Contributions of Individual Kringle Domains toward Maintenance of the Chloride-Induced Tight Conformation of Human Glutamic Acid-1 Plasminogen†

Stephen G. McCance and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

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ABSTRACT: The roles of each of the three ω-amino acid-binding kringles (K) of Glu^1-Pg, viz., [K1$_{Pg}$], [K4$_{Pg}$], and [K5$_{Pg}$], in engendering the Cl^--induced alteration to its tight (T) conformation and in effecting the ϵ-aminocaproic acid (EACA)-mediated change to the relaxed (R) protein conformation have been investigated by mutagenesis strategies wherein the ω-amino acid ligand-binding energies in the individual kringles in recombinant (r)-Glu^1-Pg were greatly reduced. This was accomplished in the most conservative manner possible by altering a critical Asp residue in each relevant kringle to Asn. The particular mutations chosen were r-[D^{139}N]Glu^1-Pg, r-[D^{413}N]Glu^1-Pg, and r-[D^{518}N]Glu^1-Pg, in which a conserved Asp residue at a homologous sequence position in each of the three kringle domains is eliminated. These changes also lead to a great reduction of the EACA-binding strength of [K1$_{Pg}$], [K4$_{Pg}$], and [K5$_{Pg}$], respectively. The $s^0_{20,w}$ of wild-type (wt) r-Glu^1-Pg in the presence of levels of Cl^- sufficient to fully occupy its binding sites on this protein was 5.9 S, a value reduced to 4.9 S as a result of addition of saturating concentrations of EACA to the Cl^-/Glu^1-Pg complex. Neither Cl^- nor EACA substantially altered the $s^0_{20,w}$ values of r-[D^{139}N]Glu^1-Pg (4.8 S) or r-[D^{413}N]Glu^1-Pg (4.5 S). On the other hand, the $s^0_{20,w}$ value of 5.2 S for r-[D^{518}N]Glu^1-Pg at saturating levels of Cl^- is slightly reduced to 4.8 S upon addition of binding maximal concentrations of EACA. Whether the disulfide bond between Cys^{169} and Cys^{297} that covalently links the [K2$_{Pg}$] and [K3$_{Pg}$] domains is a structural requisite for adoption of the Cl^--dependent T-conformation has been examined by similar studies on a mutein containing these Cys residues altered to Asp and Arg, respectively. The $s^0_{20,w}$ values obtained for this latter mutant under the conditions described above were identical to those of wtr-Pg. This demonstrated that the covalent attachment of [K2$_{Pg}$] and [K3$_{Pg}$] is unnecessary for the effector-induced conformational changes to occur in wtr-Glu^1-Pg. These results suggest that in the presence of Cl^-, or other tight binding anions, Glu^1-Pg is induced into its T-conformation, which is known to be highly activation-resistant. The T-state is most likely stabilized by insertion of lysine side chains, and/or side chain clusters mimicking ω-amino acid structures, of other regions of the protein into the ω-amino acid-binding pockets of individual kringles. Displacement of these intraprotein binding events occurs as a result of addition of EACA to the solution, thereby inducing the expanded and flexible R-conformation, which also is known to allow the Glu^1-Pg to be activated at a substantially higher rate. Additionally, we show herein that each of the ω-amino acid-binding kringles of Glu^1-Pg plays a role in these effects, with [K1$_{Pg}$] and [K4$_{Pg}$] being the most important contributors to this mechanism.

Glu^1-Pg[1] is the circulating zymogen of the serine protease Pm and exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Forsgren et al., 1987). Activation of Pg occurs as a result of activator-catalyzed cleavage of the Arg^{561}−Val^{562} peptide bond in the zymogen (Robbins et al., 1967). This step, as well as another wherein the Pm formed catalyzes a feedback cleavage of the Lys^{77}−Lys^{78} peptide bond in the remaining Glu^1-Pg, or in the initial molecules of Glu^1-Pm generated, results in Lys^{78}-Pm, a two-chain disulfide-linked enzyme (Violand & Castellino, 1976; Gonzalez-Gronow et al., 1977). The catalytic triad of amino acids of plasmin is composed of residues His^{603}, Asp^{646}, and Ser^{741}.

Both nucleotide information and amino acid sequence results support the conclusion that Glu^1-Pg consists of a series of domains (Sottrup-Jensen et al., 1978; Petersen et al., 1990). An amino-terminal span of 78 amino acid residues has been identified as the AP. Following consecutively are five homologous triple disulfide-linked regions of approximately 80 amino acids each, termed kringles, which along with the AP constitute the latent heavy chain of Pg (residues 1−561). The latent light chain of Pg (residues 562−791) is homologous to enzymes of the serine protease superfamily and is responsible for the hydrolytic activity of Pm. The kringle domains contain binding sites for positive and negative effectors of Pg activation, such as EACA and Cl^- (Sottrup-Jensen et al., 1978; Castellino et al., 1981; Urano et al., 1987a,b, 1988), and for cellular receptors of Pg and Pm (Miles et al., 1988). The kringle modules also provide extended binding sites for physiological substrates (Wiman & Wallen, 1977; Thorsen et al., 1981; Lucas et al., 1983a,b) and inhibitors (Wiman et al., 1978) of Pm. The AP region of the protein is essential for the abilities of Cl^-

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* To whom to address correspondence. Telephone: (219) 631-6456. Telefax: (219) 631-8149.

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and EACA to regulate the conformation and the concomitant activatability of Glu[1]-Pg (Violand et al., 1975, 1978; Urano et al., 1987a,b, 1988).

Glu[1]-Pg is present in a weakly activatable, compact, and internally rigid conformation (T-state) in the presence of anions, such as Cl^-, and becomes more internally flexible and expanded (R-state) as a result of its interaction with ω-amino acids (Brockway & Castellino, 1972; Castellino et al., 1973; Violand et al., 1975; Mangel et al., 1990). The conformational transition from the T- to R-state also results in a much more rapid activation of the Glu[1]-Pg (Urano et al., 1987a,b). These regulatory events require the presence of the AP and are much less pronounced in the molecular form of Pg (Lys^{78}-Pg) which lacks this region (Violand et al., 1975; Urano et al., 1987a,b).

The exosites of Pg responsible for regulation of its activation, as well as those for Pm function, are located in the ω-amino acid-binding kringles, viz., [$K1_{Pg}$] (Sottrup-Jensen et al., 1978), [$K4_{Pg}$] (Sottrup-Jensen et al., 1978), and [$K5_{Pg}$] (Castellino, 1981). Typically, ω-amino acids, e.g., EACA, displace Pg and Pm from their macromolecular binding sites. It is unclear as to which of these kringle domains contain the ligand-binding exosites that are involved in the various functions of Pg. From indirect studies with intact Pg, it has been proposed that the weaker ω-amino acid-binding sites in [$K4_{Pg}$] and [$K5_{Pg}$] are associated with the ligand-induced conformational change in the zymogen (Christensen & Molgaard, 1992). The stronger ω-amino acid-binding site in [$K1_{Pg}$] has been proposed to function by interacting with macromolecular cofactors and inhibitors of Pg and Pm but not via regulation of the conformational properties of Pg (Marshall et al., 1994).

Previous work with recombinant kringle domains and mutants thereof has allowed definition of the amino acid side chains that stabilize ω-amino acid binding to these modules (Menhart et al., 1991, 1993; De Serrano & Castellino, 1992a,b, 1993, 1994; Hoover et al., 1993; McCance et al., 1994). In order to probe the specific roles of individual kringle domains in the intact protein in regulation of the conformation and activation of Glu[1]-Pg, we employed herein existing knowledge regarding ω-amino acid binding to isolated kringles to selectively modify, by site-directed mutagenesis, the affinity of ω-amino acids for individual kringles in intact Glu[1]-Pg. This was accomplished by altering a critical Asp residue at homologous locations in [$K1_{Pg}$], [$K4_{Pg}$], and [$K5_{Pg}$] in Glu[1]-Pg to an Asn residue. In a previous study employing this same strategy, we studied the contributions of individual ω-amino acid-binding kringles in Pg to the positive and negative effector properties of EACA and Cl^- in Pg activation (Menhart et al., 1995). The current investigation, the results of which are reported herein, is focused on utilization of these same mutants to evaluate the roles of these kringle modules in defining the T- and R-states of Glu[1]-Pg and the effects of EACA and Cl^- on the stability of these conformations.

EXPERIMENTAL PROCEDURES

Proteins. Native human plasma Glu[1]-Pg was obtained from Enzyme Research Laboratories (South Bend, IN). Expression in *T. ni* cells (High Five cells; Invitrogen, San Diego, CA) of cDNAs encoding wtr-Pg, r-[D^{139}N]Glu[1]-Pg, r-[D^{413}N]Glu[1]-Pg, r-[D^{518}N]Glu[1]-Pg, and r-[C^{169}D/C^{297}R]-Glu[1]-Pg, after insertion of these heterologous genes into the baculovirus genome, and purification of the Pg gene products by affinity chromatography on Sepharose-lysine was accomplished as previously described (Menhart et al., 1995). The insect cells were infected for 72 h with the above r-baculoviruses at multiplicities of 4 plaque-forming units/cell, using infection and cell-handling protocols described earlier (Whitefleet-Smith et al., 1989). LMW-uPA was a gift of Abbott Laboratories (N. Chicago, IL). Aprotinin was purchased from the Sigma Chemical Co. (St. Louis, MO).

Genes. The construction of the wt-Pg cDNA in pUC119 (p119/Pg) has been described (Whitefleet-Smith et al., 1989). A BamHI restriction endonuclease site was inserted in the 5′-nontranslated region of p119/Pg to facilitate transfer of the Pg into the plasmid pBlueBacIII (Invitrogen). The mutagenic oligonucleotide employed for this purpose was ACTTT CTGGG atCcG CTGGC CAGTCC (the mutagenic changes are in lower case letters and the location of the restriction site is underlined).

The variants r-[D^{139}N]Glu[1]-Pg, r-[D^{413}N]Glu[1]-Pg, and [D^{518}N]Glu[1]-Pg were generated by primer-directed mutagenesis of single-stranded p119/Pg (Kunkel et al., 1987), as previously described (Menhart et al., 1995). The oligonucleotide primers used for this purpose were (1) [D^{139}N], 5′-GC AGG AAT CCg GAC AAC aAT CCG CAG GG (+*Acc*III), (2) [D^{413}N], 5′-CCA GAT GCC aAT AAg GGC CCC TGG T (+*Apa*I), and (3) [D^{518}N], 5′-CCT GAT GGT aAT GTA GGa GGT CCC TGG (+*Ppu*MI). The double mutant r-[C^{169}D/C^{297}R]Glu[1]-Pg was constructed by simultaneous addition of the following two oligonucleotides to single-stranded p119/Pg (the new restriction sites gained or lost accompanying the mutations are indicated): (4) [C^{169}D/C^{297}R], 5′-GAA TGT ATG CAC gac AGT GGA GAA A (-*Nsi*I)/5′-A GAA AAC TTC CCg cGg AAA AAT TTG G(+*Sac*II). Transformed bacterial colonies containing the cDNA for Glu[1]-Pg that simultaneously lost the *Nsi*I restriction site and gained the *Sac*II site were subjected to nucleotide sequencing to confirm the presence of the mutations.

The mutants were subcloned into in the *Bam*HI/*Hind*III polylinker sites of plasmid pBlueBacIII and inserted by homologous recombination into the baculovirus genome in

[1] Abbreviations: Pg, generic abbreviation for human plasminogen; Glu[1]-Pg, native human plasminogen with Glu, residue 1, at the amino terminus; Lys^{78}-Pg, proteolytically (plasminolytic) derived form of human plasminogen with Lys, residue 78, at the amino terminus; Pm, generic abbreviation for human plasmin; Lys^{78}-Pm, human plasmin, which arises from Glu[1]-Pg by cleavage (by plasminogen activators) at the activation site Arg^{561}-Val^{562} and also (by plasmin) at Lys^{77}-Lys^{78} or originates from Lys^{78}-Pg by cleavage at Arg^{561}-Val^{562}; LMW-uPA, low molecular weight urokinase-type plasminogen activator consisting of amino acid residues 159−411 of native urokinase; tPA, tissue-type plasminogen activator; AP, activation peptide of human Glu[1]-Pg, composed of amino acid residues 1-77 of this protein; [$K1_{Pg}$], kringle 1 region of human plasminogen, which consists of amino acid residues Cys^{84}−Cys^{162} of the intact protein; [$K2_{Pg}$], kringle 2 region (residues Cys^{166}−Cys^{243}) of human plasminogen; [$K3_{Pg}$], kringle 3 region (residues Cys^{256}−Cys^{333}) of human plasminogen; [$K4_{Pg}$], kringle 4 region (residues Cys^{358}−Cys^{435}) of human plasminogen; [$K5_{Pg}$], kringle 5 region (residues Cys^{462}−Cys^{541}) of human plasminogen; r-[$D^{XXX}N$]-Glu[1]-Pg, recombinant human Glu[1]-Pg containing an Asp to Asn mutation at amino acid sequence position *XXX*; EACA, ϵ-aminocaproic acid; S-2251, H-D-Val-Leu-Lys-*p*-nitroanilide; FPLC, fast protein liquid chromatography; $DodSO_4$/PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; wt, wild-type; r, recombinant; *T. ni*, *Trichoplusia ni*.

such a manner as to interrupt the polyhedrin gene (Whitefleet-Smith et al., 1989). Recombinant viruses were detected as blue plaques in the presence of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; purchased from the Promega Corp., Madison, WI). The r-viruses were plaque purified and used to infect *T. ni* cells.

Activation Assays in the Presence of Aprotinin. The relative quantities of Glu^1-Pg and Lys^{78}-Pg in purified Pg samples were determined by analysis of the molecular mass of the heavy chain of the HPm produced after activation of the relevant Pg in the presence of the rapid Pm inhibitor aprotinin. In this assay, all Glu^1-Pg present is converted to the Glu^1-Pm heavy chain (as well as the light chain), which is readily resolvable in $DodSO_4$/PAGE from the Lys^{78}-Pm heavy chain. Because aprotinin is present to immediately inhibit the HPm formed, the Glu^1-Pm heavy chain, when present, will not be converted to the Lys^{78}-Pm heavy chain.

The activations were carried out as follows: A 5 μL aliquot of a 14 μM solution of Pg was added to 5 μL of a 0.14 μM solution of LMW-uPA in the presence of 8 μg of aprotinin. The buffer for all solutions was 10 mM Na-Hepes/100 mM NaOAc, pH 7.4. After 2 h at 37 °C, the samples were analyzed by 9% $DodSO_4$/PAGE under reducing conditions (Laemmli, 1970).

Sedimentation Velocity Analysis. Sedimentation coefficients of Glu^1-Pg were determined in different buffers using a Beckman Optima XL-A analytical ultracentrifuge, operating at 60 000 rpm and 20 °C. An absorbance scanning device set at 280 nm was employed to monitor the sedimentation characteristics of the macromolecule. Sample scans were obtained every 8 min for a total of 1.5 h. The s_{app} values obtained from these analyses were converted to their respective $s_{20,w}$ values by correction of the density and viscosity of the buffers used to those same parameters of water at 20 °C. The protein concentrations used in these experiments were <0.7 mg/mL; thus the $s_{20,w}$ in each case was assumed to be equal to the $s^0_{20,w}$. The buffers employed were 100 mM Tris-Cl/100 mM NaCl, pH 7.8; 100 mM Tris-Cl/100 mM NaCl/100 mM EACA, pH 7.8; 100 mM Tris-OAc/100 mM NaOAc, pH 7.8; and 100 mM Tris-OAc/100 mM NaOAc/100 mM EACA, pH 7.8.

RESULTS

A series of r-Glu^1-Pg mutant cDNAs were constructed, inserted into the baculoviral genome, expressed in lepidopteran insect cells, and purified. These variants each possessed a defect in their kringle domains which greatly diminished the binding energy of ω-amino acid effectors to the kringle 1 (r-[D^{139}N]Glu^1-Pg), kringle 4 (r-[D^{413}N]Glu^1-Pg), and kringle 5 (r-[D^{518}N]Glu^1-Pg) domains. The major use of these mutants in the present study was to evaluate their conformational states in the presence and absence of the negative and positive activation effectors Cl^- and EACA, respectively. Sedimentation velocity analysis presented a very convenient method for such an analysis in these particular cases, since the changes in this parameter with Cl^- and EACA have been demonstrated to be particularly large (Brockway & Castellino, 1972; Violand et al., 1975; Urano et al., 1987a,b), and effector-related alterations have been shown to correlate with the activation rates of the zymogen (Urano et al., 1987b). An example of the type of data obtained in these analyses is shown in Figure 1, and a summary of all $s^0_{20,w}$ values obtained are listed in Table 1. At least three ultracentrifuge experiments were conducted in each case with variations <0.1 S.

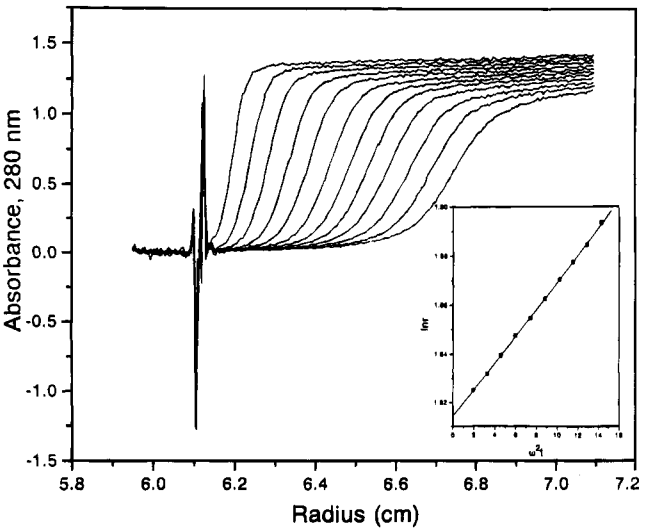

FIGURE 1: Sedimentation velocity profiles of wtr-[Glu^1]Pg in a buffer of 100 mM Tris-Cl/100 mM NaCl, pH 7.8. The position of the protein boundary in the cell was recorded every 8 min for 1.5 h and is shown sequentially for these times in the figure. The inset is a secondary plot of the movement of the midpoints of the protein boundaries as a function of their radial positions in the cell. The $s_{20,app}$ values were calculated from the slopes of these plots.

Table 1: Sedimentation Velocity Parameters for Human Glu^1-Pg Mutants

	$s^0_{20,w}$			
protein	Tris-Cl/NaCl[a]	Tris-Cl/EACA[b]	Tris-OAc/NaOAc[c]	Tris-OAc/EACA[d]
human plasma Pg	5.9	4.9	5.0	4.8
wtr-Glu^1-Pg	5.8	4.8	5.0	4.8
r-[D^{139}N]Glu^1-Pg	4.8	4.8	4.7	4.7
r-[D^{413}N]Glu^1-Pg	4.5	4.6	4.5	4.5
r-[D^{518}N]Glu^1-Pg	5.2	4.8	4.8	4.8
r-[C^{169}D/C^{297}R]Glu^1-Pg	5.8	4.8	4.8	4.7

[a] The buffer was 100 mM Tris-Cl/100 mM NaCl, pH 7.8. [b] The buffer was 100 mM Tris-Cl/100 mM NaCl/100 mM EACA, pH 7.8. [c] The buffer was 100 mM Tris-OAc/100 mM NaOAc, pH 7.8. [d] The buffer was 100 mM Tris-OAc/100 mM NaOAc/100 mM EACA, pH 7.8.

It was imperative in this study to demonstrate that all Pg variants used were present as their Glu^1-Pg forms. The relative ease of removal of the highly labile AP by traces of Pm is problematic, and were such a hydrolytic event to occur during centrifugation, confounding results would be obtained. Results from $DodSO_4$/PAGE analyses under reducing conditions, of the protein samples taken from the centrifuge cell at the conclusion of the experiment clarified this point. The gels obtained are shown in Figure 2. In order to maximize the ease of identification of Glu^1-Pg and Lys^{78}-Pg, the muteins were activated in the presence and absence of the rapid inhibitor of HPm, aprotinin, which does not significantly inhibit the activity of the Pg activator LMW-uPA. As is observed and expected, the Lys^{78}-Pg heavy chain is present in the absence of aprotinin, since the HPm formed catalyzes removal of the 77-residue AP, a step that does not occur when the HPm generated during the activation is rapidly inhibited. The observation of the Glu^1-Pm heavy

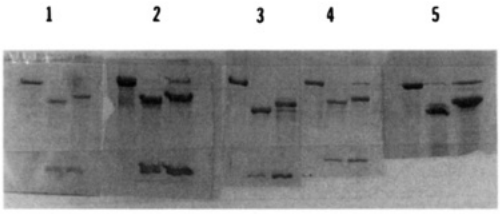

FIGURE 2: $DodSO_4$/PAGE gels of proteins removed from the ultracentrifuge cells at the conclusion of the analyses of Figure 1. Plate 1: left-to-right, wtr-Pg, activation of wtr-Pg in the absence of aprotinin, and activation of wtr-Pg in the presence of aprotinin. Plate 2: as in plate 1, except that r-[$D^{139}N$]Pg was the Pg sample employed. Plate 3: as in plate 1, except that r-[$D^{413}N$]Pg was the Pg sample employed. Plate 4: as in plate 1, except that r-[$D^{518}N$]Pg was the Pg sample employed. Plate 5: as in plate 1, except that r-[$C^{169}D/C^{297}R$]-Pg was the Pg sample employed.

chain in all of the samples proves that at least 90% of each of the Pg samples was present as their respective Glu^1-Pg forms.

DISCUSSION

The purpose of this investigation was to determine which of the ω-amino acid-binding kringles of Glu^1-Pg were responsible for the regulation by positive and negative effectors of the conformation of this zymogen. The strategy employed was to mutate essential amino acids in kringle domains 1, 4, and 5 that were found to be essential for ω-amino acid binding and to examine the conformational properties of the muteins that were generated. In all kringle domains that we have examined to date, an Asp residue present in the first segment of the inner loop of these modules (Asp^{56} in the case of [$K1_{Pg}$]—numbering from the first Cys residue of the kringle) has been found to be essential for ω-amino acid binding, and this was the residue selected for alteration in [$K1_{Pg}$], [$K4_{Pg}$], and [$K5_{Pg}$]. To confirm that no other EACA site of comparable binding energy was present in Glu^1-Pg, we constructed the triple mutant r-[$D^{139}N/D^{413}N/D^{518}N$]-Pg and demonstrated that it did not interact with Sepharose-lysine affinity chromatography resin.

A summary of current knowledge regarding the effector roles of Cl^- and EACA in Pg activation is provided in the following. In the presence of Cl^-, or other anions of this type, Glu^1-Pg exists in the compact T-conformation which is very resistant to activation (Urano et al., 1987a,b). Binding of ω-amino acid ligands, such as EACA, in the presence of these anions places Glu^1-Pg in a highly activatable (Claeys & Vermyelin, 1974; Violand et al., 1978; Urano et al., 1987a,b) and more expanded R-conformation (Brockway & Castellino, 1972; Castellino et al., 1973; Violand et al., 1975, 1978). Lys^{78}-Pg already exists in the R-conformational state (Violand et al., 1975) by virtue of the loss of the AP region that results from its formation; thus there are no significant effects of Cl^- or EACA on the conformation (Violand et al., 1975; Urano et al., 1987a) or the activatability (Claeys & Vermyelin, 1974; Urano et al., 1987a,b) of Lys^{78}-Pg. The anion, acetate, does not bind as strongly to the anion-binding sites on Glu^1-Pg as does Cl^- (Urano et al., 1987b) and therefore does not modulate as effectively the production of the T-state of Glu^1-Pg. Concomitantly, acetate does not inhibit activation of Glu^1-Pg to the same extent as Cl^- at the concentrations employed herein. Thus, acetate can be employed to provide ionic strength while still essentially maintaining the R-state of Glu^1-Pg.

These proposed relationships between the structure, effector response, and activatability of Pg have been in part fortified by a previous investigation, using the same r-Glu^1-Pg mutants described herein (Marshall et al., 1994; Menhart et al., 1995). In that study, the activation rates of Pg were examined in the presence of Cl^- and EACA, in relation to the integrity of the EACA-binding sites in individual kringle regions. We discovered that neither stimulation by EACA nor inhibition by Cl^- took place with r-[$D^{139}N$]Glu^1-Pg ([$K1_{Pg}$] mutant) or r-[$D^{413}N$]Glu^1-Pg ([$K4_{Pg}$] mutant), demonstrating that elimination of the ω-amino acid-binding site in either [$K1_{Pg}$] or [$K4_{Pg}$] of Glu^1-Pg led to a loss of the control features of these two effector molecules. With r-[$D^{518}N$]Glu^1-Pg ([$K5_{Pg}$] mutant), both stimulation by EACA and inhibition by Cl^- were found but to a less striking extent than for wtr-Glu^1-Pg. An interesting facet of the study described was the observation of the much higher activation rates in Cl^- of r-[$D^{139}N$]Glu^1-Pg, r-[$D^{413}N$]rGlu^1-Pg, and, to a lesser extent, r-[$D^{518}N$]Glu^1-Pg, as compared to that of wtr-Glu^1-Pg. These findings demonstrate that the R-state may fully or partially exist in Cl^- for Glu^1-Pg mutants defective in EACA binding, especially in the cases where such a deficiency existed in [$K1_{Pg}$] or [$K4_{Pg}$]. This hypothesis was examined in the current investigation.

The data of Table 1 show that these predictions have been mostly borne out and that excellent correlations exist between the T- and R-states produced by effectors and the activatability of Glu^1-Pg. Diminishment of the EACA-binding site from either the [$K1_{Pg}$] (r-[$D^{139}N$]Glu^1-Pg) or [$K4_{Pg}$] (r-[$D^{413}N$]-Glu^1-Pg) domains in wtr-Glu^1-Pg leads to production of the R-state in this zymogen, even in the presence of Cl^-. Addition of EACA does not significantly alter this conformation. In the case of [$K5_{Pg}$], modulation of the EACA site in this domain (r-[$D^{518}N$]Glu^1-Pg) leads to a partial formation of the T-state in the presence of Cl^- and hydrodynamic expansion to the R-state upon addition of EACA. All of these conformational transitions parallel the higher activatability of plasminogen when present in its R-state. It has been previously suggested from indirect data that the ω-amino acid-binding site in [$K1_{Pg}$] does not regulate the conformation of Glu^1-Pg (Christensen & Molgaard, 1992; Marshall et al., 1994). On the basis of the current work, we cannot offer support for that conclusion. We show that [$K1_{Pg}$] is likely an important determinant of the adoption of the T-state of Glu^1-Pg.

A model of the forces that stabilize the T-state of Glu^1-Pg can be forwarded, along with possible manners of disruption of these forces that lead to its conversion to the R-state. In the presence of Cl^- and like anions, which have binding sites within kringles that are independent of those of ω-amino acids (Urano et al., 1987a; de Vos et al., 1991; Wu et al., 1991, 1994), it is likely that folding to the T-state is determined by the ability of a lysine side chain from one region of the molecule to insert into the binding pocket of a lysine-binding kringle. Additionally, a suitably spaced amino group from a Lys side chain along with a carboxylate from a Glu or Asp side chain could mimic an ω-amino acid ligand for suitable kringle binding sites. Indeed, a peptide region of the AP consisting of Ala^{44}-F-Q-Y-H-S-K-Glu^{51} has been shown to stimulate Glu^1-Pg activation and proposed to

interact with the ω-amino acid-binding pocket of either [$K1_{Pg}$] or [$K4_{Pg}$] (Takada et al., 1993). These intraprotein forces are competitively disrupted when ω-amino acids are included in the medium, and expansion to the R-state results. From the studies herein, we propose that both [$K1_{Pg}$] and [$K4_{Pg}$] are involved in these events, with lesser participation from [$K5_{Pg}$]. There may be a degree of interdependence involved among the kringle binding sites because disruption of the ω-amino acid-binding site in any one of these kringles leads to a complete (in the cases of [$K1_{Pg}$] and [$K4_{Pg}$]) or nearly complete (in the case of [$K5_{Pg}$]) inability of the other ω-amino acid-binding kringles to support the transition to the T-state of Glu^1-Pg in the presence of Cl^- (Table 1). Evidence for such cooperativity in the T- to R-transition in Glu^1-Pg has been presented (Christensen & Molgaard, 1992). That [$K4_{Pg}$] may be the most important determinant of folding characteristics of the T-state is evidenced by the larger expansion of the structure of Glu^1-Pg when the ω-amino acid-binding site is mutated out of [$K4_{Pg}$] (Table 1). The plausibility of such a mechanism is fortified by the finding that the unit cell of crystals of the kringle 2 region of tPA contains three molecules of this domain, stabilized in part by insertion of the Lys^{49} side chain of one kringle into the ω-amino acid-binding site of another (de Vos et al., 1991). The inability of Lys^{78}-Pg to adopt the T-state under any conditions reflects the direct or indirect importance of the AP in these events. This may be due to the need for an essential Lys residue in the AP, or a possible grouping of amino acid side chains in this region that mimics an ω-amino acid, for insertion into a kringle binding pocket or for other key interactions between residues in the AP and kringle domains.

Adoption of this model requires that another point is addressed. Specifically, we believed it important to consider the question as to whether the disulfide bond between Cys^{169} and Cys^{297}, which covalently links [$K2_{Pg}$] and [$K3_{Pg}$], plays a role in providing a structural element necessary for Glu^1-Pg to attain its Cl^--induced T-state, only after which ω-amino acid ligands could cause a conformational alteration to the R-state. This could be the case if such a bond, which is the only known inter-kringle covalent bond found in any protein containing multiple kringle modules, allowed a precursor conformation to be adopted which is needed to place kringles in sufficient proximity for side chain residue pseudoligand insertion into the kringle binding pocket. To examine this question, we prepared a double mutant, r-[$C^{169}D/C^{297}R$]Glu^1-Pg, in which this disulfide bond was disrupted. The data of Table 1 clearly show that such a change did not result in any alteration of the mutant to adopt the T- and R-states in the presence of Cl^- and Cl^-/EACA, respectively. Thus, this inter-kringle disulfide bond does not place Glu^1-Pg in a required conformation for such changes to occur.

In summary, we show herein that anion binding to Glu^1-Pg stimulates the adoption of the T-state of this zymogen, which is highly resistant to activation. It appears as though the ω-amino acid-binding kringles, especially [$K1_{Pg}$] and [$K4_{Pg}$] to a greater extent than [$K5_{Pg}$], mediate this conformational transition by means of their abilities to interact with side chain Lys residues, and/or clusters of side chains mimicking an ω-amino acid structure, through their ligand-binding sites. An alteration to the R-state occurs when these intraprotein interactions are disrupted, leading to a zymogen conformation that is more susceptible to activation. Thus, any situation *in vivo* that isolates populations of Glu^1-Pg from Cl^- in a suitable microenvironment, such as possible binding to cells or other surfaces, should lead to a plasminolytic potential increases in the activation rate of native plasminogen.

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REFERENCES

Brockway, W. J., & Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194−199.
Castellino, F. J. (1981) *Chem. Rev. 81*, 431−446.
Castellino, F. J., Brockway, W. J., Thomas, J. K., Laio, H., & Rawitch, A. B. (1973) *Biochemistry 12*, 2787−2791.
Castellino, F. J., Ploplis, V. A., Powell, J. R., & Strickland, D. K. (1981) *J. Biol. Chem. 256*, 4778−4782.
Christensen, U., & Molgaard, L. (1992) *Biochem. J. 285*, 419−425.
Claeys, H., & Vermyelin, J. (1974) *Biochim. Biophys. Acta 342*, 351−359.
De Serrano, V. S., & Castellino, F. J. (1992a) *Biochemistry 31*, 11698−11706.
De Serrano, V. S., & Castellino, F. J. (1992b) *Biochemistry 31*, 3326−3335.
De Serrano, V. S., & Castellino, F. J. (1993) *Biochemistry 32*, 3540−3548.
De Serrano, V. S., & Castellino, F. J. (1994) *Biochemistry 33*, 1340−1344.
de Vos, A. M., Ultsch, M. H., Kelley, R. F., Padmanbhan, K., Tulinsky, A., Westbrook, M. L., & Kossiakoff, A. A. (1991) *Biochemistry 31*, 270−279.
Forsgren, M., Raden, B., Israelsson, M., Larsson, K., & Heden, L.-O. (1987) *FEBS Lett. 213*, 254−260.
Gonzalez-Gronow, M., Violand, B. N., & Castellino, F. J. (1977) *J. Biol. Chem. 252*, 2175−2177.
Hoover, G. J., Menhart, N., Martin, A., Warder, S., & Castellino, F. J. (1993) *Biochemistry 32*, 10936−10943.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983a) *J. Biol. Chem. 258*, 4249−4256.
Lucas, M. A., Straight, D. L., Fretto, L. J., & McKee, P. A. (1983b) *J. Biol. Chem. 258*, 12171−12177.
Mangel, W. F., Lin, B., & Ramakrishnan, V. (1990) *Science 248*, 69−73.
Marshall, J. M., Brown, A. J., & Ponting, C. P. (1994) *Biochemistry 33*, 3599−3606.
McCance, S. G., Menhart, N., & Castellino, F. J. (1994) *J. Biol. Chem. 269*, 32405−32410.
Menhart, N., Sehl, L. C., Kelley, R. F., & Castellino, F. J. (1991) *Biochemistry 30*, 1948−1957.
Menhart, N., McCance, S. G., Sehl, L. C., & Castellino, F. J. (1993) *Biochemistry 32*, 8799−8806.
Menhart, N., Hoover, G. J., McCance, S. G., & Castellino, F. J. (1995) *Biochemistry 34* (in press).
Miles, L. A., Dahlberg, C. M., & Plow, E. F. (1988) *J. Biol. Chem. 263*, 11928−11934.
Petersen, T. E., Martzen, M. R., Ichinose, A., & Davie, E. W. (1990) *J. Biol. Chem. 265*, 6104−6111.
Robbins, K. C., Summaria, L., Hsieh, B., & Shah, R. J. (1967) *J. Biol. Chem. 242*, 2333−2342.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3*, 191−209.
Takada, Y., Urano, T., & Takada, A. (1993) *Thromb. Res. 70*, 151−159.
Thorsen, S., Clemmensen, J., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668*, 377−387.

Urano, T., Chibber, B. A. K., & Castellino, F. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031−4034.
Urano, T., De Serrano, V. S., Chibber, B. A. K., & Castellino, F. J. (1987b) *J. Biol. Chem. 262*, 15959−15964.
Urano, T., De Serrano, V. S., Gaffney, P. J., & Castellino, F. J. (1988) *Biochemistry 27*, 6522−6528.
Violand, B. N., & Castellino, F. J. (1976) *J. Biol. Chem. 251*, 3906−3912.
Violand, B. N., Sodetz, J. M., & Castellino, F. J. (1975) *Arch. Biochem. Biophys. 170*, 300−305.
Violand, B. N., Byrne, R., & Castellino, F. J. (1978) *J. Biol. Chem. 253*, 5395−5401.
Whitefleet-Smith, J., Rosen, E., McLinden, J., Ploplis, V. A., Fraser, M. J., Tomlinson, J. E., McLean, J. W., & Castellino, F. J. (1989) *Arch. Biochem. Biophys. 271*, 390−399.
Wiman, B., & Wallen, P. (1977) *Thromb. Res. 10*, 213−222.
Wiman, B., Boman, L., & Collen, D. (1978) *Eur. J. Biochem. 87*, 143−146.
Wu, T.-P., Padmanabhan, K., Tulinsky, A., & Mulichak, A. M. (1991) *Biochemistry 30*, 10589−10594.
Wu, T. P., Padmanabhan, K. P., & Tulinsky, A. (1994) *Blood Coagulation Fibrinolysis 5*, 157−166.

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