Amino Acids of the Recombinant Kringle 1 Domain of Human Plasminogen That Stabilize Its Interaction with ω-Amino Acids†

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ABSTRACT: A series of strategically designed recombinant (r) mutants of the kringle 1 region of human plasminogen ([$K1_{HPg}$]) have been constructed and the resulting gene products employed to reveal the identities of the residues that contribute to stabilization of the binding of ω-amino acid ligands to this domain. On the basis of determinations of the binding constants of the ligands, 6-aminohexanoic acid and *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid, to a variety of these mutants, we find that the anionic site of the polypeptide responsible for stabilization of the amino group of the ligands consists of both D^{54} and D^{56} and the cationic site of the polypeptide that interacts with the carboxylate group of the ligand is composed solely of R^{70}. The main hydrophobic interactions that stabilize binding of these ligands, likely by interactions with the ligand hydrophobic regions, are principally due to W^{61}, Y^{63}, and Y^{71}. The results obtained are consistent with conclusions that could be made from analysis of the X-ray crystal structure of r-[$K1_{HPg}$] and from previous studies from this laboratory regarding the binding of ligands of this type to the kringle 2 region of tissue-type plasminogen activator ([$K2_{tPA}$]). It thus appears as though a common ligand binding site has evolved in different kringles with ligand specificity differences between r-[$K2_{tPA}$] and r-[$K1_{HPg}$] perhaps explainable by the different nature of the cationic sites on these polypeptides that are involved in coordination to the ligand carboxylate groups.

Several proteins involved in blood coagulation and fibrinolytic pathways contain discrete modules known as kringles. These homologous protein regions consist of approximately 80 amino acids, and all contain three disulfide bonds in rigidly conserved locations. This structural motif was originally identified in prothrombin (Magnusson et al., 1975) and later discovered in the noncatalytic chains of a variety of other proteases involved in these pathways. The number of kringle domains found in proteins is variable: one such region has been identified in urokinase-type plasminogen activator (Steffens et al., 1982), vampire bat plasminogen activator (Gardell et al., 1989), and coagulation factor XII (McMullen & Fujikawa, 1985); two have been discovered in prothrombin (Magnusseon et al., 1975) and tPA[1] (Pennica et al., 1983); four have been located in human hepatocyte growth factor (Nakamura et al., 1989) and human (Han et al., 1991) and mouse (Degen et al., 1991) growth factor-like proteins; five exist in various species of plasminogen (Sottrup-Jensen et al., 1978; Schaller et al., 1985; Degen et al., 1990); and 10 and 38 kringle regions, respectively, occur in the cloned versions of the rhesus monkey (Tomlinson et al., 1989) and human (McLean et al., 1987) apolipoprotein (*a*) genomic variants.

It is generally accepted that kringles play regulatory roles in the functions of the proteins in which they have been identified. In the case of HPg, evidence suggests that the kringle regions interact with certain HPg activation effectors, *viz.*, Cl^- (Urano et al., 1987) and ω-amino acids (Sottrup-Jensen et al., 1978). In addition, it is of functional consequence that HPg and HPm interact with fibrinogen, fibrin, and plasminolytic cleavage fragments of these proteins (Thorsen, 1975; Thorsen et al., 1981; Lucas et al., 1983a,b), as well as with plasma histidine–proline-rich glycoprotein (Lijnen et al., 1980), thrombospondin (De Poli et al., 1982), α_2-antiplasmin (Moroi & Aoki, 1976), and a variety of cell surfaces (Miles & Plow, 1987; 1988; Miles et al., 1988, 1991). The ω-amino acid binding sites of kringles are at least partially involved in these interactions, since most of the above interactions are inhibited by ω-amino acids, such as lysine and EACA.

From the above considerations, it is clear that detailed knowledge of the nature of the binding site within the kringle domains for ω-amino acids is highly relevant to an understanding of their structure–function relationships. Of additional significance are the observations that, despite the high levels of homology between various kringles, they do not equally interact with ω-amino acids. Thus, by nature of their differing abilities to bind to ω-amino acids, kringle domains profoundly influence the properties of the proteins in which they are found.

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[1] Abbrevations: HPg, human plasminogen; HPm, human plasmin; MPg, mouse plasminogen; tPA, tissue-type plasminogen activator; fXa, bovine coagulation factor Xa; [$K1_{HPg}$], the kringle 1 region (residues C^{84}–C^{162}) of human plasminogen (McLean et al., 1987); [$K1_{BPg}$], the kringle 1 region (residues C^{84}–C^{162}) of bovine plasminogen (Schaller et al., 1985); [$K1_{MPg}$], the kringle 1 region (residues C^{84}–C^{162}) of mouse plasminogen (Degen et al., 1990); [$K1_{PPg}$], the kringle 1 region (residues C^{84}–C^{162}) of porcine plasminogen (Schaller et al., 1987); [$K1_{RPg}$], the kringle 1 region (residues C^{84}–C^{162}) of rhesus monkey plasminogen (Tomlinson et al., 1989); [$K4_{HPg}$], the kringle 4 region (residues C^{358}–C^{435}) of human plasminogen (McLean et al., 1987); [$K5_{HPg}$], the kringle 5 region (residues C^{462}–C^{541}) of human plasminogen (McLean et al., 1987); [$K2_{tPA}$], the kringle 2 region (residues C^{180}–C^{261}) of human tissue-type plasminogen activator (Pennica et al., 1983); 4-ABuA, 4-aminobutyric acid; 5-APnA, 5-aminopentanoic acid; EACA, ε-aminocaproic acid; 7-AHpA, 7-aminoheptanoic acid; 8-AOcA, 8-aminooctanoic acid; AMCHA, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; bp, base pair; r, recombinant; wt, wild type; PCR, polymerase chain reaction; NaDodSO$_4$/PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; DSC, differential scanning calorimetry; DTC, differential titration calorimetry.

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In the case of HPg, only [$K1_{HPg}$], [$K4_{HPg}$], and [$K5_{HPg}$] display these interactions to a significant degree, with the strongest binding energies occurring with the [$K1_{HPg}$] module (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991). On the basis of molecular modeling of various kringle domains (Tulinsky et al., 1988) and extended by X-ray crystallographic analysis of the [$K4_{HPg}$]/EACA complex (Wu et al., 1991), it is proposed that the binding site for ω-amino acids on kringles consists of a hydrophobic channel that contains residues that stabilize interactions with the aliphatic backbone of these ligands, with anionic and cationic sites at either end of this channel, these latter of which interact with charged head groups of the ligands (Tulinsky et al., 1988). In an attempt to define the importance of particular amino acid residues in [$K1_{HPg}$] involved in interactions with ω-amino acid ligands, we have mutated those residues that seemed most likely to contribute to these interactions on the basis of known predictions from NMR (De Marco et al., 1982; Motta et al., 1987) and X-ray crystallographic investigations[2] of r-[$K1_{HPg}$] and from mutagenesis studies with r-[$K2_{tPA}$] (Kelley & Cleary, 1989; De Serrano & Castellino, 1992a,b, 1993). The effects of these mutations on the ω-amino acid binding properties of the resulting r-[$K1_{HPg}$] variants were then studied in order to more exactly define the relative importance of the various primary structural elements of this polypeptide in ligand binding. This paper is a summary of the findings of this investigation.

MATERIALS AND METHODS

Proteins. Restriction endonucleases were purchased from the Fisher Scientific Co. (Springfield, NJ) and BRL (Gaithersberg, MD). Recombinant Taq DNA polymerase (AmpliTaq) was obtained from Perkin-Elmer Cetus (Norwalk, CT). Bovine factor Xa was provided by Enzyme Research Laboratories (South Bend, IN).

Construction of the cDNAs for Expression of wtr-[$K1_{HPg}$] and Its Variants. The cDNA for wtr-[$K1_{HPg}$] was excised from the cDNA of HPg and ligated into the *Nsi*I/*Sph*I sites of the *Escherichia coli* expression vector phGH4R as described earlier (Menhart et al., 1991). This resulted in expression plasmid pSTII[$K1_{HPg}$]. Some of the r-[$K1_{HPg}$] variants were prepared by *in vitro* site-directed mutagenesis using synthetic oligonucleotides with the template pSTII[$K1_{HPg}$].

Construction of Plasmid p[$K4_{HPg}XaK1_{HPg}$]. In several cases, especially those in which it might be reasonably predicted that the ligand binding site of r-[$K1_{HPg}$] might be disrupted, it was desirable for purification of r-[$K1_{HPg}$] variants to fuse these variants to a kringle region of HPg that strongly interacted with insolubilized ω-amino acid columns. The r-[$K4_{HPg}$] domain was found to be a satisfactory fusion partner.

Several steps were required to produce a suitable fusion vector. First, the [$K4_{HPg}K5_{HPg}$] region of HPg was amplified from plasmid p119-HPg (McLean et al., 1987) and inserted in plasmid phGH4R (Menhart et al. 1991) by PCR. This provided plasmid pSTII[$K4_{HPg}K5_{HPg}$], which encoded the amino acid sequence NH_2-SVQD[$K4_{HPg}K5_{HPg}$]CAAP. The intervening stretch of amino acids between [$K4_{HPg}$] and [$K5_{HPg}$] that is present in HPg (residues S^{436}–D^{461}) is also contained in this cDNA construct. A full description of the steps used in construction of this plasmid has been provided (Menhart et al., 1993).

[2] The coordinates for the r-[$K1_{HPg}$] crystal structure have been provided to this laboratory by Dr. Alexander Tulinsky (East Lansing, MI). A complete manuscript detailing this structure is under preparation by that laboratory.

A fXa-specific cleavage site (in boldface) was created between the [$K4_{HPg}$] and [$K5_{HPg}$] domains in the translated product by site-directed mutagenesis on a template of pSTII-[$K4_{HPg}K5_{HPg}$]. The primer employed was 5′-TGC TCA GGA **AtA GAA GgG AGa** GTT Gat GCA tT CCG CCT GTT G, which begins at the last C-residue of [$K4_{HPg}$] (C^{435} of HPg). An *Nsi*I restriction site (underlined) was also inserted just downstream of the fXa cleavage site in order to facilitate construction of the fusion proteins. These steps yielded the plasmid pSTII[$K4_{HPg}$]Xa[$K5_{HPg}$]. A previously existing *Nsi*I site in the 5′ region of [$K4_{HPg}$] was removed, without affecting the amino acid sequence, from this latter plasmid by oligonucleotide-directed mutagenesis, using the synthetic oligonucleotide primer 5′-ATGCCTAcGCgTCGGTCCAG. This provided the vector pSTII[$K4_{HPg}$]Xa[$K5_{HPg}$]ΔN.

A 4-kbp fragment produced by digestion of this vector with *Nsi*I/*Sph*I (the *Sph*I site is present at the end of the [$K5_{HPg}$] region; Figure 1) was then ligated to a 0.3-kbp *Nsi*I/*Sph*I fragment encoding the [$K1_{HPg}$] region of the plasmid pSTII-[$K1_{HPg}$]. This resulted in the plasmid pSTII[$K4_{HPg}XaK1_{HPg}$], which was used as the template with synthetic mutagenic oligonucleotides for production of several of the mutant r-[$K1_{HPg}$] variants.

The r-polypeptide produced by this plasmid was NH_2-SVQD[$K4_{HPg}$]SGIEGR▼VDASE[$K1_{HPg}$]D-COOH, which after cleavage with fXa (at ▼) yielded a r-[$K1_{HPg}$] fragment, NH_2-VDASE[$K1_{HPg}$]D-COOH.

Construction of the cDNA for Expression of wtr-[$K1_{MPg}$]. Plasmid MP33B, containing a cDNA fragment from MPg encoding amino acid residues −18 to 505 inserted at the *Eco*RI site of pBR322 (Degen et al., 1990), was obtained from Dr. Sandra Degen (Cincinnati, OH). This plasmid was used as the template for a PCR reaction to obtain the cDNA for [$K1_{MPg}$]. The 5′-coding PCR primer was 5′-TGT-ATgcaTCAGAATGTAAG, which also incorporated a *Nsi*I restriction site (underlined) two amino acid residues upstream of C^1 of [$K1_{MPg}$]. The 3′-PCR anticoding primer was 3′-CACTTCTCCTTAttcgtaCgccg, which also introduced a stop codon four amino acid residues downstream of C^{79} of wtr-[$K1_{MPg}$] as well as a *Sph*I restriction site (underlined).

After digestion of the 0.3-kbp PCR product with *Nsi*I/*Sph*I, followed by ethanol precipitation, this fragment was ligated into the 4-kbp fragment of the plasmid pSTII[$K4_{HPg}$-$XaK1_{HPg}$], similarly digested with *Nsi*I/*Sph*I. This allowed construction of a gene encoding the following double kringle-containing polypeptide: NH_2-SVQD[$K4_{HPg}$]SGIEGR▼-DKASE[$K1_{MPg}$]EEE-COOH. After cleavage with fXa, a polypeptide containing r-[$M1_{HPg}$] was obtained, with the exact amino acid sequence of NH_2-VDASE[$K1_{MPg}$]EEE-COOH.

Purification of the r-[$K1_{HPg}$] Variants. The FPLC-based purification procedures were grounded on the specific adsorption of kringle-containing samples to Sepharose–lysine affinity chromatography columns. The wtr-[$K1_{HPg}$] (as well as those mutants that retain their ω-amino acid binding sites) directly interacts with this column and can be purified by this technique according to published procedures (Menhart et al., 1991). However, in many cases herein, the mutated r-[$K1_{HPg}$] contains a compromised ω-amino acid binding site. In these situations, the fusion protein was expressed, which can be readily purified on the basis of the affinity of r-[$K4_{HPg}$] to this affinity column.

In these latter cases, the sample was loaded onto Sepharose–lysine at a rate of 0.5 mL/min, and the column was washed with a solution of 25 mM Tris-HCl, pH 7.9, until the absorbance at 280 nm was negligible. The fusion polypeptide was then eluted with a linear gradient of EACA. The solutions

Table I: Construction of r-[$K1_{HPg}$] Variants

mutation[a]	primer[b]	screen
$H^{31}Q$	5′-CT TCT CCC CAG AGg CCT AGA TTC T	+*Stu*I
$R^{32}Q$	5′-TCT CCC CAC caA CCT AGg TTC TCA CCT	+*Sty*I
$R^{34}Q$	5′-C CAC AGA CCT caA TTC TCg CCg GCT ACA CAC C	+*Cfr*10I
$F^{35}V$	5′-CAC AGA CCT AGg gtC TCA CCT GCT ACA	+*Avr*II
$F^{35}Y$	5′-AC AGA CCT AGg TaC TCA CCT GCT ACA A	+*Avr*II
$F^{35}W$	5′-AC AGA CCT AGg Tgg TCA CCT GCT AC	+*Bst*EII
$D^{54}N$[c]	5′-AGG AAT CCA aAC AAC GAT CCG CAG GGt CCC TGG TGC	–*Ban*II
$D^{56}N$	5′-GC AGG AAT CCg GAC AAC aAT CCG CAG GG	+*Acc*III
$W^{61}A$[c]	5′-G CAG GGG CCa gct TGC TAT ACT A	–*Apa*I
$W^{61}F$[c]	5′-G CAG GGG CCt Ttt TGC TAT ACT A	–*Apa*I
$Y^{63}L$	5′-CCC TGG TGC ctT ACT ACT GAT	+*Ban*I
$Y^{63}H$	5′-CAG GGG CCa TGG TGC cAT ACT ACT G	+*Sty*I
$Y^{63}F$	5′-CAG GGG CCa TGG TGC TtT ACT ACT GA	+*Sty*I
$Y^{63}F$	5′-CAG GGG CCa TGG TGC Tgg ACT ACT GAT C	+*Bxt*XI
$R^{70}Q$	5′-GC TAT ACT ACg GAT CCA GAA AAG caA TAT GAC TAC	+*Bam*HI
$Y^{71}L$	5′-T CCA GAA AAG cGt cta GAC TAC TGC G	+*Xba*I
$Y^{71}F$	5′-GAT CCA GAg AAG AGA TtT GAC TAC TGC GA	+*Ear*I
$Y^{71}W$	5′-GAT CCA GAA AAa cGt Tgg GAC TAC TGC G	+*Mae*II

[a] The column below indicates the wild-type amino acid in r-[$K1_{HPg}$] and its sequence position beginning from C^1 of the kringle, followed by the amino acid replacement at that position. See Figure 4 for the numbering system of amino acids. [b] The mutated bases are represented by lower-case lettering. The underlined region represents the location of the restriction site that has been mutated into the cDNA. [c] The underlined region represents the location of a restriction site in the wt cDNA that was mutated out.

employed were 25 mM Tris-HCl, pH 7.9 (start solution), to 25 mM Tris-HCl/20 mM EACA, pH 7.9 (limit solution).

Cleavage of the Fusion Polypeptides. To liberate the r-[$K1_{HPg}$] domain from the [$K4_{HPg}$]-containing double kringle constructions, we employed immobilized factor Xa. The factor Xa was equilibrated by dialysis against a buffer of 200 mM $NaHCO_3$, pH 8.0. Sepharose CL-4B, activated with *p*-nitrophenol/chloroformate (Wilchek et al., 1984), was filtered dry and added directly to the factor Xa solution at a concentration of 2 mL of resin/mg of factor Xa. The pH was maintained at 8.5 by small additions of solid Na_2CO_3 over 30 min. The coupling reaction was then allowed to proceed at 4 °C overnight. The resin was then washed with a solution of 100 mM Tris-HCl/5% NH_2OH and stored in a buffer of sodium phosphate/10% glycerol/0.01% NaN_3, pH 7.0. The specific activity of insolubilized factor Xa was assayed with the chromogenic substrate *N*-BzO-IEGR-*p*-nitroanilide (S2222). Typically, 40%–66% of the factor Xa added was found to be bound to the resin.

The r-polypeptide to be cleaved was dialyzed against a buffer consisting of 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$, pH 7.4. The immobilized factor Xa was equilibrated with the same buffer, and the protein solution was added to this resin. Typically, 2 mL of resin (ca. 0.5 mg of factor Xa) was sufficient to catalyze cleavage of 1 mg of fusion protein in 4 h at room temperature. Longer incubations at 37 °C were employed to cleave larger amounts of fusion proteins. Upon completion of the cleavage reaction, the resin was filtered, washed with a solution containing 300 mM sodium citrate, pH 7.0, and then reequilibrated with the storage buffer, above. The filtrate and citrate washings were dialyzed against a buffer of 25 mM Tris-HCl, pH 7.9, in preparation for final purification using FPLC with a Mono Q resin (Menhart et al., 1991).

Binding of ω-Amino Acids to Kringle-Containing HPg Domains. Intrinsic fluorescence titrations were employed for the measurement of thermodynamic binding constants of r-[$K1_{HPg}$] mutants. Our exact procedures have been published (Menhart et al., 1991; De Serrano & Castellino, 1992a,b). Generally, the buffer employed was 150 mM sodium phosphate, pH 7.0, at 25 °C. Dissociation constants (K_d) characterizing the binding of ω-amino acids to the r-[$K1_{HPg}$] variants were calculated from the fluorescence titrations by nonlinear least-squares iterative fitting, as described earlier (Menhart et al., 1991).

Analytical Methods. All procedures for DNA manipulations, which include oligonucleotide synthesis, cDNA sequencing, cell transformations, plasmid minipreparations, large-scale plasmid preparations, generation of single-stranded DNAs, *in vitro* mutagenesis, 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., 1992a). Methods for analysis of the recombinant kringle domains by amino-terminal amino acid sequence analysis have been published (Sehl & Castellino, 1990).

RESULTS

In order to identify the amino acid residues in [$K1_{HPg}$] that are involved in binding of ω-amino acid ligands to this protein domain, we have mutated amino acids in the polypeptide that have been implicated in stabilizing binding of the ligands by chemical modification studies, X-ray crystallography, and NMR analysis and from homology with residues known to be involved in these interactions in r-[$K2_{tPA}$]. The mutations were accomplished by oligonucleotide-directed mutagenesis with the synthetic primers listed in Table I, and transformed bacterial colonies were screened with the indicated restriction endonuclease enzymes also provided in Table I. To assure that the proper mutations were incorporated into the cDNAs, the relevant portions of the genes were subjected to oligonucleotide sequence analysis. In all cases, the cDNA sequences were as predicted.

Expressions were conducted in *E. coli* DH5α cells as described previously for wtr-[$K1_{HPg}$], and periplasmic and oxidatively refolded fractions were examined for the desired materials (Menhart et al., 1991). In all cases, at least 70% of the r-[$K1_{HPg}$] mutants were found in the cell periplasm. Consequently, this cell fraction was used as the source of the expressed r-polypeptides. The expression levels of the mutants varied between approximately 0.05 mg/100 g (wet weight) of cells in the case of r-[$K4_{HPg}XaK1_{HPg}/W^{61}A$] to approximately 5 mg/100 g (wet weight) of cells for r-[$K4_{HPg}XaK1_{HPg}/Y^{71}W$]. Because purification of these polypeptides

FIGURE 1: Construction of the expression system for the factor Xa-cleavable fusion vector. A detailed description of the initial plasmid pSTII[$K4_{HPg}K5_{HPg}$] is provided in the Materials and Methods section of this paper. A region of HPg comprising its [$K4_{HPg}K5_{HPg}$] region was amplified from plasmid p119-HPg (McLean et al., 1987) by PCR and inserted into the *NsiI*/*SalI* sites of plasmid phGH4R (Menhart et al., 1991). This resulted in plasmid pSTII[$K4_{HPg}K5_{HPg}$]. Next, a fXa-specific cleavage site (IEGR) was created between the [$K4_{HPg}$] and [$K5_{HPg}$] domains in the translated product by site-directed mutagenesis on a template of pSTII[$K4_{HPg}K5_{HPg}$] using primer 1. This primer also allowed placement of a *NsiI* restriction site immediately downstream of the fXa cleavage site in order to facilitate construction of the fusion proteins. A previously existing *NsiI* site in the 5′ region of [$K4_{HPg}$] was removed from this plasmid by site-directed mutagenesis using primer 2. This provided the vector pSTII[$K4_{HPg}$]Xa[$K5_{HPg}$]ΔN. The desired mutations in [$K1_{HPg}$] were performed in the plasmid pSTII-[$K1_{HPg}$], and the cDNA encoding the [$K1_{HPg}$] region of this latter vector was excised with *NsiI*/*SphI*. This fragment was then ligated into the *NsiI*/*SphI* restriction sites of plasmid pSTII[$K4_{HPg}$]Xa[$K5_{HPg}$]ΔN. This led to the final expression plasmid pSTII[$K4_{HPg}$]Xa[$K1_{HPg}$].

depended on adsorption to and elution from lysine–Sepharose columns, those mutants with compromised ω-amino acid binding sites could not be expected to be satisfactorily purified in this manner. Therefore, in cases where we might reasonably anticipate problems in this regard, we inserted their cDNAs into a r-[$K4_{HPg}$]-containing factor Xa-cleavable fusion polypeptide and employed the ligand binding properties of the r-[$K4_{HPg}$] portion of the fusion polypeptide for purification purposes. In addition, some cDNAs which were difficult to express singly were expressed much better in the fusion vector. The particular cDNAs placed in the fusion vector were those containing the following mutations: $R^{32}Q$, $R^{34}Q$, $F^{35}V$, $D^{54}N$, $D^{56}N$, $W^{61}A$, $W^{61}F$, $Y^{63}L$, $Y^{63}H$, $R^{70}Q$, $Y^{71}L$. In those cases in which the fusion vector was employed, the isolated fusion polypeptide was first subjected to specific cleavage by factor Xa. Following this, the target r-[$K1_{HPg}$] mutant was readily resolved from r-[$K4_{HPg}$] by FPLC methodology. The essential elements of this latter strategy are diagrammed in Figure 1. All purified r-[$K1_{HPg}$] variants were subjected to amino-terminal amino acid sequence analysis through at least 10 residues to assure integrity of signal polypeptide cleavage or proper fXa cleavage. In all cases reported herein, the amino-terminal sequences were as expected.

The intrinsic fluorescence alterations accompanying binding or ω-amino acid ligands to a variety of isolated kringle domains (Kelley & Cleary, 1989; Kelley et al., 1991; Menhart et al., 1991; De Serrano & Castellino, 1992a,b, 1993) have been employed to measure dissociation constants (K_d) of two representative ligands, EACA and AMCHA, to all r-[$K1_{HPg}$] variants. The types of intrinsic fluorescence changes encountered varied with the nature of the kringle variant under examination, with either positive or negative changes observed. Examples of such diverse behavior are illustrated in Figures 2 and 3, as observed with titration of r-[$K1_{HPg}/Y^{63}W$] by AMCHA (Figure 2), which displayed a positive fluorescence change, and r-[$K1_{HPg}/Y^{63}L$] (Figure 3), which resulted in a negative fluorescence change as a result of titration with EACA. While the types of fluorescence alterations which

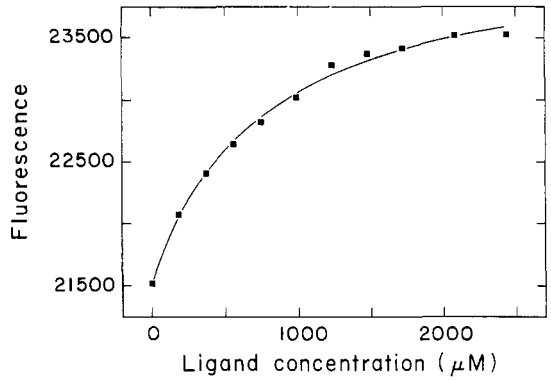

FIGURE 2: Instrinsic fluorescence titration of r-[K1$_{HPg}$/Y^{63}W] by AMCHA. The experimental points (■) were best fit to a line characterized by n = 1.0, K_d = 759 μM, and a maximal relative fluorescence change (ΔF_{max}) or 12.63%. The buffer was 150 mM sodium phosphate, pH 7.0, at 25 °C. The excitation and emission wavelengths were 290 and 340 nm, respectively. The concentration of r-[K1$_{HPg}$/Y^{63}W] was 1.5 μM.

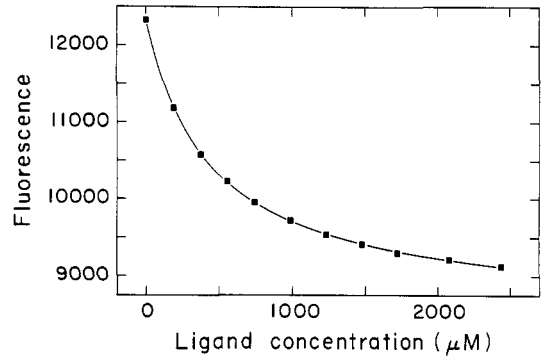

FIGURE 3: Intrinsic fluorescence titration of r-[K1$_{HPg}$/Y^{63}L] by EACA. The experimental points (■) were best-fit to a line characterized by n = 1.0, K_d = 435 μM, and a maximal relative fluorescence change (ΔF_{max}) of 30.17%. The buffer was 150 mM sodium phosphate, pH 7.0, at 25 °C. The excitation and emission wavelengths were 290 and 340 nm, respectively. The concentration of r-[K1$_{HPg}$/Y^{63}W] was 1.5 μM.

Table II: Dissociation Constants of ω-Amino Acids to r-[K1$_{HPg}$] Mutants

variant[a]	dissociation constant (μM) for EACA	AMCHA	variant[a]	dissociation constant (μM) for EACA	AMCHA
wt	11	1.4	W^{61}A	98	2.8
H^{31}Q	15	2.5	Y^{63}W	nd[b]	759
R^{32}Q	11	1.2	Y^{63}H	418	27
R^{34}Q	10	1.1	Y^{63}F	3800	2600
F^{35}V	16	0.8	Y^{63}L	435	529
F^{35}Y	15	1.0	R^{70}Q	446	29
F^{35}W	10	0.6	Y^{71}L	no fluorescence change	
D^{54}N	no fluorescence change		Y^{71}F	8	3.0
D^{56}N	920	1700	Y^{71}W	1.5	0.1
W^{61}F	61	3.6			

[a] See Figure 4 for the numbering system of amino acids. [b] Not determined.

occurred differed among the mutants, in all cases these changes were sufficiently large for analysis and were saturable with the ligands. Thus, K_d values could be readily obtained. A summary of all K_d values obtained in this work is provided in Table II.

As a more broadly based means of confirming the importance of certain of these residues in ω-amino acid binding to r-[K1$_{HPg}$], we examined known sequences of other species of plasminogen in order to evaluate whether species-dependent sequence differences could be revealing in assessing the nature of the ligand binding site. Mouse plasminogen offered the possibility of further assessment of the cationic locus for binding the carboxyl group of the ligand, since in [K1$_{MPg}$] there exist R^{32}V and R^{34}N substitutions (Figure 4). This domain of MPg was expressed in the fusion vector, and after cleavage and isolation of r-[K1$_{MPg}$], the binding (dissociation) constants of a series of ω-amino acid ligands were determined by intrinsic fluorescence titrations. The data obtained are summarized in Table III and demonstrate that no large ligand binding differences were found in the comparison of r-[K1$_{HPg}$] and r-[K1$_{MPg}$].

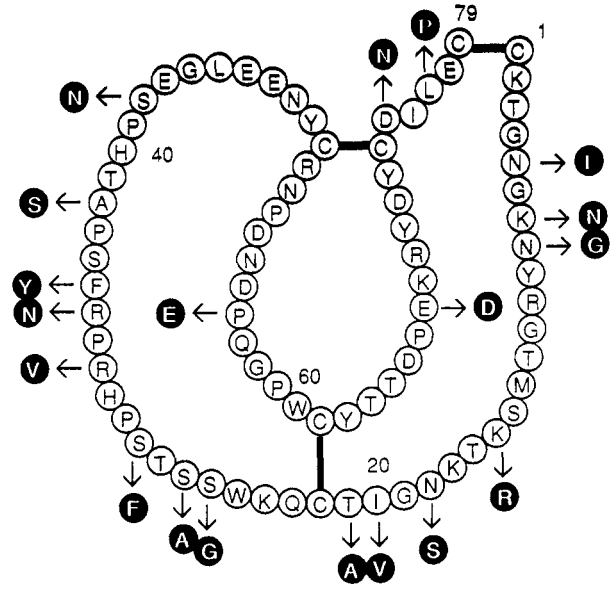

FIGURE 4: Comparisons of the amino acid sequences of r-[K1$_{HPg}$] and r-[K1$_{MPg}$]. The sequence provided is for the human polypeptide with the amino acid residues in color reversals indicating the changes in the mouse polypeptide sequence.

Table III: Dissociation Constants of ω-Amino Acids to r-[K1$_{MPg}$] at 25 °C

ligand	dissociation constant (μM) for wtr-[K1$_{HPg}$]	wtr-[K1$_{MPg}$]
4-ABuA[a]	350	220
5-APnA[b]	24	40
EACA[c]	11	10
7-AHpA[d]	250	370
8-AOcA[e]	480	1000
AMCHA[f]	1.4	1.0

[a] Refers to 4-aminobutyric acid. [b] Refers to 5-aminopentanoic acid. [c] Refers to 6-aminohexanoic acid. [d] Refers to 7-aminoheptanoic acid. [e] Refers to 8-aminooctanoic acid. [f] Refers to *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid.

DISCUSSION

The objective of this investigation was to define those amino acid side-chain residues of r-[K1$_{HPg}$] that contribute to the stabilization of the interaction of ω-amino acid ligands with this domain. Knowledge of the nature of the [K1$_{HPg}$]/ligand binding interaction is of great importance to HPg function, since this site is the strongest of the ω-amino acid binding sites of HPg (Lerch & Rickli, 1980) and has been implicated in the interaction of HPg with fibrin (Lucas et al., 1983a), with α_2-antiplasmin (Wiman et al., 1978), and with cells, such as platelets and monocytoid U937 cells (Miles et al., 1988).

Some previous studies have been performed regarding the influence of certain [K1$_{HPg}$] residues as contributors to the binding stability of ω-amino acids. Chemical modification experiments suggested that H^{31} (Lerch & Rickli, 1980) and/

or R^{34} (Vali & Patthy, 1984) were principal cationic sites for the carboxyl group of the ligand. Other indications that R^{34} might be important in this regard revolve around studies with other kringle domains. In r-[$K2_{tPA}$], the homologous residue K^{33} has been shown by site-directed mutagenesis to be the sole cationic locus on the kringle that stabilizes ligand binding (De Serrano & Castellino, 1992a; De Serrano et al., 1992b). In another kringle, [$K4_{HPg}$], chemical modification studies have demonstrated that R^{69} is a dominant ligand binding site (Trexler et al., 1982). In this latter case, the equivalent residue in [$K1_{HPg}$] is R^{70}. The anionic ligand binding locus is believed to involve a pair of D-residues, D^{54} and D^{56}, that are highly conserved in all kringle domains that interact with ω-amino acid ligands. This was proven by site-directed mutagenesis experiments with r-[$K2_{tPA}$], where it was discovered that mutation of either of the these D-residues to E-residues resulted in greatly weakened ligand binding (De Serrano & Castellino, 1993). Chemical modification studies with [$K4_{HPg}$] were also suggestive of the involvement of D^{56} in ω-amino acid binding.

NMR investigations of the binding of these ligands to kringle domains support the participation of hydrophobic amino acids, particularly aromatic residues, in stabilizing ligand binding. In the case of r-[$K1_{HPg}$], it is proposed that the most important residues in this regard are F^{35}, W^{61}, and Y^{71} (Motta et al., 1987). The results of chemical modification studies provide support for the involvement of the homologous W^{70} residue of [$K4_{HPg}$] in this binding (Hochswender & Laursen, 1981). Direct evidence that the homologous W^{74} residue of r-[$K2_{tPA}$] is a critical residue for ligand binding has been obtained from site-directed mutagenesis experiments (De Serrano & Castellino, 1992b).

The X-ray crystal structure of r-[$K1_{HPg}$] has been determined and provides support for the possibility that some of these amino acid side chains of this domain are involved in ligand binding. An appropriate view of this structure is illustrated in Figure 5, which shows a putative ligand binding pocket, along with steric relationships of the residues mutated in the current study. On the basis of comparison with other kringles, *viz.*, r-[$K2_{tPA}$] (de Vos et al., 1992), which has been crystallized with a lysine residue from a neighboring molecule presumably located at the ligand binding site, and [$K4_{HPg}$] (Mulichak & Tulinsky, 1990; Mulichak et al., 1991; Wu et al., 1991), which has been crystallized with EACA in place, it can be reasonably proposed that the amino group of the ligand interacts with both D^{54} and D^{56} and the carboxyl moiety of the ligand can interact with the positively charged cluster of residues (H^{31}, R^{32}, R^{34}, and/or R^{70}) at the top of the cavity. The methylene groups of the ligand can then be stabilized by interactions with F^{35}, W^{61}, Y^{63}, and/or Y^{71}, all of which seem to be in position to possibly interact with this region of the substrate.

Most of the above studies, while of great value, have not clearly defined the relative importance of these residues in stabilizing the binding of ω-amino acids to this kringle domain. The chemical modification investigations were highly qualitative in nature and contained the obvious problems of incorporation of large bulky reagents into the polypeptide. These could have led to indirect effects on ligand binding. The NMR investigations could not distinguish between direct and secondary effects of ligand binding on the residues in question, and the X-ray studies, while predictive of the residues that could be critical to ligand binding, could not evaluate the relative importance of each.

These points are more clearly evident upon examination of the kringle 1 sequences from other species of plasminogen,

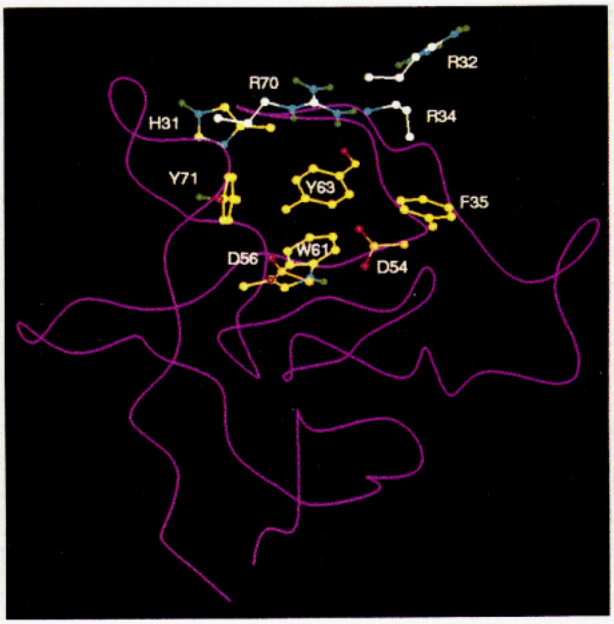

FIGURE 5: X-ray structure of wtr-[$K1_{HPg}$]. Selected amino acid side chains are displayed on the backbone structure, as indicated. The numbering of amino acids begins at C^1 of the r-[$K1_{HPg}$] sequence and continues consecutively to the last C (C^{79}) of this domain. Each amino acid is displayed from its β-carbon (most hydrogen atoms are excluded to minimize overcrowding). The backbone conformation is in magenta. Hydrophobic amino acid side-chain carbon atoms (including that of H^{31}) are in yellow, charged amino acid side-chain carbons (at physiological pH) are in white, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms are in green. With regard to R^{34}, electron density was only observed up to the ε-nitrogen. Thus, an incomplete side chain is shown for this residue.

with the assumption that this domain plays the same functional role in each. In addition to the known squence of r-[$K1_{HPg}$], amino acid sequences have been published for r-[$K1_{BPg}$] (Schaller et al., 1985), r-[$K1_{MPg}$] (Degen et al., 1990), [$K1_{PPg}$] (Schaller et al., 1987) r-[$K1_{PPg}$] (Nagamine et al., 1984), and r-[$K1_{RPg}$] (Tomlinson et al., 1989). In these cases, there is rigid conservation of the $D^{54}N^{55}D^{56}$ sequence, along with that of H^{31} and R^{70}. On the other hand, as compared to r-[$K1_{HPg}$], R^{32} is not conserved in all species, being altered to V in the cases of r-[$K1_{BPg}$] and r-[$K1_{MPg}$] and to I in r-[$K1_{PPg}$]. There is a similar lack of conservation of R^{34}, with K appearing at this position in r-[$K1_{BPg}$] and r-[$K1_{PPg}$], N present at that location in r-[$K1_{MPg}$], and T in r-[$K1_{RPg}$]. This argues for a nonessential role in ligand binding for R^{32} and R^{34}. Regarding the aromatic residues that have been implicated in ligand binding, F^{35} is altered to Y in [$K1_{MPg}$] and r-[$K1_{PPg}$], indicating a degree of tolerance at that residue. On the other hand, W^{61} and Y^{63} are strictly conserved, suggesting that they may be essential residues. The Y^{71} side chain appears as an F in r-[$K1_{PPg}$] and r-[$K1_{RPg}$], again indicating that some flexibility is tolerated. In order to evaluate these general principles of ω-amino acid ligand binding, we expressed r-[$K1_{MPg}$] and determined its binding properties to a series of such ligands in comparison to r-[$K1_{HPg}$]. The results, listed in Table III, show that great similarities are observed between these two species in the K_d values for these ligands. This more strongly suggests that R^{32} and R^{34} are not involved in stabilizing these ligand interactions to r-[$K1_{HPg}$] and that minor alterations at F^{35} and Y^{71} do not result in changes in the K_d values for these ligands. We cannot rule out that minor perturbations in binding result from these changes, since the K_d values do possess some small differences when the two species of kringle 1 domains are compared. However, these are certainly not large and may result from small conformational differences

due to the large number of amino acid substitutions between r-[$K1_{HPg}$] and r-[$K1_{MPg}$].

At this point, we believed that a more refined analysis of the polypeptide residues of r-[$K1_{HPg}$] involved in ligand binding was possible from site-directed mutagenesis experiments. Regarding the cationic residues, while no data existed suggesting that H^{31} was not a critical residue, radical mutation of this residue to a Q did not have significant effects on the ligand binding properties of the $H^{31}Q$ mutant (Table II). This suggests that H^{31} is not of importance for ligand binding. In support of this observation, it is noted that, for H^{31} to function as a cationic locus, this residue would need to possess an abnormally high pK value. Similarly, mutagenesis experiments confirmed the nonessential nature of R^{32} and R^{34} in ligand binding, since the data of Table II clearly demonstrate that alteration of these residues to a Q in each case did not influence the ligand binding properties of the mutants. On the other hand, mutation of R^{70} to Q led to a 20-fold increase in the K_d for EACA and a 20-fold increase in that for AMCHA. These effects clearly point to the essential role of R^{70} as the principal cationic site for ligand binding, despite the fact that the X-ray structure could just as easily implicate R^{34} in that regard. However, in considering a possible role for R^{34} as a possible ligand binding site, some steric assumptions had to be made. In the diffraction pattern, electron density was only observed up to the ϵ-nitrogen of R^{34}, and no electron density was found that could be assigned to the guanidino group of this residue.[3] Thus, the position of the guanidino group of R^{34} in the crystal structure is disordered, and our results suggest that it probably is not located directly in the binding pocket. On the other hand, the fact that the equivalent K^{33} is the sole cationic site for ligand binding stabilization in r-[$K2_{tPA}$] (De Serrano & Castellino, 1992a; De Serrano et al., 1992b) is consistent with the X-ray crystal structure of the intermolecular pseudo-ligand/r-[$K2_{tPA}$] complex (de Vos et al., 1992), which shows in that case that none of the residues involved in the positively charged cluster containing K^{68}, R^{70}, and R^{71} are in proximity to the location of the carboxyl group of the ligand. Perhaps the fact that the ligand specificity of r-[$K2_{tPA}$] is different from all other ω-amino acid binding kringles (Kelley et al., 1991; De Serrano & Castellino, 1992a,b, 1993) results from the fact that an alternate cationic group is employed to stabilize ligand binding.

Considering the anionic locus of the ligand binding site on r-[$K1_{HPg}$], there is a clear large consequence of alteration of D^{56} to N, with K_d increases of >80-fold for EACA/r-[$K1_{HPg}$] binding and 1500-fold in the case of AMCHA/r-[$K1_{HPg}$] binding. Similarly, we could not detect ligand binding with the $D^{54}N$ mutant, insofar as this is revealed by alterations in the intrinsic fluorescence of the mutant that accompanies this binding. Of course, this could be reflective of this mutation influencing the fluorescence properties of this particular variant, but this is unlikely to be so. Solvent (guanidine hydrochloride) denaturation concentration profiles, which undergo large changes upon ω-amino acid ligand binding for all mutants, are not altered by the ligand in the case of the $D^{56}N$ variant.[4] In addition, r-[$K1_{HPg}/D^{56}N$] does not bind to lysine–Sepharose columns. These results suggest that the ligand does not bind to this mutant. We thus conclude that both D^{54} and D^{56} are critical to these binding events, similar to the situation found in the case of r-[$K2_{tPA}$] (DeSerrano & Castellino, 1993).

Regarding the aromatic residues that have been implicated in ligand binding from other studies, we first consider the case of F^{35}. Three mutations were made at this location, two of those being more conservative, *viz.*, Y and W, and a third of a more radical nature, *viz.*, V. None of these changes influenced ligand binding, demonstrating that this residue is not primarily involved in this particular function. Thus, the perturbations seen in F^{35} in the NMR analysis of the ligand/r-[$K1_{HPg}$] complex (Motta et al., 1987) are most likely due to secondary effects of ligand binding. In the case of W^{61}, we have made both conservative (F) and nonconservative (A) mutations, and each adversely affected the binding of EACA, with a slightly smaller influence on that for AMCHA. Thus, this residue appears to play a role in governing binding specificity, perhaps due to a role in conformational maintenance of the kringle, since both types of mutations inhibit the binding almost equally. Therefore, a W at this position appears to be very relevant to the integrity of the kringle. Its likely structural importance is confirmed by the fact that, with minor exceptions in apolipoprotein(a) kringles (wherein a Y is substituted at this position), all kringle domains in all species of proteins, even those that do not bind ω-amino acids, contain a W at this position. Substitutions do not appear to be tolerated at this location.

The next set of mutations to be considered are those at Y^{63}. While all four mutants constructed resulted in dramatic decreases in the affinity of the ligands for the mutant kringle domains (Table II), the unusual finding here is that the most conservative alterations, *viz.*, $Y^{63}W$ and $Y^{63}F$, led to the greatest losses in binding capacities. More nonconservative mutations (to H and to L) resulted in smaller effects on binding. This is consistent with the strict conservation of this residue in all species of kringle 1 sequences that have been determined and leads to the conclusion that a Y at this position is a defining characteristic of the kringle 1 domain. The final set of mutations were constructed at Y^{71}. Mutation of this residue to F did not greatly influence the binding of the two ligands, consistent with the observations that F-residues are found at this location in two other species of plasminogen kringle 1 domains. That an aromatic residue is required at this location is confirmed from the observations that such a residue is present in a homologous location in all ω-amino acid binding kringles and the fact that mutation to a nonaromatic residue (to L) eliminates ligand binding, insofar as such binding is reflected by the loss of a ligand-induced intrinsic fluorescence alteration. In addition, the lack of binding of this mutant to lysine–Sepharose and the absence of ligand effects on the solvent (guanidine hydrochloride) denaturation stability of r-[$K1_{HPg}/Y^{71}L$],[4] as was the case with the $D^{54}N$ mutant discussed above, argue strongly that these ligands do not interact with this mutant. Of great interest is the effect on ligand binding of the $Y^{71}W$ mutation. Here, a large (>10-fold) increase in affinity is noted for each of the ligands, suggesting that ligand binding site affinities could also be increased by mutagenesis. It is clear from these results, and from the observation that all ligand binding kringles contain an aromatic amino acid at this location, that such a residue is critical for such interactions to occur.

In conclusion, this study has clearly defined the amino acid residues critical to ω-amino acid binding of the r-[$K1_{HPg}$] domain. While other residues not examined here could of course be important in this regard, we have taken the most reasonable approach possible, in examination of those residues that could be predicted by a variety of means to be of importance in ligand binding stabilization. From this work, and similar detailed mutagenesis investigations with r-[$K2_{tPA}$], which are highly complementary with X-ray crystallographic studies of r-[$K2_{tPA}$], r-[$K1_{HPg}$], and r-[$K4_{HPg}$], a rigorous

[3] A. Tulinsky, personal communication.

[4] N. Menhart and G. J. Hoover, unpublished experiments.

understanding of the nature of ligand/kringle domain binding has occurred. This is of great relevance not only to the fields of coagulation and fibrinolysis but also to fundamental issues of protein/ligand interactions.

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