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

Charles S. COCKELL^{*1}, Julian M. MARSHALL^{†2}, Keith M. DAWSON[‡], Stewart A. CEDERHOLM-WILLIAMS[§] and Chris P. PONTING^{||3}

^{*}University of Oxford, Laboratory of Molecular Biophysics, Department of Biochemistry, Rex Richards Building, South Parks Road, Oxford OX1 3QU, U.K., [†]Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, U.K., [‡]British Biotech, Watlington Road, Cowley, Oxford OX4 5LY, U.K., [§]Oxford Bioresearch Laboratory, Magdalen Centre, Oxford Science Park, Oxford OX4 4GA, U.K., and ^{||}University of Oxford, Oxford Centre for Molecular Sciences, Fibrinolysis Research Unit, The Old Observatory, South Parks Road, Oxford OX1 3RH, U.K.

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 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 Lys⁵⁰ and Lys⁶². In contrast, molecules in which NTP residues Lys²⁰ or Lys³³ 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 Lys⁵⁰ and/or Lys⁶². 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-Val⁵⁶² 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 Glu₁-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

Abbreviations used: 6-AHA, 6-aminohexanoic acid; DMEM, Dulbecco's modified Eagle's medium; NTP, N-terminal peptide; Plg, plasminogen; S-2251, D-valyl-L-leucyl-L-lysine-p-nitroaniline; SE-HPLC, size-exclusion HPLC; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

¹ Present address: Carnegie Institution, 260 Panama Street, Stanford, CA 94305-1297, U.S.A.

² Present address: Oxford Bioresearch Laboratory, Magdalen Centre, Oxford Science Park, Oxford OX4 4GA, U.K.

³ To whom correspondence should be addressed (e-mail Ponting@Bioch.ox.ac.uk).

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 (Lys²⁰, Lys³³, Lys⁵⁰ and Lys⁶², but not Lys⁷⁷) were considered as possible ligands of a kringle lysine-binding site. Lys⁷⁷ 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 alanine in four Plg variants: Plg(K20A), Plg(K33A), Plg(K50A) and Plg(K62A). This approach of replacing single residues contrasts with a previous study of variants containing multiple substitutions of Arg⁶¹, Lys⁶², Arg⁶⁸, Arg⁷⁰, Lys⁷⁷ and/or Lys⁷⁸ [27].

Mutagenesis

A Plg cDNA coding for a Glu-Plg protein of length 791 residues [2] was cloned from a human liver cDNA library (λ GT11 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 *SphI* fragment (0.85 kb), replacing the wild-type sequence. The full-length gene was cloned into a pGW1HG 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 *SphI* 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 μ g linearized with *NotI*) 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 μ M mycophenolic acid/0.1 mM hypoxanthine/16 μ M 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 aprotinin 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)/50 mM 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 $A_{280}^{1\%} = 16.8$ [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]

Kringle 5 mutants	Mutagenesis primer and corresponding primary sequence
D518Q	5' - G A C C A C C T A C C T G A C C A T C A G G G T T A C - 3' C-term G G V D→Q G D P N N-term
D516,518Q	5' - G A C C A C C T A C C T G A C C C T G A G G G T T A C - 3' C-term G G V D→Q G D→Q P N N-term
NTP mutants	
K20A	5' - C T C C C A G C T G C G C C T T A G T G A C A C - 3' C-term G L Q K→A K T V N-term
K33A	5' - G T C C T C C T C A C A T G C T G C T G C A C A T T C - 3' C-term D E E C K→A A A C T N-term
K50A	5' - C A T T G T T G C T C T G C A C T G T G A T A T T G - 3' C-term Q Q E K→A S H Y Q N-term
K62A	5' - G A T T A T G G A G G A C G C C C T G T T T T C A G C - 3' C-term I I S S K→A R N E A N-term

Proteins

Plasma-derived Plgs were obtained from the Plasma Fractionation Laboratory (Oxford, U.K.) and from Kabi 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 $A_{280}^{1\%}$: 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-plasmin(ogen) (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 ($10\ \mu\text{l}$) at a final concentration of $0.16\ \mu\text{M}$ with $30\ \mu\text{l}$ of 50 mM sodium phosphate buffer, pH 7.4, and $10\ \mu\text{l}$ of streptokinase at $0.16\ \mu\text{M}$ was mixed with $50\ \mu\text{l}$ of the chromogenic substrate S-2251 (D-valyl-L-leucyl-L-lysine-*p*-nitroaniline) (Chromogenix, Molndal, Sweden) 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 ($30\ \text{cm} \times 0.78\ \text{cm}$ internal diam.) was used to characterize the conformations of variants and wild-type molecules [5]. Samples of $25\ \mu\text{l}$ (concentrations $250\ \mu\text{g}/\text{ml}$ 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, τ_e , were obtained with the Hewlett-Packard integrator. Changes in τ_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 ($10\ \mu\text{l}$) sample at a concentration of $0.65\ \mu\text{M}$ was added to $90\ \mu\text{l}$ 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 ($100\ \mu\text{l}$) 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 (τ_e) and X values for native and recombinant plasminogens

Times are for elutions in the absence or presence of 10 mM 6-AHA for duplicate runs; X is the ratio of the rate of u-PA-mediated Plg activation in the presence of 5 mM 6-AHA relative to that in the absence of 6-AHA. Abbreviations: p, plasma-derived protein; r, recombinant protein.

Protein	τ_e (min)		Δ (min)	X
	Without 6-AHA	With 10 mM 6-AHA		
p-Glu-Plg	8.87	8.26	0.61	4.15 ± 0.12
	8.90	8.36	0.54	
p-Lys-Plg	8.47	8.22	0.25	1.33 ± 0.04
	8.77	8.56	0.21	
r-Glu-Plg	8.91	8.37	0.59	3.75 ± 0.13
	8.93	8.36	0.57	
r-Lys-Plg	8.42	8.22	0.20	1.23 ± 0.03
	8.46	8.27	0.19	
r-Glu-Plg(D518Q)	8.59	8.44	0.15	1.18 ± 0.04
	8.56	8.44	0.12	
r-Glu-Plg(D516Q,D518Q)	8.75	8.58	0.17	1.37 ± 0.05
	8.72	8.56	0.16	
r-Glu-Plg(K20A)	8.89	8.33	0.56	3.61 ± 0.12
	8.88	8.35	0.53	
r-Glu-Plg(K33A)	8.90	8.34	0.56	2.83 ± 0.15
	8.93	8.35	0.58	
r-Glu-Plg(K50A)	8.66	8.31	0.35	1.29 ± 0.04
	8.49	8.28	0.21	
r-Glu-Plg(K62A)	8.94	8.69	0.25	1.25 ± 0.03

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 τ_e values) represent elutions of Plgs in the absence of 6-AHA; left-hand profiles (lower τ_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 Glu₁-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

eluted 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 Lys⁵⁰ or Lys⁶² 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 Lys⁵⁰ and Lys⁶² 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 ($\Delta = 0.25$ 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; kringles 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 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 inability to adopt the 'closed' α 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 kringles 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 kringles 1 and 4, and to a smaller extent

that of kringle 5, are important determinants of the adoption of the compact state of Glu₁-Plg. However, when it is considered that (1) Glu₁-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 -Glu₁-Plg or β -Glu₁-Plg conformations is likely to induce the γ conformation in a manner that involves no direct participation by the NTP. It is clear that interchange between the conformations of Plg is regulated co-operatively 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 Lys²⁰ or Lys³³; in addition these variants retained the ability of the wild-type Glu-Plg molecule to undergo a large conformational change in the presence of 6-AHA. It is concluded that despite the conservation of Lys²⁰ and Lys³³ 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 Lys⁵⁰ and Lys⁶² 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 partly 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 Lys⁵⁰ 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 Lys⁵⁰, 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 Lys⁵⁰ as a kringle-binding determinant [48,49].

Our conclusion that kringle 5 is involved in an intramolecular interaction with Lys⁵⁰ and/or Lys⁶² of the NTP has implications for understanding the specificity of Plg activation during the early

stages of fibrinolysis. Of all the Plg kringles 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 ($\alpha \rightarrow \beta$) 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 ($\beta \rightarrow \gamma$) 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 ($\beta \rightarrow \gamma$) Plg conformational change.

C.S.C. was supported by the Science and Engineering Research Council under a SERC studentship with British Biotech Plc. C.P.P. is a Wellcome Trust Fellow.

REFERENCES

- Wallén, P. and Wiman, B. (1970) *Biochim. Biophys. Acta* **221**, 20–30
- Forsgren, M., Råden, B., Israelsson, M., Larsson, K. and Heden, L. O. (1987) *FEBS Lett.* **213**, 254–260
- Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. and Magnusson, S. (1978) *Prog. Chem. Fibrinol. Thromb.* **3**, 191–209
- Markus, G. (1996) *Fibrinolysis* **10**, 75–85
- Marshall, J. M., Brown, A. J. and Ponting, C. P. (1994) *Biochemistry* **33**, 3599–3606
- Lucas, M. A., Fretto, L. J. and McKee, P. A. (1983) *J. Biol. Chem.* **258**, 4249–4256
- Claeys, H. and Vermeylen, J. (1974) *Biochim. Biophys. Acta* **342**, 351–359
- Thorsen, S., Kok, P. and Astrup, T. (1974) *Thromb. Diath. Haem.* **32**, 325–340
- Markus, G., Priore, R. L. and Wissler, F. C. (1979) *J. Biol. Chem.* **254**, 1211–1216
- Hoylaerts, M., Rijken, D. C., Lijnen, H. R. and Collen, D. (1982) *J. Biol. Chem.* **257**, 2912–2919
- Tranqui, L., Prandini, M. and Chapel, A. (1979) *Biol. Cell.* **34**, 39–42
- Ponting, C. P., Holland, S. K., Cederholm-Williams, S. A., Marshall, J. M., Brown, A. J., Spraggon, G. and Blake, C. C. F. (1992) *Biochim. Biophys. Acta* **1159**, 155–161
- Weisel, J. W., Nagaswami, C., Korsholm, B., Petersen, L. C. and Suenson, E. (1994) *J. Mol. Biol.* **235**, 1117–1135
- Sjöholm, I., Wiman, B. and Wallén, P. (1973) *Eur. J. Biochem.* **39**, 471–479
- Thewes, T., Constantine, K., Byeon, I. L. and Llinás, M. (1990) *J. Biol. Chem.* **265**, 3906–3915
- Váradi, A. and Patthy, L. (1981) *Biochem. Biophys. Res. Commun.* **103**, 97–102
- Wiman, B. and Wallén, P. (1975) *Eur. J. Biochem.* **50**, 489–494
- Lerch, P. G., Rickli, E. E., Lergier, W. and Gillesen, D. (1980) *Eur. J. Biochem.* **107**, 7–13
- Cummings, H. S. and Castellino, F. J. (1985) *Arch. Biochem. Biophys.* **236**, 612–618
- Menhart, N., Hoover, G. J., McCance, S. G. and Castellino, F. J. (1995) *Biochemistry* **34**, 1482–1488
- McCance, S. G. and Castellino, F. J. (1995) *Biochemistry* **34**, 9581–9586
- Christensen, U. and Mølgaard, L. (1991) *FEBS Lett.* **278**, 204–206
- Wu, T., Padmanabhan, K., Tulinsky, A. and Mulichak, A. M. (1991) *Biochemistry* **30**, 10589–10594
- de Vos, A., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L. and Kossiakoff, A. A. (1992) *Biochemistry* **31**, 270–279
- Tulinsky, A., Park, C. H., Mao, B. and Llinás, M. (1988) *Proteins* **3**, 85–96
- Weening-Verhoeff, E. J. D., Quax, P. H. A., VanLeeuwen, R. T. J., Rehberg, E. F., Marotti, K. R. and Verheijen, J. H. (1990) *Protein Eng.* **4**, 191–198
- Horrovoets, A. J. G., Smilde, A. E., Fredenburgh, J. C., Pannekoek, H. and Nesheim, M. E. (1995) *J. Biol. Chem.* **270**, 15770–15776
- Dawson, K. M., Edwards, R. M. and Forman, J. M. (1991) *International Patent Application WO 91/09118*
- Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- Dawson, K. M., Marshall, J. M., Raper, R. H., Gilbert, R. J. and Ponting, C. P. (1994) *Biochemistry* **33**, 12042–12047
- Deutsch, D. G. and Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Wallén, P. and Wiman, B. (1972) *Biochim. Biophys. Acta* **257**, 122–134
- Christensen, U. (1988) *Biochim. Biophys. Acta* **957**, 258–265
- White, W. F. and Barlow, G. H. (1970) *Methods Enzymol.* **19**, 665–672
- Taylor, Jr., F. B. and Botts, J. (1968) *Biochemistry* **7**, 232–242

-
- 36 Panyim, S. and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346
37 Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
38 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
39 Walther, P. J., Hill, R. L. and McKee, P. A. (1975) *J. Biol. Chem.* **250**, 5926–5933
40 Violand, B. N., Byrne, R. and Castellino, F. J. (1978) *J. Biol. Chem.* **253**, 5395–5401
41 Markus, G., Evers, J. L. and Hobika, G. H. (1978) *J. Biol. Chem.* **253**, 733–739
42 Bock, P. E., Day, D. E., Verhamme, I. M., Bernardo, M. M., Olson, S. T. and Shore, J. D. (1996) *J. Biol. Chem.* **271**, 1072–1080
43 Brockway, W. J. and Castellino, F. J. (1972) *Arch. Biochem. Biophys.* **151**, 194–199
44 Christensen, U. (1984) *Biochem. J.* **223**, 413–421
45 Mølgaard, L., Ponting, C. P. and Christensen, U. (1997) *FEBS Lett.* **405**, 363–368
46 Holleman, W. H., Andres, W. W. and Weiss, L. J. (1975) *Thromb. Res.* **7**, 683–693
47 Zhou, H., Mazzulla, M. J., Kaufman, J. D., Stahl, S. J., Wingfield, P. T., Rubin, J. S., Bottaro, D. P. and Byrd, R. A. (1998) *Structure* **6**, 109–116
48 Urano, T., Takada, Y. and Takada, A. (1991) *Thromb. Res.* **61**, 349–359
49 Takada, Y., Urano, T. and Takada, A. (1993) *Thromb. Res.* **70**, 151–159
50 Thorsen, S., Clemmensen, I., Sottrup-Jensen, L. and Magnusson, S. (1981) *Biochim. Biophys. Acta* **668**, 377–387
51 Wu, H., Chang, B. I. and Wu, D. H. (1990) *J. Biol. Chem.* **265**, 19658–19664
52 Peltz, S. W., Hardt, T. A. and Mangel, W. F. (1982) *Biochemistry* **21**, 2798–2804
53 Ponting, C. P., Marshall, J. M. and Cederholm-Williams, S. A. (1992) *Blood Coagul. Fibrinol.* **3**, 605–614
-

Received 21 November 1997/12 March 1998; accepted 6 April 1998