Articles

Roles of Individual Kringle Domains in the Functioning of Positive and Negative Effectors of Human Plasminogen Activation†

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ABSTRACT: In order to identify the indivudal contributions of the kringle (K) domains of human plasminogen (Pg) to the ϵ-aminocaproic acid (EACA) induced stimulation of Pg activation by low-molecular-weight urokinase-type plasminogen activator (LMW-uPA) and inhibition of this same activation by Cl^-, we constructed the most conservative recombinant- (r-) Pg mutants possible that would greatly reduce the strength of the EACA binding site in the ω-amino acid binding kringles, [$K1_{Pg}$] ([D^{139}→N]r-Pg), [$K4_{Pg}$] ([D^{413}→N]r-Pg), and [$K5_{Pg}$] ([D^{518}→N]r-Pg). In each case, this involved mutation of a critical Asp (to Asn) within these three kringle domains in intact Pg. The three r-mutants were expressed in r-baculovirus-infected lepidopteran insect *(Trichoplusia ni)* cells. In the presence of Cl^-, the positive activation effector, EACA, first stimulated and then inhibited the LMW-uPA-catalyzed initial activation of wild-type (wt) r-[Glu^1]Pg and, to a lesser extent, the [$K5_{Pg}$] mutant, [D^{518}→N/Glu^1]r-Pg. The concentration of EACA that produced 50% stimulation of activation (C_{50}) occurred at 3.3 mM for wtr-[Glu^1]Pg and at 0.7 mM for [D^{518}→N/Glu^1]r-Pg. Subsequent inhibition by EACA occurred with a C_{50} of approximately 15 mM and is likely due to inhibition of the amidolytic activity of plasmin generated during the activation. Similar initial activation rates of both [D^{139}→N]r-Pg and [D^{413}N]r-Pg did not display this initial EACA-mediated stimulatory phase but did undergo ultimate inhibition with a C_{50} for this process that was similar to wtr-[Glu^1]Pg and [D^{518}→N/Glu^1]r-Pg. The initial activation rates of both wtr-[Glu^1]Pg and, to a lesser degree, [D^{518}→N/Glu^1]r-Pg were inhibited by Cl^-, but those same rates of [D^{139}→N]r-Pg and [D^{413}→N]r-Pg were not inhibited by this anion. These results demonstrate that both K1 and K4 contain functionally relevant binding sites for the activation effectors, EACA and Cl^-, respectively, and that the integrity of both of these ligand binding sites is required to produce the EACA-induced stimulation and Cl^--mediated inhibition of [Glu^1]Pg activation. On the other hand, the binding site of ω-amino acids on K5 is not of similar importance, since its elimination only partially affects the influence of these effectors on Pg activation.

Pg[1] is the circulating zymogen of the fibrinogenolytic and fibrinolytic serine protease, Pm, and exists in the circulation as a single-chain glycoprotein containing 791 amino acids (Forsgren et al., 1987). A simple biantennary bisialylated N-linked glycan assembled on Asn^{289} is present on approximately 60% of the Pg molecules of the plasma-derived protein, and an O-linked trisaccharide is contained on Thr^{346} of all molecules (Hayes & Castellino, 1979a−c). Activation of Pg occurs as a result of cleavage of the Arg^{561}−Val^{562} peptide bond in the zymogen (Robbins et al., 1967), yielding a two-chain disulfide-linked enzyme. The catalytic triad of amino acids of plasmin exists at residues His^{603}, Asp^{646}, and Ser^{741}.

Sequence information, such as the placement of introns in the genomic DNA of Pg, as well as amino acid sequence homologies with other proteins, suggests that this protein consists of a series of domains. Five regions of approximately 80 amino acids each are present in the latent heavy chain of Pg (residues 1−561) that share sequence homology to similar modules, termed kringles (Sottrup-Jensen et al., 1978), that are also present in other proteins. The latent light chain of Pg (residues 562−791) is homologous to enzymes of the serine protease superfamily. While this latter polypeptide segment is responsible for the hydro-

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[1] Abbreviations: Pg, a generic abbreviation for human plasminogen; [Glu^1]Pg, native human plasminogen with Glu, residue 1, at the amino terminus; [Lys^{78}]Pg, a proteolytically (plasminolytic) derived form of human plasminogen with Lys, residue 78, at the amino terminus; Pm, a 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}; LMW-uPA, low-molecular-weight urokinase-type plasminogen activator (amino acid residues 159−411); tPA, tissue-type plasminogen activator; [$K1_{Pg}$], the kringle 1 region (amino acid residues C^{84}−C^{162}) of human plasminogen, which consists of amino acid residues C^{84}−C^{162} of the intact protein; [$K2_{Pg}$], the kringle 2 region (residues C^{166}−C^{243}) of human plasminogen; [$K3_{Pg}$], the kringle 3 region (residues C^{256}−C^{333}) of human plasminogen; [$K4_{Pg}$], the kringle 4 region (residues C^{358}−C^{435}) of human plasminogen; [$K5_{Pg}$], the kringle 5 region (residues C^{462}−C^{541}) of human plasminogen; [D^{XX}→N]r-Pg, recombinant human plasminogen containing an Asp to Asn mutation at amino acid sequence position *XX*; EACA, ϵ-aminocaproic acid; S-2251, H-D-Val-Leu-Lys-*p*-nitroanilide; FPLC, fast protein liquid chromatography; NaDodSO$_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; wt, wild type; r, recombinant; *T. ni*, *Trichoplusia ni*.

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lytic activity of Pm, the kringle-containing region contains binding sites for positive and negative effectors of Pg activation (Sottrup-Jensen et al., 1978; Castellino et al., 1981; Urano et al., 1987a) and for cellular receptors of Pg and Pm (Miles et al., 1988). These kringle regions also provide extended binding sites for physiological substrates (Wiman & Wallen, 1977; Thorsen et al., 1981; Lucas et al., 1983) and inhibitors (Wiman et al., 1978) of Pm.

It is well established that [Glu^1]Pg is folded in a highly nonactivatable and compact conformation in the presence of anions such as Cl^-. The conformation of this protein becomes more flexible upon binding of ω-amino acids, leading to its much more rapid activation (Urano et al., 1987a,b). These modulation events require the presence of an intact amino terminus in Pg ([Glu^1]Pg) and are much less pronounced in the molecular form of Pg with a truncated amino terminus ([Lys^{78}]Pg) (Violand et al., 1978; Urano et al., 1987a,b). The exo sites of Pg responsible for regulation of its activation, as well as those for Pm function, are located in individual kringles that provide binding sites for ω-amino acids. In most cases, ω-amino acids, such as EACA, displace Pg and Pm from their binding sites on macromolecular effectors. The significant ω-amino acid binding kringles of Pg are contained in [K1$_{Pg}$] (Sottrup-Jensen et al., 1978), [K4$_{Pg}$] (Sottrup-Jensen et al., 1978), and [K5$_{Pg}$] (Castellino et al., 1981). The [K2$_{Pg}$] domain has been shown to interact with ligands of this type, but the binding constant is not sufficiently strong to have functional consequence, and the [K3$_{Pg}$] module does not measurably interact with ω-amino acids (Marti et al., 1994). It is unclear as to which of the ω-amino acid binding sites are involved in the various functions of Pg that are modulated by their presence. On the basis of indirect studies with intact Pg, it has been proposed that these weaker sites in [K4$_{Pg}$] and [K5$_{Pg}$] are associated with a ligand-induced conformational change in the zymogen (Christensen & Molgaard, 1992). Further, the strong ω-amino acid binding site in [K1$_{Pg}$] has been postulated to function by interacting with macromolecular cofactors and inhibitors of Pg and Pm but not to serve to regulate the conformational properties of Pg (Marshall et al., 1994).

The [K1$_{Pg}$] (Menhart et al., 1991; Hoover et al., 1993) and [K4$_{Pg}$] (Menhart et al., 1993) domains, as well as a number of mutants of these modules, have been expressed, and some general features of their ω-amino acid binding sites have been identified. These advances, coupled with the determination of X-ray crystal structures of [K1$_{Pg}$] (Wu et al., 1994) and of the EACA/[K4$_{Pg}$] complex (Wu et al., 1991), along with high-resolution multidimensional NMR investigations of these domains (De Marco et al., 1986, 1989; Thewes et al., 1987; Rejante & Llinas, 1994), clearly implicate certain amino acid residues common to all ω-amino acid binding kringles in stabilizing ligand/kringle binding. One amino acid residue critical to binding of EACA in all kringles is an Asp residue at sequence positions homologous to Asp^{56} (numbering from the first Cys residue of the kringle) of [K1$_{Pg}$] (Asp^{139} in Pg), and this was the focus of the mutations designed herein.

With firmly grounded knowledge of the manner in which best to eliminate, or greatly reduce, the effectiveness of the ω-amino acid binding site in Pg kringles, while at the same time maintaining protein conformational integrity, we believed that we could directly examine the roles of the individual ω-amino acid binding sites in regulation of Pg activation. Accordingly, we undertook a study of 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. This paper is a summary of the important features of this investigation.

EXPERIMENTAL PROCEDURES

Proteins. Native human plasma [Glu^1]Pg was obtained from Enzyme Research Laboratories (South Bend, IN).

For generation of wtr-Pg and the r-Pg mutants, *T. ni* cells (High Five, Invitrogen, San Diego, CA), maintained as monolayers in serum-free Excell 400 medium (JRH Biosciences, Lexena, KS) at 27 °C, were infected with r-baculoviruses at multiplicities of four plaque-forming units/cell, using infection and cell-handling protocols described earlier (Whitefleet-Smith et al., 1989). The r-baculoviruses containing wtr-Pg, [D^{139}→N]r-Pg, [D^{413}→N]r-Pg, or [D^{518}→N]r-Pg were constructed from cDNA clones of Pg containing these mutations.

After infection of the insect cells with the r-baculoviruses for 72 h, the cell-conditioned media were dialyzed against a buffer of 100 mM sodium phosphate, pH 7.0, and purified by lysine−Sepharose affinity chromatography as described earlier (Whitefleet-Smith et al., 1989), except that FPLC methodology was employed rather than conventional chromatography. After elution with 100 mM EACA, the r-Pg preparations were dialyzed against a solution of 100 mM NH_4HCO_3 and lyophilized.

LMW-uPA was a gift of Abbott Laboratories (North 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 *Bam*HI restriction endonuclease site was inserted in the 5′-nontranslated region of p119/Pg to facilitate transfer of the Pg into the pBlueBacIII (Invitrogen) shuttle vector. The mutagenic oligonucleotide employed for this purpose was ACTTT CTGGG atCcG CTGGC CAGTC C (the mutagenic changes are in lower-case letters, and the location of the restriction site is underlined). All mutations were made from this construct.

The [D^{139}→N] mutation (GAT→AAT, *vide infra*) that abolished the EACA binding site in [K1$_{Pg}$] was accomplished with the following oligonucleotide: 5′-GC AGG AAT CCg GAC AAC aAT CCG CAG GG.

Screening of the bacterial transformants for this mutant Pg gene was facilitated by analysis for the presence of the newly generated restriction site, *Acc*III (underlined).

Similarly, the [D^{413}→N] mutation (GAT→AAT, *vide infra*), which resulted in abolition of the EACA binding site in [K4$_{Pg}$], was incorporated with use of the primer 5′-CCA GAT GCC aAT AAg GGC CCC TGG T, which concomitantly generated a new restriction site for *Apa*I (underlined) in the mutant Pg.

Lastly, the [D^{518}→N] mutation (GAT→AAT, *vide infra*), which eliminated the EACA binding site in [K5$_{Pg}$], was accomplished with the oligonucleotide 5′-CCT GAT GGT aAt GTA GGa GGT CCC TGG, which also allowed screening of the bacterial transformants with the enzyme, *Ppu*MI.

All of these changes were confirmed by DNA sequencing.

Each of these mutants was inserted in the B*am*HI/*Hin*dIII polylinker sites of the plasmid pBlueBacIII and homologously recombined into the baculovirus genome (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. In order to estimate relative quantities of [Glu^1]Pg and [Lys^{78}]Pg in purified Pg samples, activation of the relevant Pg was accomplished in the presence of the rapid Pm inhibitor aprotinin, followed by electrophoretic analysis to evaluate the sizes of the Pm heavy chains produced. Although the differences in molecular masses of [Glu^1]Pg and [Lys^{78}]Pg are the same (*ca.* 8 kDa) as the differences of these molecular forms of the heavy chains of Pm, the lower overall molecular weights of the latter make the electrophoretic separation much greater and thus easier to detect. 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% NaDodSO$_4$/PAGE under reducing conditions (Laemmli, 1970).

Activation Assays of Pg in the Presence of Activation Effectors. To determine the effects of Cl^- on activation of the Pg mutants, solutions of Pg (final concentration, 10 nM) and the Pm chromogenic substrate S-2251 (final concentration, 0.625 mM) were incubated for 5 min in a buffer of 10 mM Na-Hepes/0.1% bovine serum albumin, pH 7.4. A total volume of 105 μL of this solution was added to a number of wells of a 96-well microtiter plate which contained 15 μL of solutions of NaCl/NaOAc. These latter solutions possessed sufficient Cl^- to provide final concentrations of 0, 0.05 mM, 0.5 mM, 10 mM, 20 mM, 50 mM, 80 mM, 100 mM, and 150 mM, with constant ionic strength of 150 mM maintained by appropriate concentration adjustments with NaOAc. To this was added a volume of 30 μL (0.5 nM, final concentration) of LMW-uPA, prepared in a buffer of 10 mM Na-Hepes/0.1% bovine serum albumin, pH 7.4. The mixture was incubated at 37 °C, and the ensuing reaction of the generated Pm with S-2251 was automatically monitored every 9 s for 50 min by measuring the liberated *p*-nitroanilide at 405 nm.

The ability of EACA to reverse the inhibition by Cl^- on each of the mutant Pg preparations was measured in a similar manner. Mixtures of Pg (final concentration, 10 nM) and S-2251 (final concentration, 0.625 mM) at a total volume of 105 μL were incubated for 5 min in the buffer 10 mM Na-Hepes/0.1% bovine serum albumin/100 mM NaCl, pH 7.4. Aliquots (105 μL) of this solution were added to various wells of a 96-well microtiter plate containing 15-μL solutions with sufficient EACA to provide final concentrations of 0, 0.03 mM, 0.1 mM, 0.3 mM, 1 mM, 3 mM, 10 mM, and 30 mM. LMW-uPA (30 μL, 0.5 nM final concentration) was added, and the absorbances were monitored as described above.

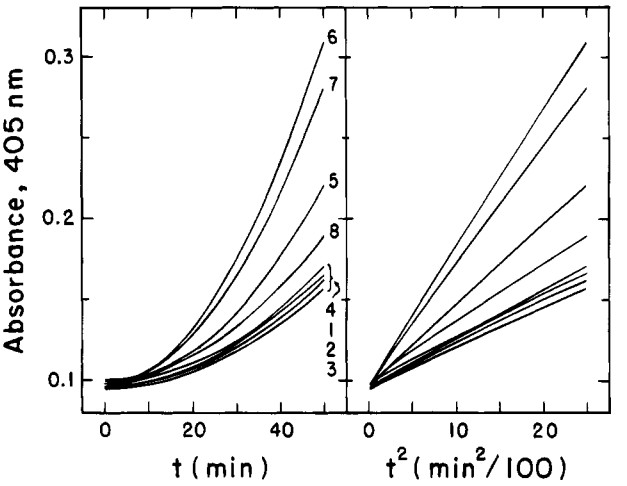

FIGURE 1: Activation rates of Pg by LMW-uPA. (A, left) Human plasma [Glu^1]Pg (10 nM, final concnetration) was activated by LMW-uPA (0.5 nM, final concentration) in the presence of the substrate S-2251 in a buffer consisting of 10 mM Na-Hepes/100 mM NaCl/0.1% bovine serum albumin, pH 7.4 at 37 °C. The Pm generated as a function of time was detected by liberation of *p*-nitroanilide from the substrate, and its concentration is provided by the absorbancy at 405 nm. The different lines are assays conducted in the presence of various concentrations of EACA. The EACA concentrations were (1) 0, (2) 0.03 mM, (3) 0.1 mM, (4) 0.3 mM, (5) 1 mM, (6) 3 mM, (7) 10 mM, and (8) 30 mM. (B, right) The same data plotted as a function of (time)^2.

RESULTS

A series of mutant r-Pgs have been constructed, expressed in insect cells, and purified. These muteins contain a single alteration resulting in greatly diminished ω-amino acid binding properties in each of the kringle domains that interact significantly with ligands of this type. Of importance to this investigation, each r-Pg was purified containing its intact amino terminus as evidenced by examination of the molecular weight of the heavy chain of Pm subsequent to complete activation with LMW-uPA. As observed in NaDodSO$_4$/PAGE, under reducing conditions, the heavy chain of Pm that results from activation of the relevant r-Pg in the presence of the potent Pm feedback inhibitor aprotinin was consistent with the sole (>95%) presence of the [Glu^1] heavy chain, with no observable [Lys^{78}] heavy-chain molecules. We thus conclude that all r-Pgs were full-length molecules.

Comparative activation rates of r-Pg mutants in which the EACA binding site in each of its three ligand kringles, *viz.*, [K1$_{Pg}$], [K4$_{Pg}$], and [K5$_{Pg}$], was individually compromised has been assessed. Figure 1 illustrates the types of primary (A) and secondary (B) data obtained in these experiments. In the example provided, the effect of EACA on the activation rate of [Glu^1]Pg by LMW-uPA was measured as a function of the concentration of EACA in a continuous assay system. The upward curving lines in Figure 1A reflect both the time-dependent generation of Pm and the consumption of a diminishing concentration of the Pm substrate, S2251. Replots of the data according to time^2 (Figure 1B) allow the activation rates of the Pg to be calculated at each EACA concentration. The linearity of these secondary plots demonstrates that [Glu^1]Pg activation is being measured over the times chosen, and other reactions such as the conversion

[2] S. McCance, N. Menhart, and F. J. Castellino, manuscript in preparation.

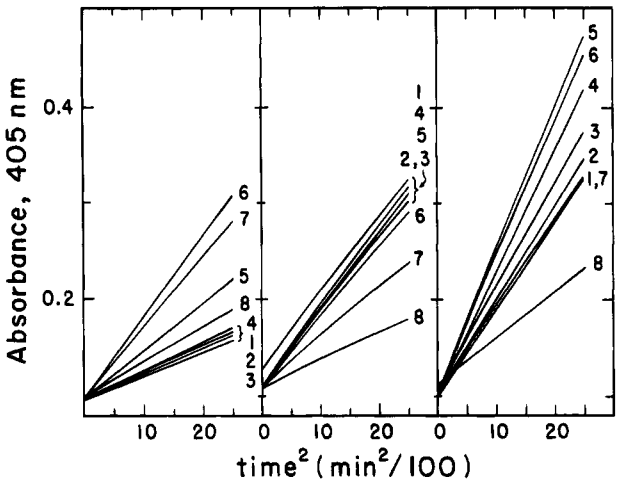

FIGURE 2: Activation rates of r-Pg mutants by LMW-uPA. As in Figure 1B, except that the following Pg samples were employed: (A, left) human plasma [Glu¹]Pg; (B, center) human plasma [Lys⁷⁸]-Pg; (C, right) [D⁵¹⁸→N]r-Pg ([K5Pg] mutant). The EACA concentrations were (1) 0, (2) 0.03 mM, (3) 0.1 mM, (4) 0.3 mM, (5) 1 mM, (6) 3 mM, (7) 10 mM, and (8) 30 mM.

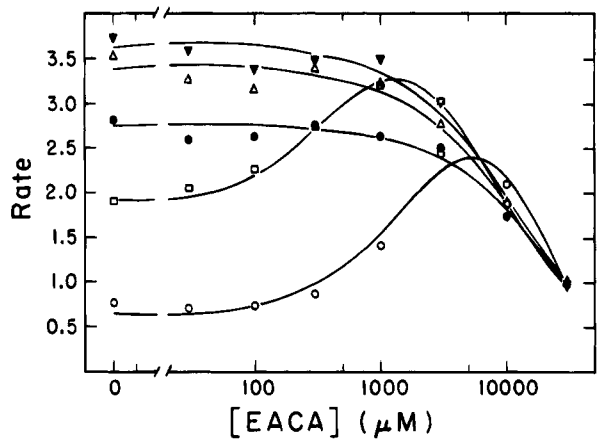

FIGURE 3: Effect of EACA on the initial activation rates of r-Pg mutants by LMW-uPA. The buffer was 10 mM Na-Hepes/100 mM NaCl/0.1% bovine serum albumin, pH 7.4 at 37 °C. The conditions were as in Figure 1. The rates were normalized so that the activation rate at 30 mM EACA was 1.0. Curves: (○) [Glu¹]Pg; (●) [Lys⁷⁸]Pg; (△) [D¹³⁹→N]r-Pg ([K1Pg] mutant); (▼) [D⁴¹³→N]r-Pg ([K4Pg] mutant); (□) [D⁵¹⁸→N]r-Pg ([K5Pg] mutant).

of [Glu¹]Pg to [Lys⁷⁸]Pg are minimal over this period. This was true in all cases reported herein, as shown by the additional examples of Figure 2.

Replots of the type of data of Figures 1 and 2 are presented in Figure 3 for all mutants studied. In the cases of r-[Glu¹]-Pg and, to a lesser extent, the [K5Pg] ([D⁵¹⁸→N]r-Pg) mutant, the data demonstrate that EACA first stimulates the activation and, at higher concentrations, shows inhibitory properties. This initial stimulatory phase is not present in the mutants wherein the EACA sites have been eliminated in the [K1Pg] ([D¹³⁹→N]r-Pg) and [K4Pg] ([D⁴¹³→N]r-PG) domains, nor is it present in [Lys⁷⁸]Pg.

The data of Figure 3 also show that, in the presence of Cl^- and absence of EACA, those mutants that do not exhibit stimulation by EACA, *viz.*, the [K1Pg] ([D¹³⁹→N]r-Pg) and [K4Pg] ([D⁴¹³→N]r-Pg) mutants, as well as [Lys⁷⁸]Pg, also activate more rapidly than those that do display this ultimate stimulation. This is likely due to the fact that Cl^- does not inhibit activation of the [D¹³⁹→N]r-Pg and [D⁴¹³→N]r-Pg mutants. This is verified by the results illustrated in Figure

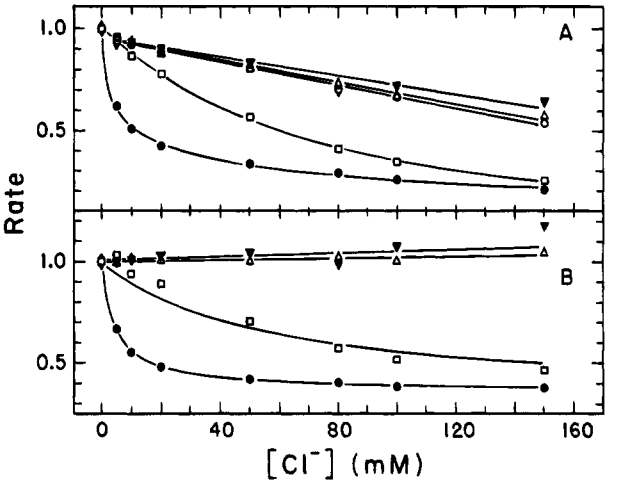

FIGURE 4: Effect of Cl^- on the initial relative activation rates of r-Pg mutants by LMW-uPA. The buffer was 10 mM Na-Hepes/0.1% bovine serum albumin, pH 7.4 at 37 °C. Constant ionic strength was maintained at 150 mM by compensatory addition of NaOAc. The rates were normalized so that the rate at 0 mM Cl^- was 1.0. (A) The following Pg variants were used: (●) [Glu¹]]-Pg; (○) [Lys⁷⁸]Pg; (△) [D¹³⁹→N]r-Pg ([K1Pg] mutant); (▼) [D⁴¹³→N]r-Pg ([K4Pg] mutant); (□) [D⁵¹⁸→N]r-Pg ([K5Pg] mutant). (B) As in (A) except that the rates were normalized to that of [Lys⁷⁸]-Pg, which showed only a small effect of Cl^- on its activation rate. Curves: (○) [Glu¹]Pg; (△) [D¹³⁹→N]r-Pg ([K1Pg] mutant); (▼) [D⁴¹³→N]r-Pg ([K4Pg] mutant); (□) [D⁵¹⁸→N]r-Pg ([K5Pg] mutant).

4, which indicate that, at constant ionic strength, the [K1Pg] ([D¹³⁹→N]r-Pg) and [K4Pg] ([D⁴¹³→N]r-Pg) mutants do not display the Cl^--induced inhibition of activation that characterizes the activation of wtr-Pg ([Glu¹]Pg) and, to a lesser extent, the [K5Pg] ([D¹⁵¹⁸→N]r-Pg) mutant.

DISCUSSION

The objective of this investigation was to show which of the particular ω-amino acid binding sites of [Glu¹]Pg were of importance to the inhibition of its activation by Cl^- and the reversal of this effect by ω-amino acids, such as EACA (Urano et al., 1987a,b). The [K1Pg] (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991; Hoover et al., 1993), [K4Pg] (Lerch et al., 1980; Sehl & Castellino, 1990), and [K5Pg] (Castellino et al., 1981; Novokhatny et al., 1989) domains of Pg, as well as the [K2tPA] (Cleary et al., 1989; De Serrano et al., 1992), all interact with ω-amino acids, as well as with anions such as Cl^- (Urano et al., 1987b; Tulinsky et al., 1988; de Vos et al., 1991; Mulichak et al., 1991). Thus, in order to execute this study, we first needed to selectively remove these ligand binding sites from the intact molecule using the most subtle of mutations that would not be expected to greatly disrupt the conformation of the kringle domains. On the basis of site-directed mutagenesis with r-[K1Pg] (Hoover et al., 1993), r-[K4Pg],[2] and r-[K5Pg],[2] as well as with r-[K2tPA] (De Serrano & Castellino, 1993), a change of Asp to Asn at an amino acid sequence position homologous to Asp⁵⁶ of [K1Pg] would virtually eliminate binding of ω-amino acids to these domains. Specifically, the [D⁵⁶→N] (numbered from the first Cys residue of the relevant kringle) mutant of r-[K1Pg] resulted in a change of the K_d for EACA from 11 μM for wtr-[K1Pg] to 920 μM for the mutant. The same mutations in r-[K4Pg] and r-[K5Pg] altered the K_d values of the wt kringles from 29 and 140 μM, respectively, to approximately 17 mM for the mutants.[2]

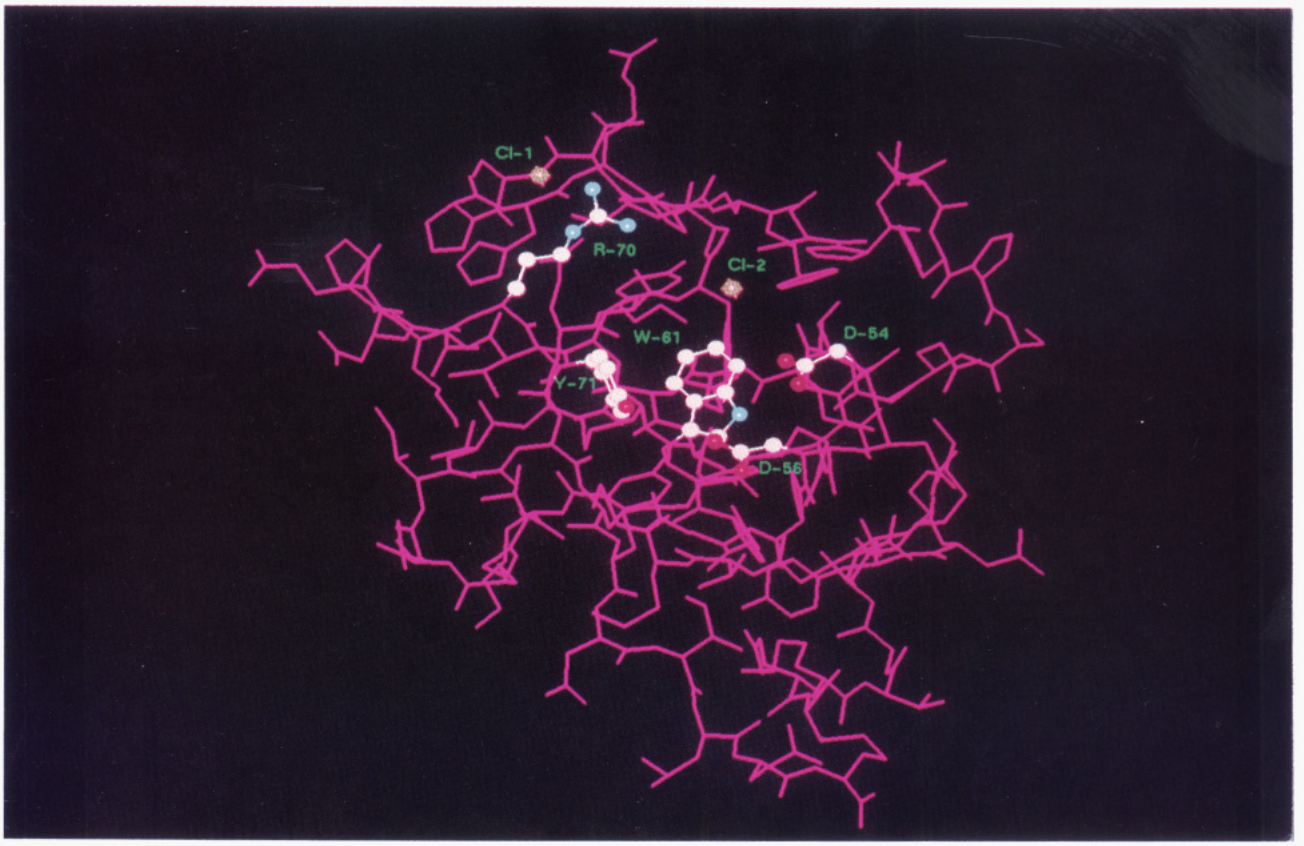

FIGURE 5: Schematic representation of the three-dimensional structure of the [$K1_{Pg}$] (Pg residues 82−164). The magenta skeleton shows the amino acid residues within this structure, with highlighted areas being of specific importance to the current study. The amino acid side chains most likely involved in binding ω-amino acids to this domain are indicated, along with two Cl^- ions crystallized in the unit cell. Color code: red, oxygen; white, carbon; light blue, nitrogen; yellow, Cl^-. Single-letter abbreviations are employed for the amino acid side chains.

A crucial aspect of this study was developing a capability to express r-Pg containing an intact amino terminus, *i.e.*, [Glu^1]Pg. It has long been realized that a polypeptide(s) containing approximately 77 residues is readily hydrolyzed, chiefly by Pm (Violand & Castellino, 1976; Gonzalez-Gronow et al., 1977), from the amino terminus of Pg, providing [Lys^{78}]Pg, a form of the zymogen that is much more easily activated to Pm than [Glu^1]Pg (Claeys & Vermylen, 1974; Violand et al., 1978). Additionally and importantly, [Lys^{78}]Pg also does not undergo the ω-amino acid-mediated conformational alteration characteristic of [Glu^1]Pg that is of such importance to the increase in activation rates of [Glu^1]Pg in the presence of this class of positive activation effectors (Violand et al., 1975). Thus, we chose a lepidopteran insect cell expression system for the purpose of Pg expression, since we have previously demonstrated that an intact [Glu^1]Pg is produced in such cell types (Whitefleet-Smith et al., 1989). While most of our previous studies with r-Pg were performed using *Spodoptera frugiperda* (SF-21AE) cells for expression, we changed here to a *T. ni* (High Five) cell line that has been commercially developed for higher expression levels. Indeed, expression levels increased from approximately 1−2 mg/L to >5 mg/L using this latter cell line. Similar to the r-Pg produced in SF-21AE cells, the r-Pg expressed from the *T. ni* cell line was at least 95% [Glu^1]Pg, as estimated from analysis by reduced $NaDodSO_4$/PAGE of the size of the Pm heavy chain produced upon complete activation of r-Pg in the presence of the Pm feedback inhibitor, aprotinin. This latter agent prevents any Pm-catalyzed release of the amino-terminal polypeptide from Pg or Pm and is an excellent manner of assessing the molecular nature of the r-Pg used as the starting material. We found, however, that with this latter cell line mainly trimannosyl- and fucosylated-trimannosyl-N-linked glycans were present on Asn^{289} of r-Pg, in contrast to the more highly processed oligosaccharides previously found with SF-21AE-expressed r-Pg (Davidson et al., 1990). However, despite the fact that the nature of the glycan assembled on Asn^{289} of Pg does influence somewhat the binding strength of ω-amino acids to this protein (Davidson & Castellino, 1993), these small differences do not affect the major conclusions of this investigation.

Current knowledge concerning the effector roles of Cl^- and EACA in Pg activation can be summarized as follows. In the presence of Cl^-, and other such anions, [Glu^1]Pg exists in a compact conformation which is very resistant to activation (Urano et al., 1987a,b). Binding of ω-amino acid ligands, such as EACA, places [Glu^1]Pg in a more expanded conformation (Brockway & Castellino, 1972; Castellino et al., 1973; Violand et al., 1975) which is highly activatable (Claeys & Vermylen, 1974; Violand et al., 1978; Urano et al., 1987a,b). [Lys^{78}]Pg already exists in this latter conformation (Violand et al., 1975); thus there are no significant effects of Cl^- or EACA on the conformation (Violand et al., 1975; Urano et al., 1987b) or the activatability (Claeys & Vermylen, 1974; Urano et al., 1987a,b) of [Lys^{78}]Pg.

These conclusions can be substantially refined by the findings of the current study. As illustrated in Figure 3, in the absence of EACA and the presence of Cl^-, [Glu^1]r-Pg is activated by LMW-uPA significantly more slowly than

[Lys^{78}]r-Pg. This effect is due to the suppression of activation of [Glu^1]Pg by Cl^-. An even higher activation rate of mutant r-Pgs lacking an ω-amino acid binding site in [$K1_{Pg}$] ([D^{139}→N]r-Pg) and [$K4_{Pg}$] ([D^{413}→N]r-Pg) is observed, strongly suggesting that suppression of the activation rate by Cl^- did not occur in these mutants. In keeping with this finding, and in contrast to the situation with [Glu^1]-Pg, stimulation by EACA also did not take place with these two mutants, nor with [Lys^{78}]Pg, 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 properties of Cl^- and ω-amino acids—the integrity of the ω-amino acid ligand binding site in neither [$K1_{Pg}$] nor [$K4_{Pg}$], alone, was able to support the regulatory properties of these effectors. Thus, it appears as though intact ω-amino acid binding sites in both of these kringle domains are necessary for these types of control mechanisms of activation of [Glu^1]Pg. Another facet of these results is the observation that if this higher activation rate (compared to that of [Glu^{1}Pg]) of the [$K1_{Pg}$] mutant, in the presence of Cl^- and absence of EACA, is correlated to the less compact conformation of this mutant compared to [Glu^1]Pg, a conclusion consistent with all available data on this topic, this would indicate that the ω-amino acid binding site in [$K1_{Pg}$] does in fact regulate the conformation of this protein, in a manner similar to that same site in [$K4_{Pg}$]. This is in contrast to the suggestion that the site in [$K1_{Pg}$] does not play a role in the conformational regulation of [Glu^1]Pg (Marshall et al., 1994). In addition, these results show that only partial loss of these effector-related control properties is noted upon elimination of the ω-amino acid binding site in [$K5_{Pg}$] ([D^{518}→N]r-Pg), since partial suppression and stimulation of activation of this mutant by Cl^- and EACA, respectively, are observed. Finally, in all cases, the inhibition of activation of all Pgs noted at high concentrations of EACA is due to inhibitors of Pm and is not of importance to the major aspects of the current investigation.

The inhibitory properties of Cl^- toward activation of the r-Pg mutants have been more directly evaluated in Figure 4. In support of the interpretation of the data of Figure 3, it is clearly noted that the presence of Cl^- does not inhibit activation of the [$K1_{Pg}$] ([D^{139}→N]r-Pg) and [$K4_{Pg}$] ([D^{413}→N]r-Pg) mutants, partially inhibits the [$K5_{Pg}$] mutant ([D^{1518}→N]r-Pg), and more fully inhibits [Glu^1]r-Pg. These results parallel the findings with EACA-induced stimulation of this same activation, confirming the reciprocity of these events (Urano et al., 1987a).

It has been discovered that the unit cell of the [$K1_{Pg}$] domain (Wu et al., 1994) and the lysine/[$K2_{tPA}$] domain (de Vos et al., 1991) contained Cl^- and that of the EACA/[$K4_{Pg}$] complex contained SO_4^{2-} (Wu et al., 1991). Analysis of these structures along with the current data allows a preliminary molecular interpretation of the interactive modes of Cl^- and EACA functions. A view of the structure of the Cl^-/[$K1_{Pg}$] complex is provided in Figure 5. The ω-amino acid binding site residues that have been identified through mutagenesis studies (Hoover et al., 1993) are highlighted, as well as the two Cl^- ions found in the crystal structure. On the basis of mutagenesis results (Hoover et al., 1993) and through parallels with the mode of binding of these ligands with [$K2_{tPA}$] and [$K4_{Pg}$], which have been crystallized with the ligand binding site occupied (de Vos et al., 1991; Wu et al., 1991), it is most likely that ω-amino acids interact through the amino group of the ligand bound to both Asp^{54} and Asp^{56}, the carboxylate group of the ligand to Arg^{70}, and the methylene backbone of the ligand sandwiched by Trp^{61} and Tyr^{71}. From this model we believe that Cl^--1 could serve to compete with the carboxylate of the ω-amino acid ligand for the cationic component of the binding site (Arg^{70}) due to its proximity to the positively charged center of Arg^{70} (the distance between Cl^--1 and the NH-2 atom of Arg^{70} is only 2.6 Å). The fact that Cl^--2 is not sufficiently proximal to the ω-amino acid binding site to be so affected (and, therefore, probably not displaced by the ligand) could reconcile the findings of the reciprocal effects of Cl^- and EACA as regulators of Pg activation, with a displacement-type mechanism, with the observations from Cl^--NMR that Cl^- and EACA can bind simultaneously to the K1−3 region of Pg (Urano et al., 1987b).

In conclusion, we show herein that the integrity of the ω-amino acid binding sites of both [$K1_{Pg}$] and [$K4_{Pg}$] are required for regulation of [Glu^1]Pg activation by Cl^- and ω-amino acids. The ligand binding site of [$K5_{Pg}$] also partly supports these properties of this zymogen. These results considerably extend previous studies from our laboratory on this topic and begin to reveal the molecular nature of this important control mechanism.

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