Functional Independence of the Kringle 4 and Kringle 5 Regions of Human Plasminogen†

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ABSTRACT: As part of continuing studies to evaluate whether the kringle domain regions of human plasminogen (HPg) exhibit independent conformational properties, simple model systems are required. Toward this end, we have constructed cDNA regions of HPg encoding its kringle 4 ([$K4_{HPg}$]) and kringle 4–5 ([$K4_{HPg}K5_{HPg}$]) regions, expressed these gene fragments in bacterial cells, and purified the recombinant (r) products. The resulting r-[$K4_{HPg}K5_{HPg}$] was also employed to obtain the r-[$K5_{HPg}$] domain of HPg by limited elastolytic digestion of this double-kringle polypeptide. The ω-amino acid ligand binding properties and thermal denaturation characteristics of r-[$K4_{HPg}$], r-[$K5_{HPg}$], and r-[$K4_{HPg}K5_{HPg}$] were determined, along with those for the [$K5_{HPg}$] domain linked to the protease (P) region of HPg ([$K5_{HPg}$]P). This allowed us to evaluate whether the conformational properties of the [$K5_{HPg}$] module were influenced by the presence of its neighboring domains in HPg. The temperature midpoint of maximum heat capacity, T_m (and calorimetric enthalpy, ΔH), for thermal denaturation of r-[$K4_{HPg}$] was 57.8 °C (79.8 kcal/mol) in the absence of ε-aminocaproic acid (EACA) and 70.8 °C (93.7 kcal/mol) in the presence of that ligand. The corresponding values for isolated r-[$K5_{HPg}$] were 50.4 °C (78.4 kcal/mol) and 61.0 °C (89.8 kcal/mol), respectively. These parameters for the isolated kringles were essentially unchanged when these same kringle domains were present in the r-[$K4_{HPg}K5_{HPg}$] and [$K5_{HPg}$]P covalently linked pairs. Similarly, the thermodynamic characteristics (ΔG, ΔH, and ΔS) that describe the binding energy of EACA to r-[$K4_{HPg}$] at 25 °C were −6.3 kcal/mol, −4.5 kcal/mol, and 6.0 eu, respectively. Those for similar binding to r-[$K5_{HPg}$] were −5.3 kcal/mol, −5.8 kcal/mol, and −1.7 eu. Again, no major differences were found in these binding properties for the domains when present in the paired domains, r-[$K4_{HPg}K5_{HPg}$] and [$K5_{HPg}$]-P. These results support the conclusion that these two kringle regions of HPg likely exist as independently folded modules. Thus, meaningful investigations can take place on the structure–function relationships of these kringle domains when isolated from the intact protein. This situation eases the interpretation of results of investigations using intact HPg and allows the assignment of protein properties to discrete regions of the molecule.

Kringles are protein structural motifs initially identified in prothrombin (Magnusson et al., 1975) and human plasminogen (HPg)[1] (Sottrup-Jensen et al., 1978) and subsequently found in a variety of proteases involved in blood coagulation and fibrinolysis (Steffens et al., 1982; Pennica et al., 1983; McMullen & Fujikawa, 1985; McLean et al., 1987). These highly homologous regions contain approximately 80 amino acids and are highly cross-linked by three intrakringle disulfide bonds. Their locations in the non-protease chains of the proteins in which they are present suggest that kringles play a regulatory role in the functions of the relevant proteins. In the cases of HPg and HPm, five kringles exist, some of which are necessary for the binding of these proteins to effector molecules. As examples, certain of the kringle regions of HPg and HPm are responsible for their macroscopic interactions with Cl^- (Urano et al., 1987), with fibrinogen, fibrin, and degradation products of these proteins (Thorsen, 1975; Thorsen et al., 1981; Lucas et al., 1983), with plasma histidine–proline-rich glycoprotein (Lijnen et al., 1980), with thrombospondin (De Poli et al., 1982), with α_2-antiplasmin (Moroi & Aoki, 1976), and with a variety of cells (Miles et al., 1988).

The lysine binding sites of kringles represent an extremely important set of interactions, since lysine and a homologous series of ω-amino acids compete for the binding to kringles of the above macromolecular ligands. Not all kringles interact with ω-amino acids. In the case of HPg, only [$K1_{HPg}$], [$K4_{HPg}$], and [$K5_{HPg}$] display these interactions to a significant degree. The only other kringle domains known to bind to ω-amino acids are [$K2_{tPA}$] and some of the Lp(a) kringles. The strongest ω-amino acid binding site on HPg is on [$K1_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991), with weaker sites present on [$K4_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; De Marco et al., 1987; Novokhatny

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[1] Abbreviations: HPg, human plasminogen; [Glu[1]]Pg, intact human plasminogen containing glutamic acid at its amino terminus; HPm, human plasmin; tPA, tissue-type plasminogen activator; [$K1_{HPg}$], the kringle 1 region (residues C^{84}–C^{162}) of human plasminogen; [$K4_{HPg}K5_{HPg}$], the kringle 4 + kringle 5 region of human plasminogen—the recombinant construct consists of residues V^{354}–P^{544}(V^{354}→S); [$K4_{HPg}$], the kringle 4 region (residues C^{358}–C^{435}) of human plasminogen; [$K5_{HPg}$], the kringle 5 region (residues C^{462}–C^{541}) of human plasminogen (its preparation by limited elastase digestion from recombinant [$K4_{HPg}K5_{HPg}$] yields as a major product V^{443}–N^{791}, with lesser amounts of V^{449}–N^{791} and L^{451}–N^{791}); [$K5_{HPg}$]P (residues V^{462}–N^{791}), region of human plasminogen containing the kringle 5 domain linked to the latent protease (P) domain (V^{562}–Asn^{791}), along with the interconnecting peptide region (its preparation by limited elastase digestion from plasma HPg yields as a major product V^{443}–N^{791}, with lesser amounts of V^{449}–N^{791} and L^{451}–N^{791}); [$K1_{tPA}$], the kringle 1 region (residues C^{84}–C^{162}) of human tissue-type plasminogen activator; [$K2_{tPA}$], the kringle 2 region (residues C^{180}–C^{261}) of human tissue-type plasminogen activator; 5-APnA, 5-aminopentanoic acid; EACA, ε-aminocaproic acid; 7-AHpA, 7-aminoheptanoic acid; AMCHA, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; bp, base pair; r, recombinant; wt, wild type; PCR, polymerase chain reaction; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; DSC, differential scanning calorimetry; DTC, differential titration calorimetry.

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et al., 1989; Sehl & Castellino, 1990) and [$K5_{HPg}$] (Novokhatny et al., 1989; Sehl, 1991).

Most of the available evidence suggests that the kringle regions of HPg are independent domains. From the biological view, each of the five kringles in the HPg gene are flanked by introns (Petersen et al., 1990), leading to the proposal that the HPg gene evolved by independent assembly of kringle exons (Banyai et al., 1983). Chemically, thermodynamic evidence from scanning calorimetric examination of HPg kringles liberated from the molecule by limited proteolysis demonstrated that their folding appears to be independent of the remainder of the protein (Castellino et al., 1981). These conclusions have been supported and extended by the ability to express the cDNAs of [$K1_{HPg}$] (Menhart et al., 1991), [$K1_{tPA}$] (De Serrano et al., 1992), and [$K2_{tPA}$] (Cleary et al., 1989) and by subsequent investigation of their ligand binding and folding properties. On the other hand, some recent evidence has indicated that the kringles may not function completely independently of one another in intact HPg. Here, it has been proposed that the large conformational change in [Glu^1]Pg that accompanies its interaction with ω-amino acid ligands (Brockway & Castellino, 1972; Violand et al., 1975) involves a cooperative interaction of ω-amino acids with both the [$K4_{HPg}$] and [$K5_{HPg}$] domains of the intact protein (Christensen & Molgaard, 1992).

While the ω-amino acid ligand binding properties of r-[$K1_{HPg}$] (Menhart et al., 1991) and [$K4_{HPg}$] (Sehl & Castellino, 1990) have been studied in some detail, only a very limited investigation of this type has been performed for [$K5_{HPg}$] (Novokhatny et al., 1989), the only other ligand binding kringle region of this protein. This has mainly been due to difficulties in discovering the appropriate conditions for its proteolytic release from HPg. Because of this, we have amplified the region of the cDNA for HPg that encodes r-[$K5_{HPg}$] in order to express and purify this module, and subsequently to investigate its folding and ligand binding properties, when present alone and in combination with its neighboring domains of the protein. From this type of study, it could be determined whether significant neighboring domain interactions occurred with this region of the molecule. This communication is a report of the major findings of this study.

MATERIALS AND METHODS

Proteins. Plasma HPg was a gift of Abbott Laboratories (N. Chicago, IL). Since neither [$K5_{HPg}$] nor the protease domain contains glycosylation sites, we did not resolve the HPg glycosylation variants (Brockway & Castellino, 1972) prior to the limited elastase digestions from which we obtained [$K5_{HPg}$]P.

Restriction endonucleases were purchased from the Fisher Scientific Company (Springfield, NJ) and BRL (Gaithersberg, MD). Recombinant Taq DNA polymerase (AmpliTaq) was obtained from Perkin-Elmer Cetus (Norwalk, CT).

Construction of the cDNAs. The [$K4_{HPg}$] and [$K5_{HPg}$] regions from HPg were amplified by PCR using synthetic oligonucleotide primers on a template consisting of the cDNA of HPg. The following three primers were used for the cDNA amplifications (the lower case lettering represents nucleotides that are either not present in or altered from the wt materials): (1) 5′-amino terminal [$K4_{HPg}$], 5′-agatgCatcG GTC CAG GAC-3′; (2) 3′-carboxyl terminal [$K4_{HPg}$], 3′-G AGT CCT TGT aTT Cagctgac-5′; (3) 3′-carboxyl terminal [$K5_{HPg}$], 3′-A CGC CGG GGA AtT cAgCTgAC-5′. An *Nsi*I restriction endonuclease site was included in the 5′-PCR primer. *Sal*I restriction sites and stop codons (TAA) were incorporated into the 3′-PCR primers (the nucleotide sequences of these latter two 3′-PCR primers are complementary to the coding strand of the HPg template).

PCR amplification with primers 1 and 2, followed by restriction endonuclease cleavage with *Nsi*I/*Sal*I, resulted in a cDNA encoding the [$K4_{HPg}$] domain consisting of the amino acid sequence, SVQD[$K4_{HPg}$]SGT, where [$K4_{HPg}$] represents amino acid residues C^{358}–C^{435} of HPg. Similar amplification with primers 1 and 3, followed by *Nsi*I/*Sal*I restriction endonuclease cleavage, resulted in a cDNA encoding the amino acid sequence, SVQD[$K4_{HPg}K5_{HPg}$]CAAP, where [$K4_{HPg}$-$K5_{HPg}$] is the region of HPg corresponding to the first C residue of the K4 domain (C^{358} of HPg) and the last C residue of the K5 domain (C^{541} of HPg). The intervening stretch of amino acids between [$K4_{HPg}$] and [$K5_{HPg}$] that are present in HPg (residues S^{436}–D^{461}) is also contained in this cDNA construct.

These cDNA fragments were ligated into the *Nsi*I/*Sal*I sites of the *Escherichia coli* expression vector, phGH4R, which has been employed for the expression of r-[$K1_{HPg}$] (Menhart et al., 1991) and r-[$K2_{tPA}$] (De Serrano & Castellino, 1992b). This resulted in expression plasmids pSTII[$K4_{HPg}$] and pSTII[$K4_{HPg}K5_{HPg}$]. These constructs were subjected to nucleotide sequence analysis to assure that PCR amplification misreadings did not occur.

Expression and Purification of [$K4_{HPg}$] *and* [$K4_{HPg}K5_{HPg}$]. Expressions of pSTII[$K4_{HPg}$] and pSTII[$K4_{HPg}K5_{HPg}$] were accomplished in *E. coli* DH5α cells, as previously described (Menhart et al., 1991; De Serrano & Castellino, 1992a,b). Prior to purification, the cells were fractionated into the periplasmic and oxidatively refolded fractions (Menhart et al., 1991). Purifications of r-[$K4_{HPg}$] and r-[$K4_{HPg}K5_{HPg}$] from the periplasmic and refolded fractions were accomplished by affinity chromatography and FPLC steps as described previously (Menhart et al., 1991; De Serrano & Castellino, 1992a,b), with the exception that the gradient on the Mono S column was from 0 to 500 mM NaCl in a buffer of 25 mM NaOAc (pH 4.7).

Elastase-Catalyzed Cleavage of r-[$K4_{HPg}K5_{HPg}$]. A solution (3–5 mg/mL) of r-[$K4_{HPg}K5_{HPg}$] in 100 mM sodium phosphate/15 mM EACA (pH 7.8) was digested with elastase at a ratio of 1:250 (w/w) elastase/r-[$K4_{HPg}K5_{HPg}$]. The time course of the digestion was monitored by $DodSO_4$/PAGE. When the reaction was complete (approximately 16 h), the mixture was treated with diisopropyl phosphorofluoridate (1 mM, final concentration), dialyzed against 25 mM NaOAc (pH 4.7), and reapplied to the Mono S column, which was equilibrated and gradient eluted under the same conditions as described above for the purification of r-[$K4_{HPg}K5_{HPg}$]. Purified r-[$K5_{HPg}$] eluted from this column at a NaCl concentration of approximately 80 mM, r-[$K4_{HPg}$] at 150 mM NaCl, and r-[$K4_{HPg}K5_{HPg}$] at 250 mM NaCl.

Isolation of [$K5_{HPg}$]*P from Plasma HPg.* [$K5_{HPg}$]P was isolated from a limited elastase digestion of plasma HPg by affinity chromatography on a column of lysine–Sepharose, essentially as described (Sottrup-Jensen et al., 1978). Typically, a 3–5 mg/mL solution of HPg was digested with 0.03–0.05 mg/mL (final concentrations) elastase at room temperature. After 3 h, an aliquot of the solution was analyzed by $DodSO_4$/PAGE, and the remaining solution was stored frozen. If the gel revealed that the reaction was complete, the stored solution was warmed to room temperature, and diisopropyl phosphorofluoridate was added (final concentration, 2 mM). This solution was then dialyzed against 0.1 M sodium phosphate (pH 8.0) and applied to a 2.5 × 40 cm column of lysine–Sepharose (Brockway & Castellino, 1972).

The column was then washed with 2–3 bed vol of 0.3 M sodium phosphate (pH 8.0). This resulted in the elution of [$K5_{HPg}$]P in a purified state.

Binding of ω-Amino Acids to Kringle-Containing HPg Domains. Both intrinsic fluorescence measurements and DTC were employed for the purpose of measuring the properties of these binding reactions at 25 °C. Our procedures have been detailed previously (Sehl & Castellino, 1990; Menhart et al., 1991; De Serrano & Castellino, 1992b). For all titrations, the buffer employed was 150 mM sodium phosphate, pH 7.0, at 25 °C. Dissociation constants (K_d) characterizing the binding of the ω-amino acids to the kringle-containing domains were calculated from the fluorescence titrations by nonlinear least-squares iterative curve fitting, as described in detail earlier (Menhart et al., 1991).

DTC measurements of the heat changes accompanying the titration of ligand into solutions of the relevant polypeptide domains of HPg were performed as published previously (Sehl & Castellino, 1990; Menhart et al., 1991). A Microcal (Northampton, MA) OMEGA titration calorimeter was used for these measurements. The data were deconvoluted as described (Sehl & Castellino, 1990; Menhart et al., 1991).

Determination of Thermodynamic Parameters of Ligand Binding from Intrinsic Fluorescence and DTC Titrations. Thermodynamic parameters of binding of EACA and AMCHA to each of these kringle-containing fragments were obtained from these measurements. The strategy employed was to determine the K_d values for binding from titrations of the large perturbations of the intrinsic fluorescence of the kringle domains upon ω-amino acid ligand binding (Novokhatny et al., 1989; Kelley et al., 1991; Menhart et al., 1991) and the ΔH values from DTC titrations of the ligands. ΔS was then calculated from the thermodynamic relationships of ΔG, ΔH, and ΔS at 25 °C. While values for n (stoichiometry of binding), K_d, and ΔH could have been obtained from a single DTC experiment, more accurate deconvolution of the DTC titration curve into only the ΔH could be made if two of these parameters were known from other measurements. The value of n (1.0) for each kringle was based on the known stoichiometry of binding of ω-amino acids to kringle domains (Markus et al., 1978a,b), and K_d was determined separately from intrinsic fluorescence titrations.

This procedure also resulted in more accurate K_d values because the low kringle concentrations needed for the fluorescence titrations (~1 μM) assured that the K_d values were relatively insensitive to the kringle concentrations (at low [kringle], $[ligand]_{free} = [ligand]_{total}$). For the DTC experiments, the kringle polypeptides were present at much higher concentrations (~100 μM). These concentrations were higher than the K_d values for most of the ligands, and the K_d values obtained were more sensitive to uncertainties in the [kringle]. However, in no case did the K_d value differ by more than 20% when determined by intrinsic fluorescence or DTC titrations. This same procedure could not be employed for ligand binding analysis of r-[$K4_{HPg}K5_{HPg}$], since it was difficult to resolve the two binding sites by fluorescence titrations. This is due to the fact that the K_d values of most ligands examined for the two kringles differ by less than an order of magnitude and also due to the fact that r-[$K4_{HPg}$] possesses a larger intrinsic fluorescence than r-[$K5_{HPg}$] and a larger ligand-induced fluorescence change than the r-[$K5_{HPg}$] domain. The signal for r-[$K4_{HPg}$] thus dominates, and the K_d value obtained from these titrations was similar to that of r-[$K4_{HPg}$]. In this case, we have obtained the two K_d values from deconvolution of the DTC titrations.

Differential Scanning Calorimetry. DSC experiments were conducted by employing a Microcal MC-2 scanning calorimeter. The samples were first dialyzed against a solution of 150 mM sodium phosphate (pH 7.0) or 100 mM sodium phosphate/50 mM EACA (pH 7.0). Thermal denaturation scans were conducted between the temperatures 25 and 100 °C at scan rates of 60 deg/h. The base line for each run was determined by an identical experiment with the sample buffer placed in each chamber. The temperature of maximum heat capacity (T_m) was obtained directly from the experiment.

Analytical Methods. All of our methods for handling DNA, which include oligonucleotide synthesis, cDNA sequencing, cell transformations, plasmid minipreparations, large-scale plasmid preparations, generation of single-stranded DNAs, and purification of DNA fragments, were performed as described in prior publications (Menhart et al., 1991; De Serrano & Castellino, 1992a,b; De Serrano et al., 1992). Our exact procedures for analysis of the recombinant kringle domains by $DodSO_4$/PAGE (Laemmli, 1970) and amino-terminal amino acid sequence analysis (Chibber et al., 1990) have been published.

RESULTS

The cDNA regions corresponding to [$K4_{HPg}$] and [$K4_{HPg}$-$K5_{HPg}$] were amplified by PCR from the cDNA of HPg and inserted into an *E. coli* expression vector. The steps involved in these constructions are shown in Figures 1 and 2, respectively. After expression, the majority of these materials were found in the periplasmic cell fractions and were readily purified by affinity chromatography on lysine–Sepharose and cation exchange FPLC. From these procedures, we obtained purified r-[$K4_{HPg}$] consisting of HPg residues S^{354}VQD[C^{358}–C^{435}]SGT, where S^{354} is a replacement for the V^{354} found in HPg and all other amino acids are unchanged from those in HPg. Since this altered amino acid was outside of the [$K4_{HPg}$] domain (residues C^{358}–C^{435}), and analysis of the ligand binding properties of this r-[$K4_{HPg}$] (*vide infra*) showed them to be the same as those of [$K4_{HPg}$] isolated by limited proteolysis of HPg (Sehl & Castellino, 1990), we did not realter S^{354} to V. The above procedures also allowed us to acquire r-[$K4_{HPg}$-$K5_{HPg}$], which contained HPg amino acid residues S^{354}VQD-[C^{358}–C^{435}]S^{436}–D^{461}[C^{462}–C^{541}]AAP (the bracketed portions of the sequence are those of [$K4_{HPg}$] and [$K5_{HPg}$], respectively), where again the only alteration from the sequence of HPg is V^{354}→S. The exact amino acid sequence of this construction is provided in Figure 3. In all cases, the fidelity of the amplified cDNAs was assured by nucleotide sequence analysis, and the integrity of translation of these cDNAs was confirmed by amino-terminal amino sequence analysis through 10 amino acids. In the latter cases, these corresponded to the sequences predicted from the cDNA sequence.

Two additional polypeptide materials were obtained for these studies. The fragment, r-[$K5_{HPg}$], was liberated from r-[$K4_{HPg}K5_{HPg}$] by limited elastase digestion. The final material isolated contained heterogeneity at the amino terminus, with V^{443} and L^{451} (0.75:0.25) being found by amino-terminal amino acid sequence analysis at that location (Figure 3). Since these residues were situated upstream of the first C residue of r-[$K5_{HPg}$] and were later discovered to be unimportant differences with regard to the properties described herein, the investigations described in this communication were conducted on this mixture. Finally, another [$K5_{HPg}$]-containing HPg fragment, [$K5_{HPg}$]P (residues V^{443}–N^{791}), was generated and purified from a limited elastolytic digestion of HPg (Sottrup-Jensen et al., 1978). Amino-terminal amino

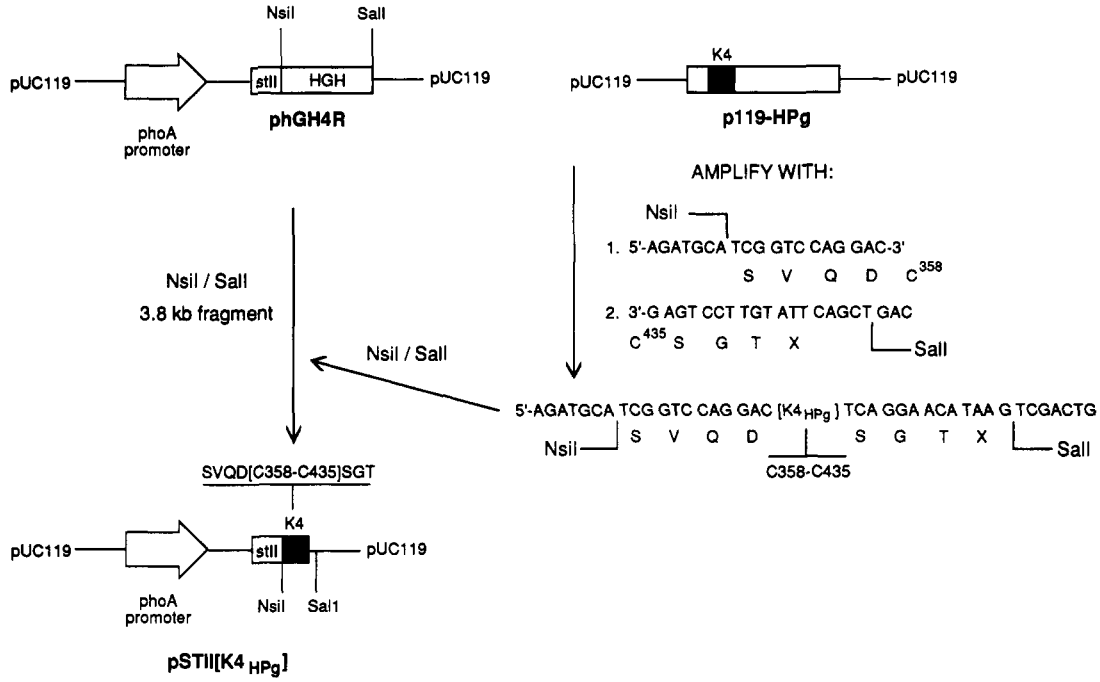

FIGURE 1: Construction of the expression plasmid for r-[$K4_{HPg}$]. A 3.8-kb fragment of an *NsiI*/*SalI* digest of plasmid phGH4R (Chang et al., 1987) was ligated to a *ca.* 0.3-kb fragment of the plasmid p119-HPg. This latter fragment was produced by PCR amplification with oligonucleotide primers 1 and 2 (5′ and 3′, respectively) and digestion with *NsiI*/*SalI*. The resulting plasmid, pSTII[$K4_{HPg}$], was employed as the expression vector in *E. coli* for r-[$K4_{HPg}$]. This expression system yielded the polypeptide fragment, SVQD[$K4_{HPg}$]SGT, where [$K4_{HPg}$] is the amino acid sequence element C^{358}–C^{435}.

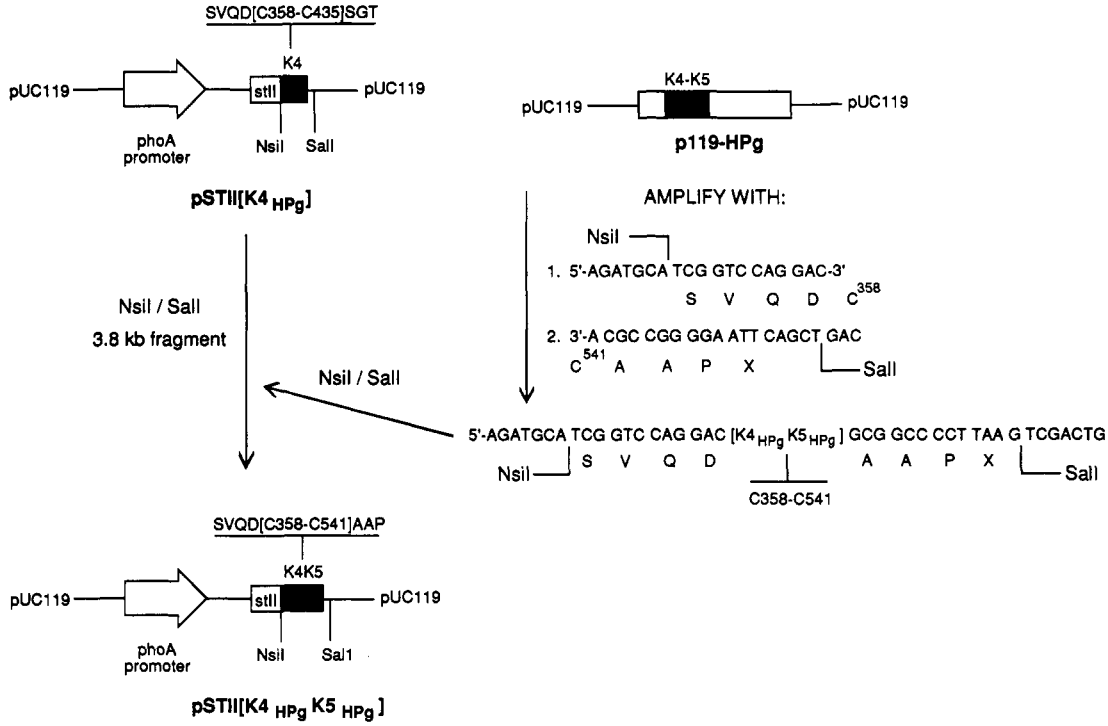

FIGURE 2: Construction of the expression plasmid for r-[$K4_{HPg}K5_{HPg}$] beginning with plasmids pSTII[$K4_{HPg}$] (Figure 1) and p119-HPg (McLean et al., 1987). The details are as for Figure 1. The same 5′ primer was used for the PCR-based amplification of the [$K4_{HPg}K5_{HPg}$] region of HPg, along with a new 3′ primer (primer 2). The expression plasmid, pSTII[$K4_{HPg}K5_{HPg}$], encoded a region of HPg consisting of SVQD[$K4_{HPg}K5_{HPg}$]AAP, where [$K4_{HPg}K5_{HPg}$] is the amino acid sequence element C^{358}–C^{541}.

acid sequence analysis of the final product again showed minor heterogeneity at the amino terminus, with V^{443}, V^{449}, and L^{451} (0.50:0.25:0.25) as amino-terminal amino acid residues (Figure 3). Since all were upstream of the first C residue of r-[$K5_{HPg}$], we did not attempt their resolution.

The characteristics of EACA and AMCHA binding to r-[$K4_{HPg}$] have been determined by intrinsic fluorescence and DTC titrations at 25 °C and compared to previously published values for binding of EACA to [$K4_{HPg}$] obtained from a limited elastolytic digest of plasma HPg (Sehl & Castellino, 1990). The results, shown in Table I, demonstrate that the recombinant material behaves nearly identically to the plasma HPg-derived kringle 4 domain. This shows that the conformation of r-[$K4_{HPg}$], insofar as such folding influences the binding

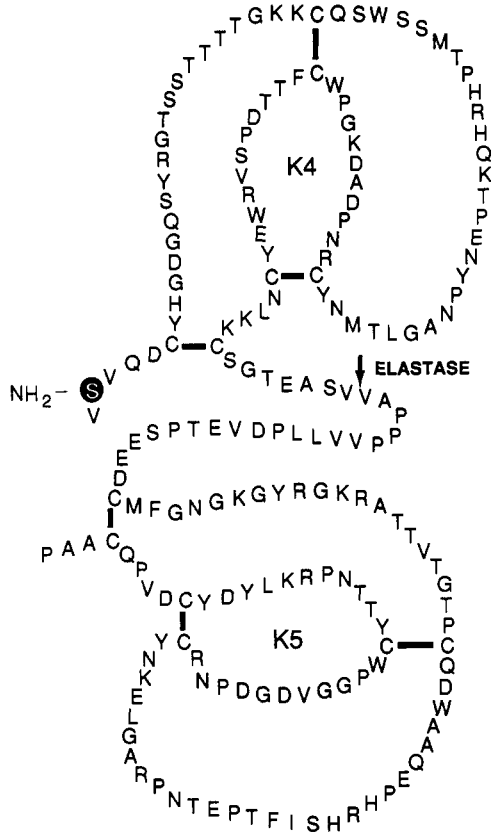

FIGURE 3: Amino acid sequence of the r-[$K4_{HPg}K5_{HPg}$] construction. The major site for elastase-catalyzed cleavage is indicated by the filled arrow.

Table I: Thermodynamic Binding Parameters of EACA and AMCHA to r-[$K4_{HPg}$] and to Human Plasma [$K4_{HPg}$] As Measured by DTC and Intrinsic Fluorescence at 25 °C

		parameter			
polypeptide	ligand	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (eu)
[$K4_{HPg}$][a]	EACA	27	−6.3	−4.5	6.0
	AMCHA	4.9	−7.3	−5.0	7.7
r-[$K4_{HPg}$]	EACA	21	−6.4	−4.5	6.0
	AMCHA	4.0	−7.2	−4.9	7.7

[a] Taken from Sehl and Castellino (1990).

pocket of the polypeptide, is the same as that of the natural material.

These binding measurements were extended to r-[$K5_{HPg}$], r-[$K4_{HPg}K5_{HPg}$], and [$K5_{HPg}$]P. An example of a calorimetric, isothermal (25 °C) titration of r-[$K5_{HPg}$] with AMCHA is provided in Figure 4, and an example of an intrinsic fluorescence titration of r-[$K4_{HPg}$] with AMCHA is illustrated in Figure 5. The thermodynamic characteristics of the binding of EACA and AMCHA to r-[$K5_{HPg}$] and [$K5_{HPg}$]P have been determined and are listed in Table II. The data show that the ω-amino acid binding site on r-[$K5_{HPg}$] is fully retained in [$K5_{HPg}$]P, with only small differences in the ΔH and ΔS values for binding.

Similar studies were conducted for the r-[$K4_{HPg}K5_{HPg}$] fragment. The binding isotherms were deconvoluted into two classes of sites, and the thermodynamic parameters that describe the binding characteristics are listed in Table II. While cumulative errors in the binding constants of each of the classes of sites occur, thereby affecting their absolute values, it is seen that the ω-amino acid binding integrities of r-[$K4_{HPg}$] and r-[$K5_{HPg}$] are reasonably well preserved in the double-

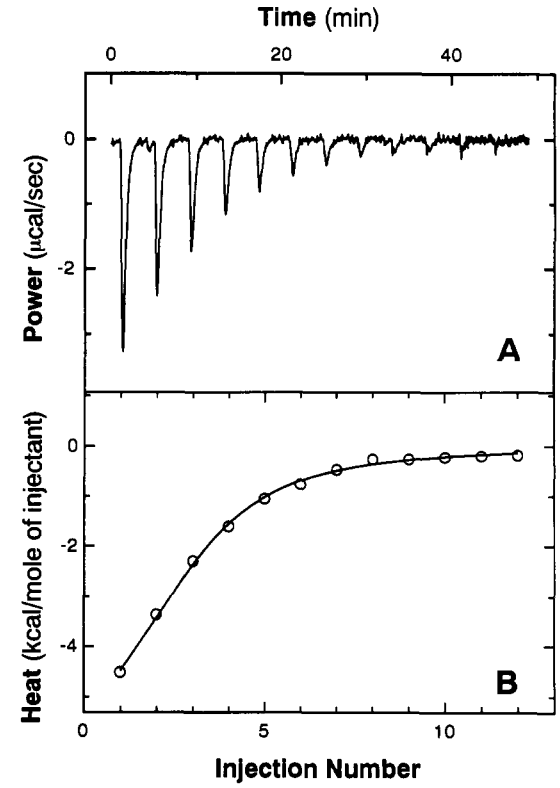

FIGURE 4: DTC analysis of the binding of AMCHA to r-[$K5_{HPg}$]. (A) Incremental heat changes accompanying the titration of r-[$K5_{HPg}$] with AMCHA at 25 °C. A sequence of 3.0-μL injections, from a stock solution (14.4 mM) of AMCHA, in 150 mM sodium phosphate (pH 7.0) was added into the sample cell containing 67 μM r-[$K5_{HPg}$] in this same buffer. Each injection took place over a 3-s time interval, with 4.0 min between injections. The cell volume was 1.394 mL. (B) Peak areas (heats) from above plotted against the injection number. The line is the best fit of the data to a binding isotherm characterized by n = 1.0, K_d = 30 μM, and ΔH = −6.4 kcal/mol.

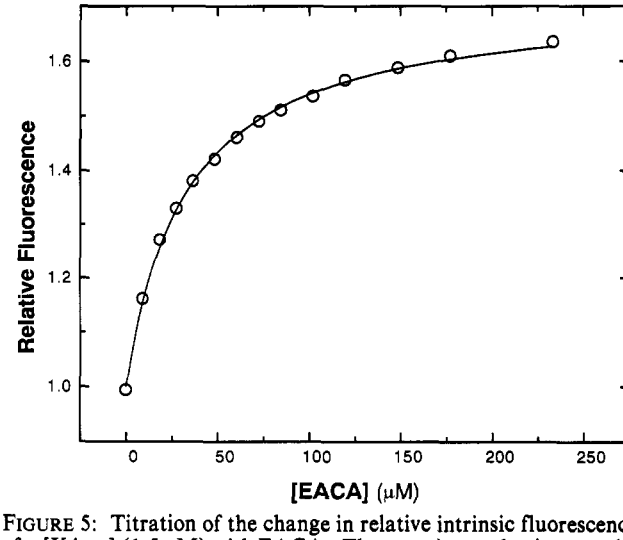

FIGURE 5: Titration of the change in relative intrinsic fluorescence of r-[$K4_{HPg}$] (1.5 μM) with EACA. The experimental points are the best fit to a line characterized by n = 1.0, K_d = 32 μM, and a maximal relative fluorescence change (ΔF_{max}) of 71%. The buffer was 150 mM sodium phosphate (pH 7.0) at 25 °C. The excitation and emission wavelengths were 290 and 340 nm, respectively.

kringle fragment, r-[$K4_{HPg}K5_{HPg}$]. Of additional relevance, as seen from Table III, is that the sums of the enthalpies of binding of EACA and AMCHA, as well as the enthalpies of two other ligands, 5-APnA and 7-AHpA, to r-[$K4_{HPg}$] and r-[$K5_{HPg}$] are close to those determined for r-[$K4_{HPg}K5_{HPg}$]. The additivities of these ΔH values, which are measured

Table II: Thermodynamic Binding Parameters of EACA and AMCHA to r-[$K5_{HPg}$], [$K5_{HPg}$]P, and r-[$K4_{HPg}K5_{HPg}$] As Measured by DTC and Intrinsic Fluorescence at 25 °C

		parameter			
polypeptide	ligand	K_d (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (eu)
r-[$K5_{HPg}$]	EACA	140	−5.3	−5.8	−1.7
	AMCHA	22	−6.3	−6.4	−0.3
[$K5_{HPg}$]P	EACA	205	−5.0	−5.0	0
r-[$K4_{HPg}K5_{HPg}$]	EACA	26	−6.3	−4.9	4.7
		160	−5.2	−6.4	4.0
	AMCHA	7	−7.1	−4.0	10.4
		20	−6.4	−5.9	1.7

Table III: Enthalpies of Binding of ω-Amino Acid Ligands to r-[$K4_{HPg}$], r-[$K5_{HPg}$], and r-[$K4_{HPg}K5_{HPg}$] As Measured by DTC at 25 °C

	ΔH (kcal/mol)			
ligand	r-[$K4_{HPg}$]	r-[$K5_{HPg}$]	r-[$K4_{HPg}K5_{HPg}$]	Σ(4-[$K4_{HPg}$] + [$K5_{HPg}$])
5-APnA	−3.6	−5.5	−9.3	−9.1
EACA	−4.5	−5.8	−11.3	−10.3
7-AHpA	−1.2	−4.6	−5.4	−5.8
AMCHA	−4.9	−6.4	−9.9	−11.3

directly from the DTC titrations, provide support for the independent nature of r-[$K4_{HPg}$] and r-[$K5_{HPg}$] in the r-[$K4_{HPg}K5_{HPg}$] fragment.

DSC measurements were conducted on all of the kringle-containing fragments prepared for this study. These investigations were designed to determine whether the T_m and ΔH values for thermal denaturation of the isolated kringle domains were retained when these kringles were present with adjoining segments of the HPg molecule. Examples of DSC thermograms for r-[$K4_{HPg}$], r-[$K4_{HPg}K5_{HPg}$], and r-[K_5HPg], in the absence and presence of saturating levels of EACA, are illustrated in Figures 6 and 7, respectively. In these cases, deconvolution of the melting transitions resulted in two separate endotherms, with relationships in T_m (Table IV) and ΔH values (Table V) that would be expected from independent melting of r-[$K4_{HPg}$] (Castellino et al., 1981) and r-[$K5_{HPg}$], both in the presence and absence of EACA. The increased thermal stability of these kringle domains in HPg in the presence of EACA has been previously observed (Castellino et al., 1981).

DISCUSSION

The interactions between ω-amino acids and kringle modules have received a great deal of attention due to the importance of protein kringle domains as regulatory elements in the activity of coagulation and fibrinolytic enzymes. ω-Amino acids compete with most of the macromolecular ligands that interact with kringle-containing proteins, thus providing significance to an understanding of the mode of binding of these amino acid effectors to kringle domains. In addition, ω-amino acid interactions with kringles are believed to be of direct importance, since the interaction of HPg, and perhaps tPA, with carboxyl-terminal lysine residues of partially degraded fibrin is thought to play a special role in activation of the clot dissolution pathway. While several proteins involved in clot formation and dissolution contain kringles, only a limited number of these domains interact with ω-amino acids. Those known to possess this property are [$K1_{HPg}$] (Lerch et al., 1980), [$K4_{HPg}$] (Lerch & Rickli, 1980; De Marco et al., 1987; Sehl & Castellino, 1990), [$K5_{HPg}$] (Castellino et al., 1981; Novokhatny et al., 1989), [$K2_{tPA}$] (Cleary et al., 1989; De

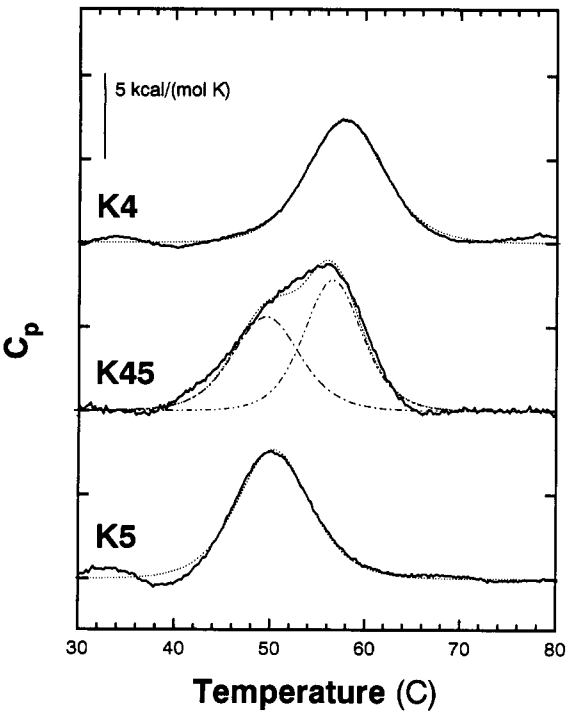

FIGURE 6: DSC thermograms of r-[$K4_{HPg}$] (K4), r-[$K4_{HPg}K5_{HPg}$] (K45), and r-[$K5_{HPg}$] (K5), illustrating the change in heat capacity at constant pressure (ΔC_p) against temperature. The buffer employed was 150 mM sodium phosphate (pH 7.0). The solid lines were derived experimentally and the broken lines are the results from deconvolution of the thermal transitions.

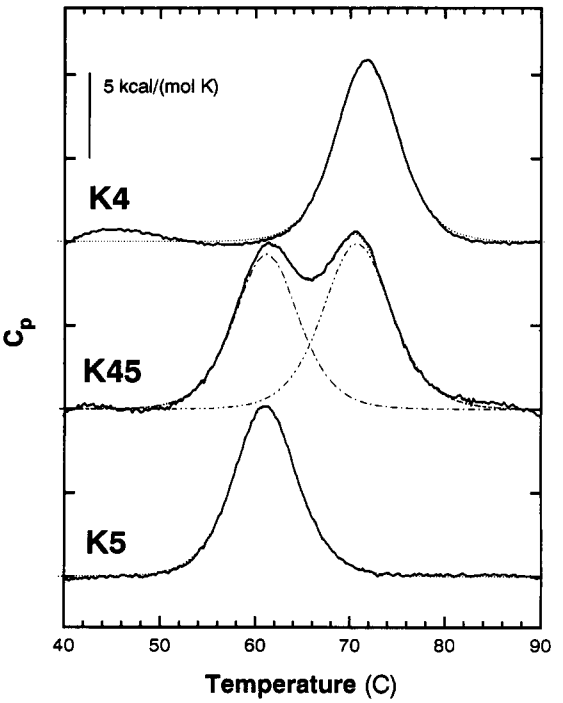

FIGURE 7: Same as for Figure 6, except that the buffer employed was 100 mM sodium phosphate/50 mM EACA (pH 7.0).

Serrano & Castellino, 1992b), and some of the Lp(a) kringles, most of which possess homology to [$K4_{HPg}$] or [$K5_{HPg}$] (McLean et al., 1987). In general, ω-amino acids bind tightest to [$K1_{HPg}$], and the specificity of ligand binding to r-[$K1_{HPg}$] demonstrates that EACA is the tightest binding analogue of a series of simple straight-chain ω-amino acids ranging from 4-aminobutyric acid to 8-aminooctanoic acid (Menhart et al., 1991). This same ligand specificity is observed with r-[$K4_{HPg}$]

Table IV: Temperatures of Maximum Heat Capacities (T_m) for the Thermal Denaturation of r-[$K4_{HPg}$], r-[$K5_{HPg}$], [$K5_{HPg}$]P, and r-[$K4_{HPg}K5_{HPg}$] As Measured by DSC

	T_m (°C)			
ligand	r-[$K4_{HPg}$]	r-[$K5_{HPg}$]	r-[$K4_{HPg}K5_{HPg}$]	[$K5_{HPg}$]P[a]
none	57.8	50.4	49.7, 57.5	51.0
+EACA	70.8	61.0	61.4, 71.7	64.0

[a] The T_m is for the peak assigned to the [$K5_{HPg}$] domain. Another thermal transition is present at T_m = 61 °C, presumably from the protease domain which does not shift upon the addition of EACA (Castellino et al., 1981).

Table V: Enthalpies of Thermal Denaturation of r-[$K4_{HPg}$], r-[$K5_{HPg}$], and r-[$K4_{HPg}K5_{HPg}$] As Measured by DSC

	ΔH (kcal/mol)[a]		
ligand	r-[$K4_{HPg}$]	r-[$K5_{HPg}$]	r-[$K4_{HPg}K5_{HPg}$]
none	79.8 (79.4)	78.4 (79.9)	72.3 (70.5), 49.5 (47.0)
+EACA	93.7 (108)	89.8 (98.4)	83.3 (96.4), 95.0 (95.9)

[a] The calorimetric enthalpy (the van't Hoff enthalpy).

and r-[$K5_{HPg}$], but is altered with r-[$K2_{tPA}$]. In this latter case, 7-AHpA binds to this protein domain more tightly than does EACA.

While isolated kringle domains represent important models to investigate ligand binding characteristics, these domains normally exist in proteins together with additional kringles, as well as with additional protein structural motifs. In order to assess the influence of other structural elements on ligand binding properties of kringle domains, we have examined the ligand binding and folding characteristics of more complex models. In particular, one construction, *viz.*, r-[$K4_{HPg}K5_{HPg}$], indicates [$K5_{HPg}$] flanked on its amino terminus by [$K4_{HPg}$], and another construction, *viz.*, [$K5_{HPg}$]P, indicates [$K5_{HPg}$] flanked on its carboxyl terminal with the P domain of HPg. The properties of the double-domain-containing polypeptide were compared to those of isolated r-[$K4_{HPg}$] and r-[$K5_{HPg}$]. It was expected that results of this investigation would shed light on the issue as to whether the kringle domains could function independently. Previous studies from this laboratory based on calorimetric (Castellino et al., 1981), ligand binding (Cole & Castellino, 1984), and immunological (Ploplis et al., 1982) evidence have demonstrated that the kringle domains of HPg exhibited behavior suggestive of their existence as independent domains in the intact protein. This question is of primary importance to the many investigations that rely on genetic engineering to shuffle kringle domains from one protein to another. Only if these kringles carry full folding information within their primary structures can it be assumed that their effector functions will also be transported into the target protein.

It was first required that we investigate the ligand binding and folding characteristics of the single r-[$K4_{HPg}$] and r-[$K5_{HPg}$] domains. While it is possible to readily obtain [$K4_{HPg}$] from limited elastolytic digests of HPg (Sottrup-Jensen et al., 1978), we nonetheless constructed and expressed a recombinant version because we ultimately desired to perform mutagenesis experiments on this domain. The ligand binding and thermal denaturation properties of r-[$K4_{HPg}$] were compared to those of [$K4_{HPg}$] obtained by limited proteolytic digestion of HPg. As is clear from the data of Table I, the thermodynamic parameters characterizing the binding of EACA and AMCHA to r-[$K4_{HPg}$], which is driven by both enthalpic and entropic contributions, were nearly identical to those obtained for the binding of these same two ligands to [$K4_{HPg}$]. In addition, the T_m of thermal denaturation of r-[$K4_{HPg}$] listed in Table IV is very similar to that of [$K4_{HPg}$] derived from HPg (Castellino et al., 1981). The significant stabilization of the native structure of r-[$K4_{HPg}$] in the presence of EACA has also been found in the case of [$K4_{HPg}$] (Castellino et al., 1981). These data support the conclusion that the folding of [$K4_{HPg}$] is self-directed since its folding properties in the intact HPg structure, as revealed by the integrity of ligand binding and conformational transitional properties, are similar to those of r-[$K4_{HPg}$], which folded independently of the remainder of the HPg structure.

These same properties for isolated r-[$K5_{HPg}$] are readily distinguishable from those of r-[$K4_{HPg}$]. As seen from the binding data of Table II, the K_d values for the interactions of EACA and AMCHA with r-[$K5_{HPg}$] are considerably higher than those for binding of these ligands to r-[$K4_{HPg}$]. This decrease in affinity is mainly due to the more unfavorable binding entropies for r-[$K5_{HPg}$], as compared to those for r-[$K4_{HPg}$]. From the T_m values that characterize the thermal unfolding of r-[$K5_{HPg}$] (Table IV), it is concluded that this latter polypeptide is structurally less stable than r-[$K4_{HPg}$]. Both EACA and AMCHA stabilize the native structure of r-[$K5_{HPg}$], as was the case for r-[$K4_{HPg}$]. It was shown in previous investigations that similar relative structural stabilizations of kringles by these ligands also occurred with other ω-amino acid ligand binding kringles, *viz.*, r-[$K1_{HPg}$] (Menhart et al., 1991) and r-[$K2_{tPA}$] (Kelley & Cleary, 1989; De Serrano & Castellino, 1992b).

In order to evaluate whether [$K5_{HPg}$] could function independently, we investigated ligand binding and thermal stability properties of this kringle module separately linked to its neighboring domains in HPg, *viz.*, [$K4_{HPg}$] and P. In the examination of the ω-amino acid binding properties of the tandem kringle construction, r-[$K4_{HPg}K5_{HPg}$], we were able to deconvolute the titration curve from DTC measurements into two binding events that possess thermodynamic binding constants similar to those of r-[$K4_{HPg}$] and r-[$K5_{HPg}$] when studied independently (Table III). Similarly, the binding parameters for EACA to r-[$K5_{HPg}$] are found in the [$K5_{HPg}$]P fragment (Table III). These studies provide good evidence that [$K5_{HPg}$] acts independently when linked to either [$K4_{HPg}$] or P and also confirm earlier work on the independent folding nature of [$K4_{HPg}$].

In the thermal denaturation studies of these plasminogen fragments, T_m values characteristic of the endothermic transitions of r-[$K4_{HPg}$] and r-[$K5_{HPg}$], when examined separately, are found in the double-kringle construction (Table IV). This suggests the absence of interdomain interactions that might alter the stabilities of [$K4_{HPg}$] and [$K5_{HPg}$]. The sensitivity of this technique to address this question can be derived from studies published on the topic of DSC behavior of a protein with two interacting domains (Wiseman et al., 1989). A model predicting the extent of the shift in the T_m value of the lower melting domain ($\Delta T_{m,a}$) as a function of the interaction energy between domains a and b (ΔG_{ab}) is related by $\Delta T_{m,a} = (T_{m,a}\Delta G_{ab})/\Delta H_a$, where ΔH_a is the enthalpy of thermal unfolding of the lower melting domain and $T_{m,a}$ is its temperature at maximum heat capacity of the thermal transition.

From Tables IV and V, it is seen that the lower melting domain, [$K5_{HPg}$], possesses a T_m of approximately 323 K and a ΔH of approximately 80 kcal/mol. Thus, there is about a 1° shift for every 0.25 kcal/mol of stabilization energy. We could easily detect a $\Delta T_{m,a}$ of 2° and probably less, placing the upper limit of the ΔG_{ab} in both r-[$K4_{HPg}K5_{HPg}$] and

[$K5_{HPg}$]P as <0.5 kcal/mol, a very low value indeed. We did not detect any perceptible shift (<1°) in the T_m of r-[$K5_{HPg}$] in either case, suggesting that r-[$K5_{HPg}$] does not interact with either r-[$K4_{HPg}$] or P to a significant extent. Similar considerations apply to the melting of these domains in the presence of EACA (Table V). In the case of [$K5_{HPg}$]P, there is an approximate 3° shift in the T_m of the [$K5_{HPg}$] module. This may indicate a very weak interaction of approximately 0.75 kcal/mol. However, it should be noted that the DSC scan of [$K5_{HPg}$]P in EACA leads to poorly resolved transitions (Castellino et al., 1981), with concomitant difficulty in assigning T_m values. Thus, some uncertainties are present in this case. The situation is more clear with the r-[$K4_{HPg}K5_{HPg}$] construction.

While the types of data obtained in the present study are consistent with the [$K5_{HPg}$] module functioning independently of its adjacent domains, *viz.*, [$K4_{HPg}$] and P, a recent investigation (Christensen & Molegaard, 1992) has suggested that binding of ω-amino acid ligands to their weak binding sites (presumably [$K4_{HPg}$] and [$K5_{HPg}$]) in [Glu^1]Pg was cooperative. Such cooperativity in these domains, if indeed present, was not detected in these more simple model structures. This suggests that events of this nature involve other parts of the protein molecule.

In conclusion, we believe that it is of fundamental importance to assess the independence of domains in multidomain proteins in order to ascribe properties of proteins to their component regions. This is especially true in kringle-containing proteins, where a vast number of studies employing kringle fragments, kringle deletions, and kringle insertions continue to take place. In the case of HPg, while it cannot at this stage be proven conclusively that the kringles are truly independent modules, we have no evidence to date suggesting that the individual kringle conformations in the intact protein depend upon interactions with other regions of the molecule. Regarding the particular studies contained in this communication, while there may well be interactions of [$K4_{HPg}$] and [$K5_{HPg}$] with other regions of HPg, such interactions, if present, do not seem to be of much functional significance to the general folding properties of these two domains.

REFERENCES

Banyai, L., Varadi, A., & Patthy, L. (1983) *FEBS Lett. 163*, 37–41.

Brandts, J. F., Hu, C. Q., Lin, L.-N., & Mas, M. T. (1989) *Biochemistry 28*, 8588–8596.

Brockway, W. J., & Castellino, F. J. (1972) *Arch. Biochem. Biophys. 151*, 194–199.

Castellino, F. J., Ploplis, V. A., Powell, J. R., & Strickland, D. K. (1981) *J. Biol. Chem. 256*, 4778–4782.

Chang, C. N., Rey, M., Bochner, B., Heyneker, H., & Gray, G. (1987) *Gene 55*, 189–196.

Chibber, B. A. K., Urano, S., & Castellino, F. J. (1990) *Int. J. Pept. Protein Res. 35*, 73–80.

Christensen, U., & Molgaard, L. (1992) *Biochem. J. 285*, 419–425.

Cleary, S., Mulkerrin, M. G., & Kelley, R. F. (1989) *Biochemistry 28*, 1884–1891.

Cole, K. R., & Castellino, F. J. (1984) *Arch. Biochem. Biophys. 229*, 568–575.

De Marco, A., Petros, A. M., Laursen, R. A., & Llinas, M. (1987) *Eur. Biophys. J. 14*, 359–368.

De Poli, P., Bacon-Baguley, T., Kendra-Franczak, S., Cederholm, T. S., & Walz, D. A. (1982) *Blood 73*, 361–368.

De Serrano, V. S., & Castellino, F. J. (1992a) *Biochemistry* (in press).

De Serrano, V. S., & Castellino, F. J. (1992b) *Biochemistry 31*, 3326–3335.

De Serrano, V. S., Menhart, N., & Castellino, F. J. (1992) *Arch. Biochem. Biophys. 294*, 282–290.

Kelley, R. F., & Cleary, S. (1989) *Biochemistry 28*, 4047–4054.

Kelley, R. F., DeVos, A. M., & Cleary, S. (1991) *Proteins: Struct., Funct., Genet. 11*, 35–44.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lerch, P. G., & Rickli, E. E. (1980) *Biochim. Biophys. Acta 625*, 374–378.

Lerch, P. G., Rickli, E. E., Lergier, W., & Gillessen, D. (1980) *Eur. J. Biochem. 107*, 7–13.

Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255*, 10214–10222.

Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258*, 4249–4256.

Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., & Claeys, H. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) pp 123–149, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Markus, G., DePasquale, J. L., & Wissler, F. C. (1978a) *J. Biol. Chem. 253*, 727–732.

Markus, G., Evers, J. L., & Hobika, G. (1978b) *J. Biol. Chem. 253*, 733–739.

McLean, J. W., Tomlinson, J. E., Kuang, W.-j., Eaton, D. L., Chen, E. Y., Gless, G. M., Scanu, A. M., & Lawn, R. M. (1987) *Nature (London) 330*, 132–137.

McMullen, B. A., & Fujikawa, K. (1985) *J. Biol. Chem. 260*, 5328–5341.

Menhart, N., Sehl, L. C., Kelley, R. F., & Castellino, F. J. (1991) *Biochemistry 30*, 1948–1957.

Miles, L. A., Dahlberg, C. M., & Plow, E. F. (1988) *J. Biol. Chem. 263*, 11928–11934.

Moroi, M., & Aoki, N. (1976) *J. Biol. Chem. 251*, 5956–5965.

Novokhatny, V. V., Matsuka, Y. V., & Kudinov, S. A. (1989) *Thromb. Res. 53*, 243–252.

Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., & Collen, D. (1983) *Nature (London) 301*, 214–221.

Petersen, T. E., Martzen, M. R., Ichinose, A., & Davie, E. W. (1990) *J. Biol. Chem. 265*, 6104–6111.

Ploplis, V. A., Cummings, H. S., & Castellino, F. J. (1982) *Biochemistry 21*, 5891–5897.

Sehl, L. C. (1991) Ph.D. Dissertation, University of Notre Dame, Notre Dame, IN.

Sehl, L. C., & Castellino, F. J. (1990) *J. Biol. Chem. 265*, 5482–5486.

Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinol. Thrombol. 3*, 191–209.

Steffens, G. J., Gunzler, W. A., Otting, F., Frankus, E., & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 1043–1058.

Thorsen, S. (1975) *Biochim. Biophys. Acta 393*, 55–65.

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. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4031–4034.

Violand, B. N., Sodetz, J. M., & Castellino, F. J. (1975) *Arch. Biochem. Biophys. 170*, 300–305.

Wiseman, T., Williston, S., Brandts, J. F., & Lin, L.-N. (1989) *Anal. Biochem. 179*, 131–137.