Enhancement through Mutagenesis of the Binding of the Isolated Kringle 2 Domain of Human Plasminogen to ω-Amino Acid Ligands and to an Internal Sequence of a Streptococcal Surface Protein*

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In the background of the recombinant K2 module of human plasminogen (K2Pg), a triple mutant, K2Pg[C4G/E56D/L72Y], was generated and expressed in Pichia pastoris cells in yields exceeding 100 mg/liter. The binding affinities of a series of lysine analogs, viz. 4-aminobutyric acid, 5-aminopentanoic acid, ε-aminocaproylic acid, 7-aminohexanoic acid, and 4-aminomethylcyclohexan-1-carboxylic acid, to this mutant were measured and showed up to a 15-fold tighter interaction, as compared with wild-type K2Pg (K2Pg[C4G]). The variant, K2Pg[C4G/E56D/L72Y], afforded up to a 4-fold increase in the binding affinity to these same ligands, whereas the K2Pg[C4G/L72Y] mutant decreased the same affinities up to 5-fold, as compared with K2Pg[C4G]. The thermal stability of K2Pg[C4G/E56D/L72Y] was increased by approximately 13 °C, as compared with K2Pg[C4G]. The functional consequence of up-regulating the lysine binding property of K2Pg was explored, as reflected by its ability to interact with an internal sequence of a plasminogen-binding protein (PAM) on the surface of group A streptococci. A 30-mer peptide of PAM, containing its K2Pg-specific binding region, was synthesized, and its binding to each mutant of K2Pg was assessed. Only a slight enhancement in peptide binding was observed for K2Pg[C4G/E56D/L72Y], compared with K2Pg[C4G] (Kd = 460 nM). A 5-fold decrease in binding affinity was observed for K2Pg[C4G/L72Y] (Kd = 3200 nM). However, a 12-fold enhancement in binding to this peptide was observed for K2Pg[C4G/E56D/L72Y] (Kd = 37 nM). Results of these PAM peptide binding studies parallel results of ω-amino acid binding to these K2Pg mutants, indicating that the high affinity PAM binding by plasminogen, mediated exclusively through K2Pg, occurs through its lysine-binding site. This conclusion is supported by the 100-fold decrease in PAM peptide binding to K2Pg[C4G/E56D/L72Y] in the presence of 50 mM 6-aminohexanoic acid. Finally, a thermodynamic analysis of PAM peptide binding to each of these mutants reveals that the positions Asp54 and Tyr72 in the K2Pg[C4G/E56D/L72Y] mutant are synergistically coupled in terms of their contribution to the enhancement of PAM peptide binding.

Krigles are modular units found in a number of proteins that participate in the blood coagulation and fibrinolytic path-ways. These structural motifs contain approximately 80 amino acids with three disulfide bonds in a 1–6, 2–4, 3–5 arrangement. The copy numbers of krigles vary in these proteins. For example, a single kringle is found in urokinase-type plasminogen activator (1); two are present in tissue-type plasminogen activator (tPA) (2) and prothrombin (3); four are contained in hepatocyte growth factor-like protein (4, 5); five are included in plasminogen (6); and as many as 41 exist in apolipoprotein(a) (7).

The nature of this lysine-binding site (LBS) of krigles is well understood due to advances in establishment of expression systems for these modules (25, 26), generation of models of binding through x-ray crystallographic (23, 27–30) and NMR solution studies (31–33) of kringle/ligand complexes, and analysis of the effects of alterations in primary structures of krigles on ligand-kringle binding (22, 23, 34–39). These combination of studies have shown that those kringle residues most directly involved in ligand binding are Asp54 and Asp56 (K1Pg numbering) which coordinate the amino group of the ω-amino acid ligand, Arg77 (LYS77) in the case of K2tPA which stabilizes the carboxylate moiety of the ligand, and Trp61 and Tyr71 which accommodate the methylene groups of the ligand in the binding pocket.

A verification of the extent of understanding of the interactions critical to this binding event would be to design the

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1 The abbreviations used are: tPA, tissue-type plasminogen activator; K2Pg, K2 module of human plasminogen; EACA, 6-aminohexanoic acid; hPg, human plasminogen; LBS, lysine-binding site; PCR, polymerase chain reaction; bp, base pair; ITC, isothermal titration calorimetry; 4-ABA, 4-aminobutyric acid; 5-APA, 5-aminopentanoic acid; 7-AHA, 7-aminohexanoic acid; t-AMCHA, trans-4-aminomethyl-1-cyclohexanoic acid; TOF-MALDI-DE-MS, time-of-flight matrix-assisted laser-desorption ionization with delayed-extraction mass spectrometry.
binding site in a kringle module that does not contain such a site or, more modestly, to up-regulate this site in a kringle motif that possesses a weaker site. This latter feature characterizes K2r, and it was our aim to redesign this kringle unit to contain a stronger α-amino acid-binding site. The results of this investigation are reported herein.

**EXPERIMENTAL PROCEDURES**

**Construction of pPIC9k[K2r]—** A construct containing K1, linked through a factor Xla-sensitive cleavage site (IEGR) to K2r, was inserted into the yeast expression bacterial shuttle vector pPIC9k (Invitrogen, San Diego, CA) pPIC9k(K1, XamK2r), and used as the template DNA for PCR-based mutagenesis. The plasmid was provided by Hui Wang of this laboratory. The K2r was amplified by PCR for preparation of the mutants used in the work described herein.

For PCR-based amplification of K2r, the forward primer (1), 5′-CGATGAATTCCTCCAGAATGTACCTGCG-CACCCGGGG, contained the 3′ end of the K2r gene, engineered with a 3′ NotI site. This primer also coded for two Ala residues (mutated bases underlined) immediately following the final residue, Cys9, of K2r. Two Thr residues are found at these positions in the native hPg molecule, which have been replaced by Ala residues to avoid the possibility of O-linked glycosylation at these sites during the subsequent expression in yeast. This primer also encodes two stop codons (in italics) immediately following the two Ala residues and are immediately upstream of the NotI site.

By using primers P1 and P2, a 276-bp fragment (F1) was amplified, containing the wild-type K2r gene flanked by an upstream EcoRI site and a downstream NotI site, which were used to ligate F1 into plasmid pPIC9k, generating the pPIC9k(K2r) construct.

The ultimate expression of the isolated wtr-K2r gene product yielded little intact properly folded target material. This is mostly due to the existence of Cys9, which normally disulfide-linked to the Cys9 of K2r in hPg. Therefore, the Cys9 of K2r was mutated to Gly, which is the amino acid found in all ligand-binding kringles of hPg at this position. This is considered to be the wild-type K2r.

**Construction of pPIC9k[K2r/C4G]—** This plasmid was generated using a two-round PCR strategy, using the pPIC9k(K2r) as the template.

For round 1, the forward primer (P3) was 5′-ACTCATTGGGCAATGAT- CACCATG, which is positioned approximately 86 bases upstream of the 5′ terminus of the K2r gene, at the α-factor signal sequence of the pPIC9k vector. The reverse primer (P4) employed was 5′-CTCCGTGATCCATGCCATAC, which contains the sequence within the K2r gene coding for the C4G mutation (the codon for G is in bold, with the mutated base underlined).

By using primers P3 and P4, a 105-bp fragment (F2) containing the C4G mutation was amplified from the pPIC9k(K2r) template.

The forward primer (P5), 5′-GTATGCACTGGCAGTGGAG, is the complement of P4 and also contains the sequence within the K2r gene which codes for the C4G mutation (the codon for G is in bold, with the mutated base underlined).

By using primers P3 and P6, a 370-bp fragment (F3) containing the C4G mutation was amplified from the pPIC9k(K2r) template. Because fragments F2 and F3 are complementary around the region of the C4G mutation in K2r, they were employed as overlapping templates in round 2 of a PCR reaction, using primers P5 and P6 to amplify a 457-bp fragment (F4). This latter fragment is composed of the following genes: α-factor signal sequence EcoRI site-SEE[K2r/C4G]AA-NotI site-3′AOX1. The upstream EcoRI and downstream NotI sites were used to ligate the PCR-amplified DNA into the pPIC9k vector, thus creating the pPIC9k[K2r/C4G] construct.

**Construction of pPIC9k[K2r/C4G, E56D]—** Plasmid pPIC9k[K2r/C4G] was used as the template for the generation by PCR of the following mutant K2r construct, pPIC9k[K2r/C4G, E56D].

Reverse primer P7 5′-CAAGGCGGCGATCCCTATCC and forward primer P8 5′-CGATAGGGATCCGCGCTTGG are complementary primers at the region of glu9 of the K2r gene, and both contain the E56D mutation (in bold). Forward P3 and reverse primer P7 were used to amplify a 265-bp fragment (F5) containing the genes encoding the amino-terminal region of K2r, including the C4G and E56D mutations.

Forward primer P8 and reverse primer P6 were used to amplify a 213-bp fragment (F6) containing the gene encoding for the carboxy-terminal region of K2r, including the E56D mutation. The fragments F5 and F6 were annealed together to give a complementary fragment around the region of the E56D mutation, they were employed as overlapping templates in a subsequent PCR reaction, using primers P3 and P6 to amplify a 457-bp fragment (F7). F7 is comprised of the genes for the following: α-factor signal sequence EcoRI site-SEE[K2r/C4G, E56D]AA-NotI site-3′AOX1, which was ligated into pPIC9k through the upstream EcoRI and downstream NotI site.

**Construction of pPIC9k[K2r/C4G, L72Y]—** The pPIC9k[K2r/C4G] DNA was used as the template for the generation by PCR of the double mutant K2r construct, pPIC9k[K2r/C4G, L72Y].

Reverse primer P9, 5′-GATGTCGAATATCCGACGCTTT, and forward primer P10, AAGCCTGGGAATATTGGCAGCATC, are complementary primers at the region of the Leu72 of the K2r gene, and both contain the L72Y mutation (in bold). Forward primer P3 and reverse primer P9 were used to amplify a 312-bp fragment (F8) containing the genes encoding for the amino-terminal region of K2r, including the C4G and L72Y mutations.

Forward primer P10 and reverse primer P6 were used to amplify a 169-bp fragment F9 containing the gene encoding for the carboxyl-terminal region of K2r, including the L72Y mutation.

Because fragments F8 and F9 are complementary around the region of the L72Y mutation, they were used as overlapping templates in a subsequent PCR reaction, using primers P3 and P6 to amplify a 457-bp fragment (F10). Fragment F10 is comprised of the genes for the following: α-factor signal sequence EcoRI site-SEE[K2r/C4G, E56D, L72Y], which was ligated into pPIC9k through the upstream EcoRI and downstream NotI sites as above.

**Construction of pPIC9k[K2r/C4G, E56D, L72Y]—** The same PCR strategy, described above, that was used for the generation of pPIC9k[K2r/C4G, E56D, L72Y] was used to create pPIC9k[K2r/C4G, E56D, L72Y], using pPIC9k[K2r/C4G, E56D] DNA as the template.

The final fragment (F11) was a 457-bp product comprised of the genes for the following: α-factor signal sequence EcoRI site-SEE[K2r/C4G, E56D, L72Y], which was ligated into pPIC9k as above.

**Expression and Purification of the K2r Variants**—For each of these variants, 35 transformed clones were screened for expression on a small scale. The best expressing clones from plasmids linearized with either BgiII or Sall, and transformed into the GS115 strain of Pichia pastoris, were used for methylotrophic fermentation. The media were then collected and removed by centrifugation. After adjusting the supernatant to pH 7.8, the precipitated salts were removed by centrifugation. The resulting supernatant was directly percolated over a lysine-Sepharose column that was equilibrated in 50 mM Tris-HCl, 50 mM NaCl, pH 7.8.

In the case of the K2r[C4G] mutant, the protein bound weakly to the column and was eluted with extensive application of the wash buffer, viz. 50 mM Tris-HCl, 50 mM NaCl, pH 7.8. Elution with the competing ligand, EACA, was not necessary. The retarded protein, however, bound to the column to a sufficient degree to be separated from any contaminating yeast proteins produced in the fermentation. No further purification was necessary. The yield was >100 mg/liter after purification. For K2r[C4G,E56D], purification on lysine-Sepharose column was attained by chromatography with lower ionic strength buffer than above, viz. 10 mM Tris-HCl, 10 mM NaCl, pH 7.8. Under these conditions, this peptide was retained more tightly than was the case with K2r/C4G but could still be eluted from the column in a purified form with extensive washing. However, a large fraction was eluted with 0.15 M EACA. The final yield was approximately 100 mg/liter.

In the case of K2r[C4G,E56D], the lysine-Sepharose step afforded no purification since this protein was not retarded during this process. Therefore, the media were dialyzed extensively against a solution of 25 mM NaOAc, pH 4.5, and percolated over a Mono S column pre-equilibrated in the same buffer, using fast protein liquid chromatography. A 0–0.3 linear gradient of NaCl in 25 mM NaOAc, pH 4.5, was applied to the column at 1 ml/min over 60 min. The peptide was eluted in a homogeneous state at 0.5 x NaCl. The purified peptide eluted at 0.1 M NaCl.

The variant, K2r[C4G,E56D,L72Y], did adsorb to the lysine-Sepharose column in a buffer of 50 mM Tris-HCl, 50 mM NaCl, pH 7.8. After extensive washing with this same buffer, the peptide was batch-eluted with 0.15 M EACA. A homogenous product was obtained in a yield of >160 mg/liter.

**Synthesis and Characterization of the Streptococcal Surface Protein**
**Table I**

| Peptide                  | \( M_r \) (calculated) | \( M_r \) (experimental) |
|-------------------------|-------------------------|--------------------------|
| K2\(_2\)[^C4G][^E56D]   | 10,109.54               | 10,111.40                |
| -YV                     | 9,946.05                | 9,943.16                 |
| -YVF                    | 9,717.81                | 9,713.32                 |
| K2\(_2\)[^C4G/E56D]     | 9,570.64                | 9,565.83                 |
| -YV                     | 9,932.03                | 9,933.96                 |
| -YVF                    | 9,832.89                | 9,833.63                 |
| K2\(_2\)[^C4G/L72Y]     | 10,159.24               |                          |
| -YV                     | 9,896.94                | 9,900.47                 |
| -YVF                    | 9,620.65                | 9,627.91                 |
| K2\(_2\)[^C4G/E56D/L72Y]| 10,145.24               | 10,142.40                |
| -YV                     | 9,606.62                | 9,606.91                 |

**RESULTS**

To examine the importance of specific amino acid residues in the lysine-binding site of the K2\(_2\) peptide, a series of mutations, viz. C4G, C4G/E56D, C4G/L72Y, and C4G/E56D/L72Y, were incorporated into this isolated module that would be predicted to up-regulate its LBS. To obtain sufficient amounts of the variant recombinant kringles, a *P. pastoris* expression system was employed that has been successfully used by this laboratory to obtain large amounts of other kringles from hPg, as well as from tPA (23, 26, 41–43). Large scale fermentation of the recombinant *GS115* cells produced these peptides at levels >100 mg/liter. Purification was accomplished by a combination of affinity chromatography on Sepharose-lysine and fast protein liquid chromatography and yielded very high purity (>98%) materials in all cases. Analyses of the peptides by TOF-MALDI-DE-MS indicated that several molecular weight forms were present for each of the K2\(_2\) mutants. However, each of these forms is readily identifiable as a variant of K2\(_2\) that lacks one or more amino acids at its amino-terminal stretch of non-kringle residues. More specifically, the K2\(_2\) parent form, from which all mutants were generated, was constructed to express the following amino acids at its amino terminus: NH\(_2\)-YVFSEE-[K2\(_2\)]AA.

Analytical Methods—Molecular weight analysis of the K2\(_2\) peptides was performed by isotachopherography (ITC) with an OMEGA titration calorimeter (Microcal, Inc., Northampton, MA) at 25 °C in a buffer of 100 mM sodium phosphate, pH 7.4. Peptide sequences samples in concentrations from 30 to 90 \( \mu \)M in a total volume of 1.0 ml were diluted in the reaction cell. After equilibration to a given concentration of \( \alpha \)-aminoo acids or peptide VEK30 in matching buffer was delivered at discrete intervals in 5-μl aliquots. The observed heat was measured after each injection. The total observed heat effects were corrected for the heats of dilution of ligand by performing control titrations in the absence of peptide. The resulting titration curves were deconvoluted for the best-fit model using the ORIGIN for ITC software package supplied by Microcal and yielded values for stoichiometry of binding (\( n \)), \( K_r \), and \( \Delta T \).

**Intrinsic Fluorescence Titrations**—The binding of ligands to K2\(_2\) and its variants was determined by titration of the change in intrinsic fluorescence in the kringle that occurred upon ligand binding. The experiments were carried out at 25 °C in a buffer containing 50 mM Tris-OAc, 150 mM NaOAc, pH 8.0. The \( K_r \) values that characterize the ligand-kringle interaction were calculated from the fluorescence titration curves by nonlinear least squares iterative curve fitting of the titration data (20).
dynamic properties of K2Pg[C4G], but the mutation at Leu72 (L72Y) appears to be the major basis for the increased thermal stability of K2Pg[C4G/E56D/L72Y]. The addition of high levels of EACA increases the Tm of each of these peptides by approximately 7–13 °C (Fig. 1 and Table II), suggesting that EACA interacts with all of these mutant K2Pg domains. Of particular relevance is the fact that the Tm of wild-type K2Pg can be increased by more than 20 °C through the two additional mutations illustrated and by the addition of EACA.

It is well known that the binding of ω-amino acids to kringle domains induces a large change in their intrinsic fluorescence, and this property has been employed extensively to determine the steady state thermodynamic properties of these interactions (e.g. Ref. 36). In the case of K2Pg, for all of the ligands tested, maximal fluorescence changes of 7%, at the lowest, to 75%, at the highest, were observed. All fluorescence changes were saturable and were fit to simple binding isotherms for a single binding site. Kd values for a series of lysine analogues, viz. 4-aminobutyric acid (4-ABA), 5-aminopentanoic acid (5-APA), 6-aminohexanoic acid (EACA), 7-aminohexanoic acid (7-AHA), and trans-4-aminomethyl-1-cyclohexanoic acid (t-AMCHA), were determined by this method. An example titration of K2Pg[C4G/E56D/L72Y] with EACA is illustrated in Fig. 2, which yielded a Kd value of 60 μM. All Kd values obtained by this method are listed in Table III. To obtain an independent measure of these binding constants, Kd values of several of these ligands with K2Pg[C4G/E56D/L72Y] have been measured by ITC titrations. An example of one of these titrations, that of K2Pg[C4G/E56D/L72Y] with t-AMCHA, is shown in Fig. 3. The Kd value obtained was 11 μM, which is in good agreement with the same value of 7 μM obtained by fluorescence titrations (Table III).

Finally, the relationship between the LBS of K2Pg and the binding of this kringle domain to bacterial cell surfaces has been examined. For these studies, a 30-mer peptide (VEK30) was synthesized, which consists of 6 amino acids upstream of a1 region of PAM, the 13 amino acid a1 region, and 10 amino acids from the a2 region of this protein, followed by a Tyr. This peptide has been shown to constitute the K2Pg-binding site on the group A Streptococcus pyogenes surface protein, PAM (17). Thermodynamic properties describing the binding of this peptide to K2Pg variants have been determined by ITC titrations, and an illustration of the data obtained for binding of VEK30 to K2Pg[C4G/E56D/L72Y] is provided in Fig. 4. In all cases, a single binding site for VEK30 to K2Pg is observed. A similar experiment with K1Pg did not demonstrate binding of this peptide. Based on titrations of this type with each of the K2Pg mutants constructed herein, a complete list of steady state thermodynamic binding parameters have been obtained and are summarized in Table IV. Similar to the findings with the ω-amino acid class of ligands, the strongest binding is observed for K2Pg[C4G/E56D/L72Y] (Kd = 37 nM). The binding affinity of VEK30 to K2Pg[C4G/E56D/L72Y] was reduced by nearly 100-fold (Kd = 3900 nM) when the interaction of VEK30 with K2Pg[C4G/E56D/L72Y] was measured in the presence of 50 mM EACA.

**DISCUSSION**

Kringle domains of proteins are mainly involved in interactions with other soluble or insoluble proteins or with proteins on cell surfaces. Many of these interactions are mediated by the LBS of kringle modules that are able to interact with lysine and its structural analogues. Important examples of this are the binding of hPf with proteins containing carboxyl-terminal lysine residues, such as α-eno-lase (46), plasmid-digested fibrinogen (47), and α2-antiplasmin (48). Because of these important functional roles of LBS on kringle domains of hPf, and other proteins containing kringle domains capable of interacting with lysine, many studies have appeared that have effectively characterized the mode of binding of lysine analogues to these structural motifs. In the case of hPf, the kringles containing an LBS are K1Pg, K2Pg, K4Pg, and K5Pg.

X-ray crystal structures and solution structures of of K1Pg (30, 33), K4Pg, (49), and K5Pg (23) complexed to ligands, along with site-directed mutagenesis investigations (22, 37), have identified various residues as essential for binding of ω-amino acids to the LBS, which include (K1Pg numbering) Asp54 and Asp56, Arg70, Tyr63, and Tyr71, as essential to such binding interactions. An aromatic residue (preferably a Tyr) at position 73 is also a characteristic of kringle domains with a strong LBS but not through direct participation in ligand binding (39). K2Pg also binds these types of ligands (24, 50) but much more weakly than its companion kringles. In examining the amino acid sequence of K2Pg in light of known determinants of kringle/ligand binding, it appeared that changes of Glu56 to Asp and Leu72 to Tyr would provide all primary structural elements for strong ligand binding to occur.

**Table II**

| Peptide             | Tm (°C) | ΔH (kcal/K mol) |
|---------------------|---------|-----------------|
|                     | -EACA   | +EACA           | -EACA   | +EACA   |
| K2Pg[C4G]           | 60.7 ± 0.03 | 70.5 ± 0.05 | 68.8 ± 1.3 | 79.9 ± 0.5 |
| K2Pg[C4G/E56D]      | 58.8 ± 0.07 | 71.7 ± 0.07 | 72.4 ± 0.4 | 91.2 ± 1.4 |
| K2Pg[C4G/L72Y]      | 71.7 ± 0.07 | 78.2 ± 0.07 | 71.3 ± 0.4 | 82.1 ± 0.6 |
| K2Pg[C4G/E56D/L72Y] | 70.9 ± 0.07 | 83.6 ± 0.07 | 80.2 ± 2.3 | 99.0 ± 0.5 |

**FIG. 1.** Differential scanning calorimetry thermogram of K2Pg[C4G/E56D/L72Y] (dotted line) at a concentration of 1 mg/ml and its change as a result of addition of EACA (solid line). The heat capacity at constant pressure (Cp) is plotted against the temperature. The buffers employed were 100 mM sodium phosphate, pH 7.4, or 50 mM sodium phosphate, 50 mM EACA, pH 7.4. The temperature of maximum heat capacity is 71 and 84 °C in the absence and presence of EACA, respectively.
The $T_m$ for K2$_{Pg}$ is 60.7 °C, which is within the range of those found for other isolated kringle modules. The $T_m$ values for K1$_{tPA}$ (51) and K2$_{tPA}$ (34) were 61.3 and 75.4 °C, respectively. Those same values for K1$_{Pg}$ (20), K4$_{Pg}$ (52), and K5$_{Pg}$ (52) were 67.7, 57.8, and 50.4 °C, respectively. Thus K2$_{Pg}$ is in about the mid-range of kringles in its thermal stability properties. However, insertion of the Tyr at position 72, a residue present in these other kringles (except for K1$_{tPA}$, in which a Phe is found) and the presence of which is known to lead to substantial stabilization of the native structure of K2$_{tPA}$ (39), also increases the $T_m$ of K2$_{pg}$ by approximately 8 °C. Additional placement of an Asp at position 54 lends further stability to K2$_{Pg}$, characterized in this report, appear to be typical of kringle motifs. With this additional characterization of another hPg structural domain, a time is approaching when all of the properties of the individual modules can be compared with their same properties in the intact protein and the information employed to assess rigorously their structural independence.

The binding of a series of $\omega$-amino acids to wild-type-K2$_{Pg}$ (K2$_{Pg}$(C4G)) demonstrated an order of binding of $t$-AMCHA (K2$_{Pg}$(C4G/E56D/L72Y)) with 26 automated 5-$\mu$l injections of a 2 mM $t$-AMCHA stock solution. Lower panel, best-fit of the thermodynamic parameters characterizing the incremental heat change accompanying binding. The data in the upper panel were deconvoluted, and the solid line represents the least squares best-fit to the data, providing $n = 0.88$, $K_d = 11 \mu\text{M}$, and $\Delta H = -3.6 \text{ kcal/mol}$.

![Graph](image1)

### Table III

| Peptide          | 4-ABA  | 5-APA  | EACA   | 7-AHA  | $t$-AMCHA |
|------------------|--------|--------|--------|--------|-----------|
| K2$_{Pg}$(C4G)   | 1420 ± 170 | 330 ± 15 | 560 ± 32 | 2030 ± 62 | 94 ± 10   |
| K2$_{Pg}$(C4G/E56D) | NT    | 105 ± 30 | 136 ± 40 | 600 ± 50  | 12 ± 0.7  |
| K2$_{Pg}$(C4G/L72Y) | 4970 ± 1100 | 1110 ± 290 | 2110 ± 260 | 10,100 ± 2200 | 500 ± 140 |
| K2$_{Pg}$(C4G/E56D/L72Y) | 380 ± 23  | 60 ± 4  | 60 ± 2.3 | 253 ± 31  | 7 ± 0.4   |

FIG. 2. Binding of EACA to K2$_{Pg}$(C4G/E56D/L72Y) as determined by the change in intrinsic fluorescence. The buffer employed was 50 mM Tris-Oac, 150 mM NaOAc, pH 8.0, at 25 °C, at a protein concentration of 3 $\mu\text{M}$. Derived from the curve is a nonlinear least squares best-fit line at $n = 1$ and $K_d = 60 \mu\text{M}$.

Different $t$-AMCHA to K2$_{pg}$ as determined by isothermal titration calorimetry. The buffer was 100 mM sodium phosphate, pH 7.4, at 25 °C, at a protein concentration of 90 $\mu\text{M}$. Upper panel, heat change accompanying titration of K2$_{pg}$(C4G/E56D/L72Y) with 26 automated 5-$\mu$l injections of a 2 mM $t$-AMCHA stock solution. Lower panel, best-fit of the thermodynamic parameters characterizing the incremental heat change accompanying binding. The data in the upper panel were deconvoluted, and the solid line represents the least squares best-fit to the data, providing $n = 0.88$, $K_d = 11 \mu\text{M}$, and $\Delta H = -3.6 \text{ kcal/mol}$.

![Graph](image2)
**FIG. 4. Binding of the PAM-derived peptide, VEK30, to K2\textsubscript{pG}[(C4G/E56D/L72Y)] as determined by isothermal titration calorimetry.** The buffer was 100 mM sodium phosphate, pH 7.4, at 25 °C. The top panel represents incremental heat changes accompanying the titration of K2\textsubscript{pG}[(C4G/E56D/L72Y)] with VEK30 at 25 °C. A series of 5-μl injections of a 0.75 mM stock solution of VEK30 was added to the sample cell containing 30 μM K2\textsubscript{pG}[(C4G/E56D/L72Y)]. The bottom panel illustrates the peak areas (heat) from above plotted against the molar ratio of PAM to K2\textsubscript{pG}[(C4G/E56D/L72Y)]. The line is the best-fit of the data to a binding isotherm characterized by \( n = 0.9, K_d = 37 \text{ nM}, \) and \( \Delta_H = -20.3 \text{ kcal/mol}. \)

**TABLE IV**

**Thermodynamic parameters of binding of PAM peptide to K2\textsubscript{pG} variants as measured by isothermal titration calorimetry**

| Peptide         | n   | \( K_d \) | \( \Delta G \) | \( \Delta H \) | \( \Delta S \) |
|-----------------|-----|----------|--------------|-------------|-------------|
| K2\textsubscript{pG}[(C4G)] | 0.80 | 460 | -8.6 | -19.5 | -10.9 |
| K2\textsubscript{pG}[(C4G/E56D)] | 0.83 | 430 | -8.7 | -17.4 | -8.7 |
| K2\textsubscript{pG}[(C4G/L72Y)] | 0.91 | 2160 | -7.7 | -17.0 | -9.3 |
| K2\textsubscript{pG}[(C4G/E56D/L72Y)] | 0.90 | 37 | -10.3 | -19.9 | -9.6 |

Erated in this report is a synthetic peptide, VEK30, containing the a1a2 repeat of PAM. The data in Table IV clearly demonstrate that a single binding site for VEK30 exists on K2\textsubscript{pG}. The \( K_d \) value of 460 nM for the interaction of VEK30 with K2\textsubscript{pG} is virtually unchanged by the E56D mutation and is down-regulated by the single change of L72Y. These properties of the mutants are similar to those observed in Table III for their binding interactions with the \( \omega \)-amino acid group of ligands. Also similar is the fact that as a result of generation of the a1a2 repeat of PAM, the data in Table IV clearly demonstrate that binding sites are largely preformed in the K2\textsubscript{pG} domain, as is the case with other \( \omega \)-amino acid-binding kringles. Since a free carboxyl-terminal lysine group does not exist in the peptide, the nature of its interaction with the LBS of K2\textsubscript{pG} is of great interest. It is unlikely that a side chain lysine residue could provide the energy for such tight binding in the absence of the added stability that would be provided by the ligand carboxylate group. However, there are several side chain glutamates present in this peptide that may form a pseudo-lysine when appropriately positioned with the side chain lysine. Examination of the amino acid sequence of peptide VEK30 shows that Lys\textsubscript{14} and Lys\textsubscript{27} are flanked by Glu\textsubscript{15} and Glu\textsubscript{28} provided by the ligand carboxylate group. However, there are several side chain glutamates present in this peptide that may form a pseudo-lysine when appropriately positioned with the side chain lysine. Examination of the amino acid sequence of peptide VEK30 shows that Lys\textsubscript{14} and Lys\textsubscript{27} are flanked by Glu residues and, in combination, might provide a suitable ligand for the LBS.

As seen from the thermodynamic box of Fig. 5, alterations of Glu\textsubscript{56} and Leu\textsubscript{72} are not additive in their effects but synergistic. Furthermore, the coupling energy between E56D and L72Y of -2.43 kcal/mol is equal to that of approximately the generation of a single net hydrogen bond. Interestingly, we proposed earlier in the case of K2\textsubscript{tPA} (55) that the OH of Tyr\textsubscript{76} (equivalent to Tyr\textsubscript{72} of K2\textsubscript{pG}[(C4G/E56D/L72Y)]) is sufficiently close to the OD1 atom of Asp\textsubscript{59} (equivalent to Asp\textsubscript{56} of K2\textsubscript{pG}[(C4G/E56D/L72Y)]) such that a hydrogen bond is possible between these side chains, which may stabilize the binding pocket. Whether distance considerations are appropriate to allow this hydrogen bond to form in K2\textsubscript{pG} awaits more detailed structural information.

In conclusion, the results of this study have shown that strategic rational and knowledge-based minimal mutagenesis of K2\textsubscript{pG} can be employed to up-regulate its LBS, which then can be employed to engineer enhanced functionality into this kringle domain, and likely then into the parent molecule, hPg. This shows the power of approaches that investigate properties of isolated regions of proteins of this type to redesign the characteristics of these proteins.

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