CMLS, Cell. Mol. Life Sci. 55 (1999) 135–141
1420-682X/99/010135-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Expression, isolation and characterization of a mutated human plasminogen kringle 3 with a functional lysine binding site

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Received 17 November 1998; accepted 19 November 1998

Abstract. Each kringle of human plasminogen (HPg) except kringle 3 (K3) exhibits affinity for ω-aminocarboxylic acids. Assuming that the K3 domain contains a preformed but nonfunctional lysine binding site (LBS), Lys^{311} was altered by site-directed mutagenesis into Asp^{311} in accordance with the consensus sequence of the LBS. Cys^{297} involved in the interkringle disulfide bridge was mutated into Ser^{297} to minimize dimerization and aggregation. The mutated K3 TYQ[$K3_{HPg}$/C297S/K311D]DS (r-$K3_{mut}$) was expressed in *Escherichia coli*, isolated on an Ni^{2+}-nitrilotriacetic acid-agarose column, refolded and purified on a lysine Bio-Gel column. Fluorescence titration indicates affinity of r-$K3_{mut}$ for ω-aminocarboxylic acids with the following association constants (K_{ass}, mM^{-1}): 5-aminopentanoic acid: 1.3; 6-aminohexanoic acid: 4.2; 7-aminoheptanoic acid: 0.5; *trans*-(aminomethyl)cyclohexanecarboxylic acid: 12.7; *p*-benzylaminesulfonic acid: 11.8. r-$K3_{mut}$ exhibits an affinity similar to native and mutated (R220G, E221D) K2. The results indicate the presence of a preformed but nonfunctional LBS in native K3 of HPg. We were able to demonstrate for the first time that an appropriate mutation in the LBS of a kringle produced a weak but distinct affinity for ω-aminocarboxylic acids.

Key words. Kringle domain; lysine binding site; ω-aminocarboxylic acids; mutated kringle 3; human plasminogen.

Plasminogen (Pg) is the main component of the fibrinolytic system, where its activated form, plasmin (Pm), is responsible for the proteolytic degradation of fibrin clots [1]. In addition, the Pg-Pm system plays a major role in cell migration by assisting cell permeability [2]. Moreover, angiostatin, comprising the kringle 1–4 (K1–4) region of human plasminogen (HPg), has been identified as an inhibitor of angiogenesis [3, 4] and the K5 domain of HPg as an inhibitor of endothelial cell growth [5, 6].

HPg is a single-chain proenzyme of 791 amino acids [7] with a molecular weight ranging between 90 kDa and 92 kDa depending on the extent of glycosylation [1, 8]. HPg is composed of three structurally distinct regions:

1) The N-terminal region, also called preactivation peptide, corresponding to Glu^{1} to Lys^{77}. It is thought to be responsible for the closed and activation-resistant conformation of Pg [9]. The presence of two plasminogen-related genes termed PRG-A and PRG-B with a pronounced similarity to the preactivation peptide of Pg has been reported [10, 11].

2) Five homologous domains called kringles from Lys^{78} to Arg^{561} corresponding to the heavy chain of Pm. It is through these kringles that Pg interacts with its ligands.

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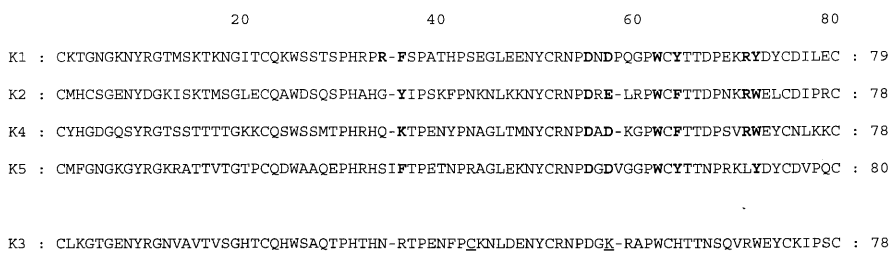

Figure 1. Sequence alignment of the five HPg kringles. The amino acids involved in the LBS are shown in bold type. The underlined Cys and Lys residues in K3 were mutated into Ser and Asp, respectively (r-$K3_{mut}$).

3) The catalytic domain from Val^{562} to Asn^{791} corresponding to the Pm light chain with the typical serine protease triad His^{603}, Asp^{646} and Ser^{741}.

The kringles are autonomous folding units [12] of about 80 amino acids containing three disulfide bonds with the typical pattern C1–C6, C2–C4, C3–C5. Pg and hepatocyte growth factors are the only kringle-containing proteins with an interkringle disulfide bridge between K2 and K3. Kringle-carrying proteins occur primarily in blood coagulation and fibrinolysis such as Pg with five copies, tissue-type plasminogen activator (tPA) [13] and prothrombin [14] with two copies, urokinase-type plasminogen activator (uPA) [15] and factor XII [16] with one copy, hepatocyte growth factor [17] and hepatocyte growth factor-like protein [18] with four copies and human apolipoprotein a [19] with up to 38 copies. The three-dimensional structure of several kringles has been determined by X-ray crystallography for HPg K1 [20, 21], HPg K4 [22, 23], HPg K5 [24], human tPA K2 [25], bovine prothrombin K1 [26] and K2 [27] and the 37th K4-type kringle of apolipoprotein a [28], and by nuclear magnetic resonance (NMR) for HPg K1 [29], HPg K4 [30] and horse Pg K4 [31], human tPA K2 [32] and uPA [33, 34]. The kringle domains are thought to be responsible for specific binding and regulatory functions in their parent proteins. The Pg kringles mediate binding to fibrin, to receptors on cell surfaces and to activators and inhibitors. Interaction with the ligand occurs through the lysine binding site (LBS): the consensus model [35] places the ligand carboxylate group in ionic interaction with Arg^{71} (and possibly with Arg^{35}/Lys^{35}), whereas the amino group of the same ligand molecule ion-pairs with the anionic side chains of Asp^{55} and Asp^{57}/Glu^{57}. Ligand binding is further stabilized via van der Waals interactions of the ligand's hydrophobic backbone with hydrophobic side chains of the LBS: Phe^{36}/Tyr^{36}, Trp^{62}, Tyr^{64}/Phe^{64} and Tyr^{72}/Trp^{62}. The association constants for 6-aminohexanoic acid (6-AHA) HPg kringles decrease in the order of (in mM^{-1}): r-K1 = 74.2, K4 = 21.0, K5 = 10.6 and r-K2 = 2.3 [36] and references therein. K3 does not show any affinity for ω-aminocarboxylic acids [37, 38]. This variation can partially be explained by sequence differences in the LBS of each kringle. The positively charged center and the hydrophobic aromatic groove of the LBS are quite well conserved in most kringles. The negatively charged center is usually composed of an aspartic acid followed by a small, neutral amino acid and another aspartic acid. In the case of K2 the neutral amino acid and the second aspartic acid are replaced by an arginine and a glutamic acid, respectively. For instance, mutated K2 (R56G and E57D, kringle numbering), exhibits an increased affinity for 6-aminohexanoic acid, $K_{ass} = 3.9\ mM^{-1}$ [36] compared with 2.3 mM^{-1} for native K2. In the case of K3 the second aspartic acid is replaced by a lysine, which leads to loss of the negative charge in the anionic center. Assuming that K3 contains a preformed LBS, the appropriate mutation should render the nonbinding K3 into a binding form. The choice of mutations is dictated by the consensus sequence of the other kringles in the LBS (fig. 1).

Materials and methods

Materials

Polyclonal goat anti-HPg antibodies and alkaline phosphatase conjugated to rabbit anti-goat immunoglobulin G (IgG) antibodies were purchased from Sigma (St. Louis, MO, USA). Restriction endonucleases were obtained from Boehringer Mannheim (Mannheim, Germany). Taq DNA polymerase, calf intestinal alkaline phosphatase and T4 DNA ligase came from Promega (Madison, WI, USA). Lysine Bio-Gel P-300 (Bio-Rad, Hercules, CA, USA) was prepared according to Brunisholz et al. [39]. Ni^{2+}-NTA-agarose was purchased from QIAgen (Basel, Switzerland). Agarose and Prep-A-Gene DNA purification came from Bio-Rad. The primers were synthesized by Microsynth (Windisch, Switzerland). For the large-scale plasmid isolation, the QIAgen tip-100 columns were used. DNA sequencing

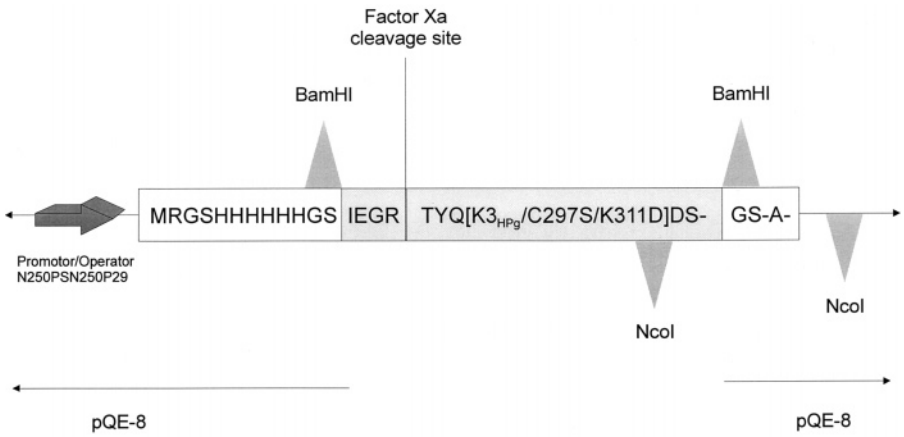

Figure 2. Construct of r-$K3_{mut}$. The FXa-sensitive cleavage site and the mutated kringle 3 were introduced into the single *Bam*HI cleavage site of the vector pQE-8. The stop codons are indicated by (-). The two *Nco*I restriction sites, one in the kringle-coding DNA and the other in the vector, help to verify the orientation of the inserted DNA.

was performed by alkaline denaturation of double-stranded DNA followed by dideoxynucleotide chain termination [40] using the Sequenase 2.0 Kit from USB (Cleveland, OH, USA). FXa was obtained from Haematologic Technologies Inc. (Essex Jct., VT, USA).

Bacterial strains and plasmids. *Escherichia coli* strain M15 (F^- Str^R $lacZ^{del}$) [41] was purchased from QIAgen and used for expression of the kringle. *E. coli* strain HB101 [42] was used for routine transformations and plasmid preparations. The plasmid pQE-8 [43], used for the expression of the mutated K3, was also from QIAgen. The plasmid pPLGKG [44], which contains the complete complementary DNA (cDNA) sequence of HPg, was kindly provided by Prof Hedén (University of Lund, Sweden).

Methods

Construction of the protein expression vector for the mutated kringle 3. Starting from pPLGKG [44], the DNA was mutated and amplified as described by Mikaelian and Sergeant [45], and the following primers were required:

The 5′ primer which binds to the noncoding strand of the HPg cDNA is used to introduce a *Bam*HI restriction endonuclease site and a cDNA segment coding for the coagulation factor Xa-sensitive cleavage site upstream of the codon for Thr^{253}.

Primer 1: 5′-GCGGATCCATCGAGGGTAGAA-CCTACCAGTGTCTGAAGGGA-3′

The 3′ primer, complementary to a region of the coding strand, is used to introduce a stop codon and a *Bam*HI restriction endonuclease site following the codon for Ser^{335}.

Primer 2: 5′-GCGGATCCCTAGGAGTCACA-GGACGG-3′

The mismatch primer which binds to the noncoding strand of the HPg cDNA is used to allow amplification of the DNA containing the mutation. It binds to a DNA region upstream of the region of the two introduced mutations. The 17 bases from the 3′ end are complementary to the noncoding cDNA strand, and the 8 bases at 5′ end do not hybridize to the cDNA.

Primer 3: 5′-ATATTTGGTGTGGCTGTTACC-GTTT-3′

The mutation primers, complementary to a region of the coding strand, contain the information for the mutations C297S (primer 4) and K311D (primer 5) with 10 bases hybridizing to the DNA at both ends.

Primer 4: 5′-AAATTTTTGGAGGGGAAGT-3′
Primer 5: 5′-CCATGGGGCCCTGTCTCCG-TCAGGATT-3′

The DNA coding for the mutated K3 is the product of several polymerase chain reactions (PCRs) using Taq DNA polymerase: (i) PCR of HPg cDNA with primers 1 and 4 and PCR of HPg cDNA with primers 3 and 2; (ii) PCR of the DNA products of (i) with primers 1 and 2; (iii) PCR of the DNA product of (ii) with primers 1 and 5 and PCR of the DNA product of (ii) with primers 3 and 2; and (iv) PCR of the DNA products of (iii) with primers 1 and 2.

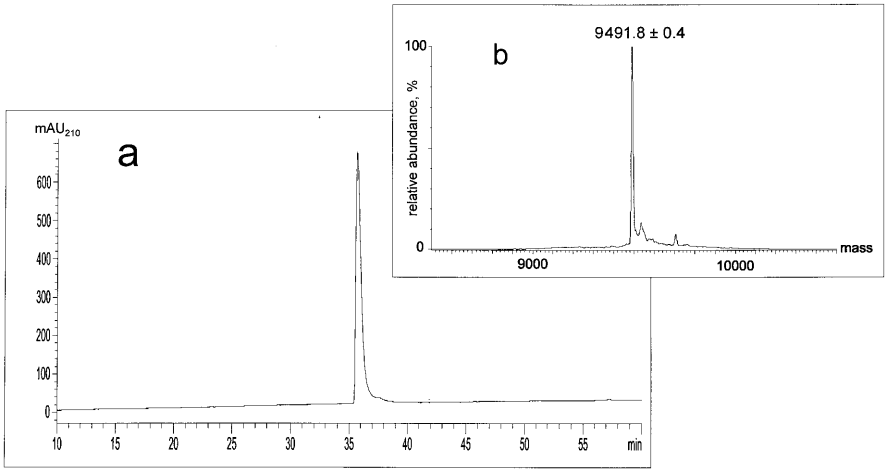

Figure 3. (*a*) RP-HPLC after final processing of r-K3$_{mut}$ with FXa using a linear acetonitrile gradient (0–50% solution B in 60 min). Solution A: 0.1% TFA in water; solution B: 0.1% TFA, 80% acetonitrile in water. (*b*) Positive ESI-MS of r-K3$_{mut}$ (calculated mass: 9492.3 Da) dissolved in H_2O/acetonitrile (v:v, 1:1) containing 0.5% formic acid

The PCR products were purified on 1.6% agarose gels before use in the next step, and PCR product (iv) was first digested with *Bam*HI and then purified. The final product was cloned into the *Bam*HI cleaved and dephosphorylated pBR322 [46] for sequence analysis of both strands [40]. The DNA coding for the mutated K3 TYQ[K3$_{HPg}$/C297S/K311D]DS (r-K3$_{mut}$) was ligated into the single *Bam*HI restriction site of pQE-8 and transformed into the *E. coli* strain M15 containing the repressor plasmid pREP4. The orientation of the insert was checked by digestion with *Nco*I.

Expression and isolation of r-K3$_{mut}$. The expression and isolation of r-K3$_{mut}$ was carried out according to a slightly modified protocol of Marti et al. [37]. The cells were grown at 37 °C in 2× YT medium (100 μg ampicillin/ml and 25 μg kanamycin/ml) to an OD_{600} of about 0.7–0.9. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM to induce the production of r-K3$_{mut}$, and the cells were grown for another 4.5 h at 37 °C and were harvested by centrifugation for 30 min (4000*g*, 4 °C). The cell paste was stored at −80 °C until use.

Table 1. Values of the K_{ass} of different ligands for r-K3$_{mut}$ measured by fluorimetric titration as described under methods.

Ligand	Dipole length (Å)	K_{ass} (mM^{-1})
5-APA	6.19	$K_{ass} = 1.28 \pm 0.11$
6-AHA	7.33	$K_{ass} = 4.16 \pm 0.15$
7-AHA	8.65	$K_{ass} = 0.52 \pm 0.08$
AMCHA	6.16	$K_{ass} = 12.66 \pm 0.46$
BASA	6.64	$K_{ass} = 11.75 \pm 0.38$

The relative amount of the r-K3$_{mut}$ was determined by SDS-polyacrylamide gel electrophoresis (PAGE) (15%) and blotted onto a nitrocellulose membrane. The proteins were visualized by Ponceau S staining, and the recombinant kringle was identified by enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies against native HPg.

For the isolation of r-K3$_{mut}$, the cell paste was thawed and suspended in the extraction buffer (6 M guanidine hydrochloride in 0.1 M sodium phosphate, pH 8; 5 ml/g cell paste). The suspension was stirred overnight at 4 °C and centrifuged for 30 min (12,000*g*, 4 °C). The supernatant was loaded on an Ni^{2+}-nitrilotriacetic acid (NTA)-agarose column equilibrated with the extraction buffer, pH 8. The column was successively washed with the extraction buffer, pH 8 and pH 6.3, and the r-K3$_{mut}$ was eluted with the extraction buffer, pH 5.

Refolding and isolation. The refolding of r-K3$_{mut}$ was carried out according to a protocol similar to that of Cleary et al. [47]. The pH of the kringle-containing solution was adjusted to pH 8, and dithiothreitol (DTT) was added to a final concentration of 5 mM. After stirring overnight at 4 °C, the solution was slowly diluted during 3 h with 4 volumes of 50 mM Tris-HCl, pH 8, containing 1.25 mM of reduced and oxidized glutathione each and stirred for an additional day at 4 °C. The protein was dialyzed against water for 4 days, lyophilized and desalted on a Sephadex G-15 column equilibrated with 50 mM NH_4HCO_3 and again lyophilized.

After dissolving the r-K3$_{mut}$ in 50 mM sodium phosphate buffer, pH 7.5, the protein was loaded onto a

lysine Bio-Gel column, washed with the loading buffer, and r-$K3_{mut}$ was eluted with the same buffer containing 50 mM 6-AHA. Finally, the protein was desalted and lyophilized as described above.

Cleavage with FXa. To cleave off the His-tag, r-$K3_{mut}$ was incubated in 50 mM Tris–HCl, pH 8, containing 100 mM NaCl (1 mg/ml) [48] for 24 h at 37 °C at an enzyme/substrate ratio 1:100 (w/w). Finally, the kringle was separated from the His-tag on a Sephadex G-50 column in 50 mM NH_4HCO_3.

RP-HPLC. Reversed-phase high-pressure liquid chromatography (RP-HPLC) was carried out on an Aquapore butyl column (2.1 mm × 100 mm, wide pore 30 nm, 7 μm, Applied Biosystems) with a Hewlett-Packard liquid chromatograph 1090. A linear acetonitrile gradient (0–50% solution B in 60 min) was used at a flow rate of 0.3 ml/min with 0.1% TFA in water as solution A and 0.1% TFA, 80% acetonitrile in water as solution B.

Amino acid analysis. Samples were hydrolyzed in the gas phase with 6 M HCl containing 0.1% (v/v) phenol for 24 h at 115 °C under vacuum according to Chang and Knecht [49]. The liberated amino acids were reacted with phenylisothiocyanate, and the resulting phenylthiocarbamyl amino acids were analyzed by RP-HPLC on a Nova Pack C18 column (3.9 mm × 150 mm, 4 mm; Waters) in a Hewlett-Packard liquid chromatograph 1090 with an automatic injection system according to Bidlingmeyer et al. [50]. The 0.14 M sodium acetate buffer, pH 6.3, was replaced by the corresponding ammonium acetate buffer.

Amino acid sequence analysis. N-terminal amino acid sequence analysis was carried out using Edman degradation in a pulsed liquid-phase sequenator 477A from Applied Biosystems using a program adapted from Hunkapiller et al. [51]. The released amino acids were analyzed on-line by RP-HPLC.

Molecular mass analysis. The mass of the r-$K3_{mut}$ was determined by electrospray ionization mass spectrometry (ESI-MS) (VG Platform, Micromass, Manchester, UK).

Fluorimetric titration. The effect of the ligand on the intrinsic fluorescence of the r-$K3_{mut}$ was measured in 50 mM sodium phosphate, pH 8, at 25 °C on a FluoroMax spectrofluorometer (SPEX Industries Inc., Edison, NJ, USA) as reported by Menhart et al. [52]. In a 5 μM protein solution the ligand concentration was enhanced in 100 μM steps for 6-AHA, *trans*-(aminomethyl)cyclohexanecarboxylic acid (AMCHA) and *p*-benzylaminesulfonic acid (BASA) and in 300 μM steps for 5-aminopentanoic acid (5-APA), 7-aminoheptanoic acid (7-AHA) until the change of the intrinsic fluorescence was constant. Fluorescence titration data were interpreted according to Scatchard [53].

Results and discussion

The construction of the DNA coding for the r-$K3_{mut}$ was carried out according to a protocol of Mikaelian and Sergeant [45]. The C297S mutation was required to avoid a free thiol group in order to minimize dimerization and aggregation. However, previous experiments with wild-type r-K3 containing a single Cys residue at position 297 indicated that the free thiol group was not interfering with the refolding process and that the three intrakringle disulfide bridges were correctly formed [37]. Although wild-type r-K3 exhibits no measurable affinity for ω-aminocarboxylic acids, we assume that K3 also possesses a preformed but nonfunctional LBS. The introduction of the K311D mutation in the anionic center of the LBS should enable us to verify this hypothesis. The applied strategy for the expression and isolation of r-$K3_{mut}$ corresponds mainly to the protocols reported by Marti et al. [37] and by Söhndel et al. [38]. Specific isolation of the expressed r-$K3_{mut}$ was ensured by fusing it at the N-terminal end to a His-tag suitable for affinity chromatography on an Ni^{2+}-NTA-agarose column. Introducing an FXa-sensitive sequence I-E-G-R between the His-tag and the N-terminal part of the kringle construct ascertained the correct processing of r-$K3_{mut}$ protein after isolation (fig. 2). The correctly refolded r-$K3_{mut}$ was finally purified on a lysine Bio-Gel column.

DNA manipulation. Analysis of the PCR products on agarose gels allowed us to control the correct length of the DNA bands as well as the required purification step for the next PCR and the restriction digest. The DNA sequence analysis confirmed that the mutations were correctly introduced and that unwanted mutations did not appear in the r-$K3_{mut}$-coding DNA [40]. Finally, the orientation of the *Bam*HI-digested insert in the single *Bam*HI restriction site of pQE-8 was controlled by restriction analysis with *Nco*I.

Purification and characterization of r-$K3_{mut}$. Preliminary identification of the recombinant protein was performed by SDS-PAGE and by immunoblotting. The results show, as expected, a new band appearing after induction with IPTG at about 14 kDa with a high level of expression in accordance with the results from other kringles. The r-$K3_{mut}$ was isolated by affinity chromatography on an Ni^{2+}-NTA-agarose column with a yield of about 1.5 mg/g of wet cell. To minimize precipitation losses during the refolding process, a slow buffer change is imminent for a successful isolation. Purification of the correctly refolded protein was achieved by affinity chromatography on a lysine Bio-Gel column, which also indicated the presence of a properly shaped LBS. The amino acid composition was in good agreement

with the theoretical numbers, and the measured mass of 11,347.4 ± 0.5 Da agreed well with the calculated value of 11,346.3 Da. After the final processing of r-$K3_{mut}$ with FXa, the purity of the material was controlled by RP-HPLC (fig. 3a). Mass analysis confirmed the complete removal of the His-tag peptide, and the measured mass of 9491.8 ± 0.4 Da (fig. 3b) is very close to the calculated mass of 9492.3 Da. N-terminal sequence analysis yielded the expected sequence T-Y-Q-X-L. These combined results confirm the correct expression, refolding and processing of the mutated K3.

Fluorimetric titration. Adsorption of r-$K3_{mut}$ on a lysine Bio-Gel column was already a good indication for the presence of a functional LBS. In order to be able to quantify the affinity of r-$K3_{mut}$ for ω-aminocarboxylic acids, fluorescence titration experiments were carried out. The intrinsic fluorescence change in proteins during addition of an appropriate ligand is based on conformational changes in the vicinity of tryptophan residues caused by ligand binding. Therefore, fluorescence measurements are a suitable method to ascertain ligand-protein interactions. The linearization of the fluorimetric titration curve allows us to calculate the affinity of the ligand for the kringle as reported by Scatchard [53].

In contrast to native K3, r-$K3_{mut}$ shows a weak but distinct affinity for ω-aminocarboxylic acids (table 1). In the case of 6-AHA, the K_{ass} value of 4.2 mM^{-1} for r-$K3_{mut}$ is between those for K5 (K_{ass} 10.6 mM^{-1}) and K2 (K_{ass} 2.3 mM^{-1}). For linear ω-aminocarboxylic acids r-$K3_{mut}$ exhibits the highest affinity for 6-AHA as in the case of K1, K4 and K5 of HPg. The rapid decrease in the K_{ass} values for 5-APA (K_{ass} 1.3 mM^{-1}) and 7-AHA (K_{ass} 0.5 mM^{-1}) indicates that the two charged centers in mutated K3 are separated by a distance of about 7 Å, which corresponds approximately to the length between the amino and carboxyl groups of the lysine side chains in natural ligands. The cyclic ligands AMCHA (K_{ass} 12.7 mM^{-1}) and BASA (K_{ass} 11.8 mM^{-1}) both have a stronger affinity for r-$K3_{mut}$ than 6-AHA, although the distance between their ionic centers is about 0.5–1.0 Å shorter. This phenomenon has already been reported recently in some detail by Marti et al. [36] for all the other kringles of HPg. Most likely, the more extended surface area of the cyclic ligands allows a more pronounced hydrophobic interaction with the aromatic residues of the LBS. Mutated K3 (K311D) is no exception, and nicely fits into this picture.

Although native K3 shows no affinity for ω-aminocarboxylic acids, an appropriate mutation in the expected LBS (K311D) converts it from a nonbinding to a binding form. The data presented are good evidence for the presence of a preformed but nonfunctional LBS in the native K3 of HPg. We have been able to show for the first time that appropriate alteration by site-directed mutagenesis of the expected but nonfunctional LBS in a kringle produced a weak but distinct affinity for ω-aminocarboxylic acids. Ongoing NMR studies of both native and mutated K3 should provide final and conclusive evidence of a preformed LBS in K3 of HPg.

Acknowledgements. We thank Mr. U. Kämpfer for expert technical assistance, Prof. E. E. Rickli for supervising this work at the early stages and Dr. S. Söhndel for expert technical support. These studies were supported by grant 31-39298.93 from the Swiss National Science Foundation.

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