td>8.56</td>
<td>8.44</td>
</tr>
<tr>
<td>Glu-Plg(K33A)</td>
<td>8.75</td>
<td>8.58</td>
</tr>
<tr>
<td>r-Glu-Plg(K33A)</td>
<td>8.72</td>
<td>8.56</td>
</tr>
<tr>
<td>Glu-Plg(K59A)</td>
<td>8.89</td>
<td>8.33</td>
</tr>
<tr>
<td>r-Glu-Plg(K59A)</td>
<td>8.88</td>
<td>8.35</td>
</tr>
<tr>
<td>Glu-Plg(K62A)</td>
<td>8.90</td>
<td>8.34</td>
</tr>
<tr>
<td>r-Glu-Plg(K62A)</td>
<td>8.93</td>
<td>8.35</td>
</tr>
<tr>
<td>Glu-Plg(K63A)</td>
<td>8.66</td>
<td>8.31</td>
</tr>
<tr>
<td>r-Glu-Plg(K63A)</td>
<td>8.49</td>
<td>8.28</td>
</tr>
</tbody>
</table>

Abbreviations: p, plasma-derived protein; r, recombinant protein.
Figure 1  SE-HPLC elution profiles for recombinant variant and wild-type Glu-Plgs and Lys-Plgs

“Native” Plgs refer to human plasma-derived material. In all instances right-hand profiles (higher \( \tau_e \) values) represent elutions of Plgs in the absence of 6-AHA; left-hand profiles (lower \( \tau_e \) values) represent elutions of Plgs in the presence of 10 mM 6-AHA. Abbreviation: mAU, \( 10^{-3} \) absorbance unit.

before and after experimentation, indicating that results relate to Glu-Plg molecules with mass 92 kDa [1]. The sole exception to this was the variant K62A, which although it could be produced and purified intact, could not be sequenced beyond its N-terminal four residues owing to its rapid degradation. The cause of this degradation is not known; it was not arrested by the addition of protease inhibitors aprotinin or \( p \)-nitrophenyl \( p \)-guanidinobenzoate.

Variant and wild-type Plgs were each recognized by all four monoclonal antibodies directed against the first eight residues of the NTP and domains K1–K3, K4 and the K5–serine protease region, providing evidence that these regions were correctly folded. All variants demonstrated activation with streptokinase at initial rates that were comparable (different by less than 2-fold) to that of wild-type Glu-Plg. These rates were not expected to be equivalent owing to known differences in affinity for different Plg conformations [42]. Repetition of these experiments subsequent to SE-HPLC and u-PA studies showed that no significant changes had occurred to any sample, with the exception of variant K62A, which showed rapid degradation. For this latter variant SE-HPLC and u-PA studies were performed immediately after the lysine-Sepharose elution purification step.

Conformations of native and wild-type Plgs assessed by SE-HPLC and by u-PA-mediated activation

In SE-HPLC experiments, plasma-derived Glu-Plg and Lys-Plg, and also Glu-Plg in the presence or absence of 6-AHA, were
eled at distinctive times (Table 2 and Figure 1), as demonstrated previously [5]. The time difference, Δ, therefore provides a convenient measure of the Plg conformational state. The measured mean Δ for plasma-derived Glu-Plg of 0.575 min is interpreted as resulting from the difference between a fully closed α conformation and a fully extended γ conformation, with the latter being adopted in the presence of saturating concentrations of 6-AHA. The smaller difference in elution time for plasma-derived Lys-Plg (a mean Δ of 0.23 min) is interpreted as having resulted from a smaller, β to γ, non-NTP-dependent conformational change involving the lysine-binding site of kringle 4 [5].

The activation of plasma-derived Plgs by u-PA was studied in the absence and presence of 5 mM 6-AHA. X, defined as the ratio of the rate of plasmin-mediated S-2251 hydrolysis in the presence of 5 mM 6-AHA relative to that in its absence, is shown in Table 2 for each of the native forms. The observed enhancement of u-PA-mediated activation of Glu-Plg and Lys-Plg with 1.78 mM S-2251 shows that this concentration of substrate was insufficient to saturate binding to the lysine-binding sites regulating Plg conformation. In the presence of 5 mM 6-AHA, Glu-Plg showed a large increase in activation (4.2-fold) in the absence of 6-AHA, whereas Lys-Plg showed a smaller increase in activation (1.3-fold). These results were qualitatively similar to those obtained for recombinant Glu-Plg and Lys-Plg with the wild-type sequence (Table 2).

Conformations of variant Plgs assessed by SE-HPLC and by u-PA-mediated activation

In SE-HPLC experiments, Glu-Plg variants Plg(D518Q) and Plg(D516Q,D518Q) yielded Δ values of approx. 0.15 min, in contrast with those obtained for Glu-Plg molecules with the wild-type sequence (approx. 0.60 min) and yet were comparable to those obtained for wild-type Lys-Plgs (approx. 0.20 min) (Table 2). Similarly, the stimulation of u-PA-mediated Glu-Plg activation by 5 mM 6-AHA was considerably less pronounced for each of the two kringle 5 variants (1.2–1.4-fold) than for wild-type Glu-Plgs (3.8–4.2-fold), and yet was comparable to the results obtained for wild-type Lys-Plgs (1.2–1.3-fold) (Table 2). Although Glu-Plg variants Plg[D518Q] and Plg[D516Q,D518Q] both retain the NTP of native wild-type Glu-Plg, it seems that substitutions within their kringle 5 lysine-binding sites result in characteristics similar to those of Plg lacking the NTP, i.e. Lys-Plg.

Glu-Plg variants with single substitutions for Lys<sup>56</sup> or Lys<sup>62</sup> within the NTP [Plg(K50A) and Plg(K62A)] also behaved similarly to wild-type Lys-Plg, and differently from wild-type Glu-Plg. Elution times (Table 2) and stimulation by 6-AHA of u-PA-mediated Plg activation (Table 2) differed significantly from those of the wild-type molecule, and more closely mirrored those of the Lys-Plg forms. In contrast, this was found not to be true of two other variants containing lysine-to-alanine substitutions within the NTP, Plg(K20A) and Plg(K33A) yielded SE-HPLC elutions and u-PA-mediated activation values that were comparable to those obtained for wild-type Glu-Plgs (Table 2).

Results therefore indicated that both human Glu-Plg residues Lys<sup>56</sup> and Lys<sup>62</sup> were required for native-like intramolecular interactions with the kringle 5 lysine-binding site. Unusually, however, variant Plg(K62A) was found to be unstable when purified because it degraded rapidly even in the presence of the plasmin inhibitor p-nitrophenyl p-guanidinobenzoate at 10-fold molar excess at −20 °C. After 1–2 h on ice following purification led to rapid degradation, which was observed with SE-HPLC by the appearance of lower-molecular-mass degradation products. The reasons for this rapid degradation are not known. Results shown in Table 2 were obtained by investigations occurring immediately after lysine-Sepharose column elution of Plg(K62A), during which period the variant was determined to be in its full-length (N-terminal residue glutamic acid) form. For conformational analysis by SE-HPLC only a single run with the full-length molecule Plg(K62A) was completed (Δ = 0.25 min; Table 2). In other runs with samples obtained from two separate harvests, degradation products were present together with a main peak, assumed to be the full-length molecule, which showed a mean Δ of 0.18 min when assessed with or without 6-AHA.

**DISCUSSION**

Glu-Plg undergoes a large conformational change that can be induced either by removal of the NTP [14] or by the addition of lysine analogues such as 6-AHA. The opening of Glu-Plg conformation by 6-AHA has been established by a variety of methods [5,43] and suggests that this lysine analogue competes with a lysine residue residing within the NTP for binding to a kringle lysine-binding site. Each of the kringle domains possesses affinity for lysine analogues; kringle 1, 4 and 5 have each been implicated as binding sites for the NTP [17–21,44]. However, the disruption of the closed conformation by benzamidine, which is known to bind kringle 5 [15], suggests that the kringle 5 lysine-binding site is the most likely NTP-binding candidate. To investigate this hypothesis, site-directed mutagenesis was used to generate variants with substitutions of conserved aspartic acid residues critical to the lysine-binding function of kringle 5 and additional variants with substitutions of conserved lysine residues present in the NTP. Conformations were assessed by using SE-HPLC and chromogenic activation assays.

Control experiments with plasma-derived and recombinant Glu-Plg and Lys-Plg provided results that were consistent with the reported ability of Glu-Plg to adopt three conformations [5]. These results indicated that the difference in glycosylation between plasma-derived and recombinant Glu-Plgs did not alter the abilities of the latter molecules to undergo the two known conformational changes. This is in accord with a recent conclusion that differences in Plg glycosylation modify rates of conformational change rather than abrogating the change itself [45]. Streptokinase-mediated zymogen activation and monoclonal antibody recognition assays suggested that recombinant molecules adopted native-like folds.

A single-site substitution variant Plg(D518Q) and a double substitution variant Plg(D516Q,D518Q) each displayed 6-AHA conformational changes that differed significantly from those of the wild-type Glu-Plg molecule yet were similar to those of wild-type Lys-Plgs. These results were obtained despite the fact that by SDS/PAGE, acid/urea PAGE, ELISA and N-terminal sequencing experiments these variants were found to be identical to wild-type Glu-Plg. These results are interpreted as indicating the abilities of these variants to undergo the β to γ conformational change, and their abilities to adopt the ‘closed’ γ conformation of native Glu-Plg. This implicates the two aspartic residues of the kringle 5 lysine-binding site in mediating the closed α conformation. This conclusion is in agreement with previous work that suggested kringle 5 is the NTP’s intramolecular binding partner [16,45,46] and is consistent with the closed right-handed spiral model of Glu-Plg that places the NTP close to kringle 5 [11,12].

Variants with substitutions within the lysine-binding sites of kringle 1 or 4 have recently been shown by Castellino and co-workers [20,21] to adopt fully extended conformations in the presence of 100 mM 6-AHA. The authors concluded that the lysine-binding sites of kringle 1 and 4, and to a smaller extent
that of kringle 5, are important determinants of the adoption of the compact state of Glu-Plg. However, when it is considered that (1) Glu-Plg can adopt an intermediate β conformation, in addition to compact (α) and fully extended (γ) conformations, (2) that this conformation is susceptible to changes on the addition of 6-AHA or benzamidine [5] or when the kringle 5 lysine-binding site residues are substituted [21], and the present study and (3) that, of all the Plg kringles, benzamidine binds only kringle 5 specifically [15,16], it is likely that the lysine-binding site of kringle 5 and not that of kringle 1 or 4 binds the NTP. Furthermore, binding by other ligands to kringle 1 or 4 lysine-binding sites in either α-Glu-Plg or β-Glu-Plg conformations is likely to induce the γ conformation in a manner that involves no direct participation by the NTP. It is clear that interexchange between the conformations of Plg is regulated cooperatively by contrasting affinities between the five kringles for intermolecular and intramolecular ligands, although we argue here that of these interactions, that involving the NTP also involves kringle 5.

Functional characterization of NTP variants demonstrated that, in the absence of 6-AHA, the closed α conformation was conserved in variants with substitutions for Lys59 or Lys59 in Glu-Plg to undergo a large conformational change in the presence of 6-AHA. It is concluded that despite the conservation of Lys59 and Lys59 in Glu-Plgs from different animal species, these residues are not involved in mediating the closed conformation.

Plg(K50A) and Plg(K62A), however, were found to display different conformational properties from those of plasma-derived and wild-type Glu-Plgs, and similar conformational properties to those of Lys-Plgs. This indicates that each of these variants is unable to adopt a closed α conformation, and in common with wild-type Lys-Plg they adopt a partially open β or a fully open γ conformation in the absence or presence of 6-AHA respectively. In the absence of additional information it seems that both Lys59 and Lys59 are involved in maintaining the closed α conformation, presumably by interaction with the kringle 5 lysine-binding site. However, the rapid rate of degradation of the Plg(K62A) variant after purification renders these results questionable. It is interesting to note that the residue analogous to Lys62 in the known X-ray crystallographic structure of hepatocyte growth factor (HGF) is in a buried position in the interface between the NTP domain and the first kringle (A. M. de Vos, Genentech, South San Francisco, CA, U.S.A., personal communication) suggesting that this might also be true of Lys62 in human Glu-Plg. If so, its replacement with alanine might disrupt the interdomain interface, rendering the variant partially unfolded, which might account for its observed susceptibility to proteolysis. Further work is necessary to examine whether this degradation is specific to the particular variant cell line or whether it is a direct result of the Plg(K62A) mutation.

HGF NTP domain crystallographic and NMR structures predict that human Glu-Plg Lys59 is positioned at the tip of a short loop connecting two consecutive β-strands, pointing into the solvent with its side chain completely exposed [47], and A. M. de Vos, personal communication). This indicates that Lys59, in contrast with Lys62, is likely to be available to bind a kringle lysine-binding site. This is consistent with previous studies involving the measurement of the rate of Glu-Plg activation by u-PA in the presence of excess synthetic peptides, which also implicated Lys59 as a kringle-binding determinant [48,49].

Our conclusion that kringle 5 is involved in an intramolecular interaction with Lys59 and/or Lys59 of the NTP has implications for understanding the specificity of Plg activation during the early stages of fibrinolysis. Of all the Plg kringle domains, kringle 5 possesses the highest affinity for intact fibrin [50,51] and has been proposed to mediate Plg’s initial binding event with fibrin [5,44]. Because this binding event increases the rate of Plg activation [4] a model [33,52,53] of the early stages of fibrinolysis can be summarized as: (1) fibrin competes with the NTP for interaction with the lysine-binding site of kringle 5, resulting in (2) a conformational change (α→β) within Glu-Plg, enabling (3) a greater accessibility of Plg activators to the Plg scissile bond, enhancing (4) Plg activation and (5) plasminolysis of fibrin, that generates (6) C-terminal lysine residues, that function as (7) additional Plg-binding sites and additionally (8) initiate the (β→γ) Plg conformational change, rendering Plg increasingly prone to activation. Further investigations should consider the validity of this model and the residues that regulate the second (β→γ) Plg conformational change.

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REFERENCES

34 Taylor, Jr., F. B. and Botls, J. (1968) Biochemistry, 7, 232–242

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