Protein–Ligand Interactions in the Lysine-Binding Site of Plasminogen Kringle 4 Are Different in Crystal and Solution. Electrostatic Interactions Studied by Site-Directed Mutagenesis Exclude Lys35 as an Important Acceptor in Solution

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ABSTRACT: Three amino acid residues previously reported to establish the interactions between lysine-like derivatives and plasminogen kringle 4 have been replaced by other residue types using the methods of site-directed mutagenesis. The effect of these modifications on the binding constant have been measured. The residues are Lys35, Asp57, and Arg71, according to the sequence numbering scheme adapted from the plasminogen kringle 5 domain. The plasminogen kringle 4 derivatives where Lys35 of the native molecule is replaced with isoleucine and methionine residues, respectively, were seen to bind the ligands, respectively, with association constants similar to those of the unmodified recombinant kringle 4 domain. The modification of Asp57 to asparagine was shown to eliminate the ability to bind to the lysine affinity column used to purify the protein. Similarly the site-directed mutagenesis for Arg71 to glutamine resulted in a 12–19-fold decrease in binding of each of the two ligands. In addition, the effect of ionic strength on the binding of 6-aminohexanoic acid to the recombinant plasminogen kringle 4 and the three single substituted derivatives was examined. For the unmodified kringle domain as well as for the two derivatives modified only at the position of Lys35, an ionic strength of 0.5 M reduced the binding constant by a factor of 3 to 0.12×10^5 M^{-1}. The derivative modified at the position of Arg71 was not effected by the ionic strength and maintained a rather low binding constant of 0.02×10^5 M^{-1}. The observations suggest that the carboxylate of Asp57 and the guanidino group of Arg71 provide the electrostatic interaction in the binding site for the ϵ-amino group and the α-carboxylate of a C-terminal lysine residue. These chemical observations are only in part in agreement with the studies by X-ray diffraction of the crystallized complex of plasminogen kringle 4 and 6-aminohexanoic acid. Whereas in solution there is no indication that Lys35 is important for the binding of these ligands, in the crystal Lys35 N^{ζ} is seen to be only 0.272 nm from one of the carboxylate oxygens of 6-aminohexanoic acid. The presence of the hydrogen bond and the electrostatic effects implied by this short distance is not in agreement with the finding that replacement of the lysine residue with an uncharged residue has no effect on binding of neither 6-aminohexanoic acid nor *trans*-(aminomethyl)-cyclohexane-1-carboxylic acid.

Plasminogen is the precursor of plasmin, a serine proteinase that is active in fibrinolyses. The active plasmin consists of two chains, a light chain forming the serine proteinase domain and a heavy chain containing five homologous, autonomous kringle domains (Claeys et al., 1976; Magnusson et al., 1976; Sottrup-Jensen et al., 1978; Trexler & Patthy, 1983). These are known to mediate the binding of plasmin to fibrinogen (Fleury & Anglés-Cano, 1991; Rouy et al., 1992; Wu et al., 1990; Christensen, 1985; Rejante et al., 1991; Váli & Patthy, 1984; Wiman et al., 1979). Studies of the binding have demonstrated that it is possible to inhibit the interaction between plasminogen and fibrin by addition of the lysine analogues 6-AHA[1] or AMCHA. These ligands are therefore often referred to as antifibrinolytic agents (Okamoto, 1957; Lucas et al., 1983). A consistent hypothesis, describing the role of lysine-binding sites in fibrinolysis, has been proposed by Wiman and Collen (1978). They assume that the lysine binding sites in plasminogen are identical to the binding sites for fibrin and α_2-antiplasmin.

The five kringles in plasminogen are members of a family of domains with a common structural motif. All members have a high identity in primary structure and have a conserved fold. They have approximately 80 amino acids organized in three loops defined by three cystine bridges. Since the kringle domain was first described by Magnusson et al. (1975), kringle domains have been found in many blood proteins. Besides the one in prothrombin (Magnusson et al., 1975), there are five in plasminogen (Sottrup-Jensen et al., 1978), one in urokinase (Steffens et al.,1982), two kringles in t-PA (Pennica et al., 1983), one in coagulation factor XII (McMullen & Fujikawa, 1985), 38 in apolipoprotein(a) (McLean et al., 1987), and three kringles in human hepatocyte growth factor (Nakamura et al., 1989). The different domains seem to originate from gene duplication (Patthy, 1985) and are all supposed to be involved in protein–protein interactions (Rejante et al., 1991; Wiman & Collen, 1978), some probably

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[1] Abbreviations: AMCHA, *trans*-(aminomethyl)cyclohexane-1-carboxylic acid; 6-AHA, 6-aminohexanoic acid; 7-AHA, 7-aminoheptanoic acid; PKr4, human plasminogen kringle 4; t-PA, tissue-type plasminogen activator; PKr1, human plasminogen kringle 1; PKr5, human plasminogen kringle 5; t-PAKr2, tissue-type plasminogen kringle 2; wt, wild type; r, recombinant; pH*, pH-meter reading uncorrected for deuterium isotope effects; the numbering used in the amino acid sequence is based on the plasminogen kringle 5 convention.

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in intramolecular interactions (Váli & Patthy, 1982; Thorsen et al., 1981; Mangel et al., 1990), and some in intermolecular interactions (Wiman & Collen, 1978; Thorsen et al., 1981; Wiman et al., 1979). Of these, some kringle domains are known to bind ω-amino acids although with different specificity; these are PKr1 (Lerch et al., 1980; Váli & Patthy, 1984), PKr4 (Lerch et al., 1980; De Marco et al., 1987; Menhart et al., 1991), PKr5 (Novokhatny et al., 1989; Thewes et al., 1990; Christensen, 1984), and t-PAKr2 (van Zonneveld et al., 1986; DeSerrano & Castellino, 1992). PKr4 interacts specifically with ω-amino acids with a distance of 0.68 nm between the anionic and cationic loci (Winn et al., 1980). The binding site has been thoroughly investigated by chemical modification (Trexler et al., 1982, 1983), by NMR (Petros et al., 1989; Trexler et al., 1983; Ramesh et al., 1987; De Marco et al., 1986, 1987), and by X-ray crystallography (Wu et al., 1991; Mulichak et al., 1991). These studies have identified the amino acids important for binding and shown Trp72 to be essential (Trexler et al., 1983; Hochschwender & Laursen, 1981) as are Arg71 and Asp57 (Trexler et al., 1982). Lys35 is also suggested to be of importance (Wu et al., 1991).

The purpose of the present study is to establish the relative role of the charged amino acid residues Lys35, Asp57, and Arg71 in the lysine binding site of PKr4. Although the determination of the three-dimensional structure of the complex of PKr4 and ligand have already suggested the role of these residues it is of interest to reexamine these observations by the use of methods that directly study the chemical binding. In this study we have applied the techniques of site-directed mutagenesis to replace the residues in question with uncharged amino acid residues and examined the effects on the binding of two antifibrinolytic agents to the modified kringle domain.

MATERIALS AND METHODS

rPKr4 and rPKr4 Mutants. Human plasminogen cDNA (Forsgren et al., 1987) was obtained as a gift from Dr. Earl Davie, Seattle, WA. The DNA fragment encoding kringle 4 (residues 354–439 of Glu-plasminogen) was excised from the cDNA clone and inserted between a DNA segment encoding a factor Xa recognition site (Nagai & Thøgersen, 1984) and a translational stop codon using standard methods of recombinant-DNA technology. This construct was then ligated into the expression vector pLcIIMLCH6 (Lorenzen et al., 1993) to obtain the rPKr4 expression vector which after transfection into *Escherichia coli* QY13 (Nagai & Thøgersen, 1987) may be induced to direct the synthesis of a fusion protein containing an N-terminal region including an affinity purification handle for single-step purification of the fusion protein (Hochuli et al., 1988) joined via the factor Xa substrate recognition sequence Ile-Glu-Gly-Arg to a C-terminal kringle 4 polypeptide that can be liberated by sequence-specific cleavage by factor Xa. Expression vectors encoding single- or double-mutant derivatives of rPKr4 were prepared using standard oligonucleotide-directed mutagenesis techniques and verified by complete DNA sequencing of each construct.

Expression, Purification, and in Vitro Folding of Recombinant rPKr4 Fusion Protein Derivatives. E. coli QY13 cells harboring the rPKr4 expression vector were grown and heat-shocked at A_{600} = 0.8 as described (Nagai & Thøgersen, 1987), harvested by centrifugation, and then resuspended by addition of 1/60 culture volume of a buffer containing 50 mM Tris-HCl, pH 8, 0.5 M NaCl, and 2 mM EDTA. The cell suspension was then mixed with 1/120 culture volume of neutralized phenol and sonicated. After separation by centrifugation, a crude protein fraction was obtained as a precipitate by addition of two volumes of ethanol to the phenol extract. The pelleted crude protein precipitate was extracted overnight by 1/120 culture volume of 50 mM Tris-HCl, pH 8, containing 6 M guanidine-HCl and 100 mM dithioerythrotreitol. The cleared extract was transferred into coupling buffer (50 mM Tris-HCl, pH 8, 8 M urea, 1 M NaCl) by gel filtration and loaded on the affinity column (1/120 culture volume of Ni^{2+}-charged nitrilotriacetic acid agarose). After removal of host cell proteins by washing with the nonreducing buffer, the urea concentration in the elution buffer was gradually reduced to zero over 2 h before releasing the purified fusion protein by addition of 10 mM EDTA to the elution buffer. In the next step the disulfide-blocked protein was transferred by gel filtration into a refolding buffer (50 mM Tris-HCl, pH 8.7, 0.5 M NaCl) and then subjected to disulfide exchange (16 h) by addition of oxidized and reduced glutathione at final concentration of 0.5 and 5 mM, respectively. After removal of reduced and oxidized glutathione by gelfiltration into 50 mM Tris-HCl, pH 8, containing 0.5 M NaCl, the authentic kringle protein was excised from the fusion protein by incubation with factor Xa (obtained from Denzyme, Denmark) (12 h, 20 °C, 1:100 w/w).

With the exception of the $rPKr4_{D57N}$, $rPKr4_{wt}$ and all the other mutant derivatives of PKr4 examined could be separated from free N-terminal fragments of the fusion protein by affinity purification on lysine-agarose, columns (Deutsch & Mertz, 1970). Following chromatography on lysine-agarose each product was subjected to final purification by gradient ion exchange chromatography on a column of S-Sepharose (Pharmacia) (20 mM sodium phosphate, pH 5, 5–500 mM NaCl) to ensure complete removal of the 6-AHA ligand. Finally, the highly purified rPKr4 preparations were transferred into ammonium bicarbonate by gel filtration and recovered in a salt-free form by lyophilization. The $rPKr4_{D57N}$ that did not bind to the affinity column was purified using the procedure of ion exchange chromatography. The protein was soluble and migrated as a monomer on SDS–polyacrylamide gel electrophoresis at nonreducing conditions, suggesting that the $rPKr4_{D57N}$ had the right kringle domain fold.

The $rPKr4_{wt}$ product was identical to the main component of kringle 4 preparations excised from natural human plasminogen by elastase treatment in molecular mass as determined by PDMS mass spectrometry and could be crystallized under identical conditions (collaboration with Dr. R. Huber).

Plasma Desorption Mass Spectrometry. Plasma desorption mass spectrometry (PDMS) was performed on a BioIon 20 plasma desorption mass spectrometer (Applied Biosystems Inc., Forster City, CA). The samples were applied on nitrocellulose covered targets (Johnson et al., 1986) using the spin technique for sample application (Nielsen et al., 1988).

Titrations Monitored by ^1H NMR Spectroscopy. The association constant for kringle binding of the lysine analogues AMCHA and 6-AHA were determined by monitoring the chemical shift changes accompanying addition of the ligand to a kringle sample. This was done as described by DeMarco et al. (1982, 1987). Samples for NMR were prepared by dissolution of the lyophilized protein in ^2H_2O to a final concentration of approximately 0.2 mM. The concentrations were measured by amino acid analysis. These were obtained from an Alpha-Plus amino acid analyzer (Pharmacia, Sweden) after 24 h of hydrolization by 5.7 M HCl at 110 °C in evacuated and sealed tubes. The pH* (pH-meter reading uncorrected for deuterium isotope effects) of the samples was adjusted to

7.2 by addition of 0.5 M ^{2}HCl. In order to keep the kringle concentration constant during the titration the ligand solutions were prepared using the protein solution to be used in the titration. Volumes between 3 and 10 μL were added to 500- or 600-μL samples contained in a 5-mm NMR tube until the concentration of the ligand was 3–14 times that of the kringle. For each addition the 1D ^{1}H NMR spectrum was measured. The spectra were recorded on a Bruker AM500 NMR spectrometer at 500-MHz spectral width set to 7042 Hz and at a temperature of 300 K. Water suppression was accomplished by gated pulse irradiation of this resonance for 1 s between scans. Enhancement of resolution was achieved by multiplication with a sine function. Acetone was used as an internal reference at 2.225 ppm.

$$\delta_{obs} = \delta_P + \frac{\delta_{PL} - \delta_P}{2[P_{tot}]}([P]_{tot} + [L]_{tot} + K_d - \sqrt{([P]_{tot} + [K]_{tot} + K_d)^2 - 4[P]_{tot}[L]_{tot}}) \quad (1)$$

Calculations of the K_d. The change in chemical shift as a function of ligand concentration was analyzed to find K_d and the chemical shift at infinite concentration of ligand. Considering the fast-exchange-limit conditions, this could be done by fitting to eq 1 (Feeney et al., 1979):

where $[K]_{tot}$ and $[L]_{tot}$ are the total concentration of kringle and ligand, respectively. K_d is the dissociation constant and δ_P is the chemical shift when there is no ligand present whereas δ_{PL} is the chemical shift of the protons sensing the ligand. The fit was carried out by the least squares method of Levenberg-Marquardt (Press et al., 1988).

Titrations Monitored by Fluorescence Spectrometry. Protein and ligand, respectively, were dissolved in a 50 mM Tris-HCl and 0.5 M NaCl buffer at pH 8.0. For a comparison the binding at low ionic strength was measured for rPKr4$_{wt}$ in 10 mM Tris-HCl, pH 8.0 at 298 K. To keep the protein concentration constant throughout the titration, both titrant (rPKr4 derivatives) and titrator solutions (6-AHA) were 14 μM in protein. The titration was performed on a Perkin Elmer LS50B fluorimeter by adding 5 μL of ligand solution to the cuvette containing 960 μL of kringle sample for each point. The sample was excited at 295 nm, and the emission was measured at 344 nm. The emission data were analyzed similarly to the chemical shifts from the NMR experiments substituting in eq 1 δ_P with the emission intensity for the free protein, δ_{PL} with the emission of the fully bound protein, and δ_{obs} with the emission intensity at given ligand concentrations.

RESULTS AND DISCUSSION

In order to investigate the relative importance of the amino acid residues Lys35, Asp57, and Arg71 for the binding of lysine and lysine analogues, the following mutants were constructed. The lysine at position 35 was replaced by methionine and isoleucine residues, respectively, to form rPKr4$_{K35M}$ and rPKr4$_{K35I}$. Arg71 was replaced by glutamine to form rPKr4$_{R71Q}$, and both the double mutants rPKr4$_{K35M-R71Q}$ and rPKr4$_{K35I-R71Q}$ were constructed. Asp57 was replaced by an asparagine to form rPKr4$_{D57N}$. The relative mass of the derivatives were examined by plasma desorption mass spectrometry, and the results were shown to agree with theoretical values in Table I, only the five proteins listed were measured. This and DNA sequencing of the constructs documented the formation of the correct derivatives.

The 1D ^{1}H NMR spectra of the rPKr4$_{wt}$ and all the derivatives of this are very similar to the spectra published by DeMarco et al. (1985) and Ramesh et al. (1986) recorded for the nonrecombinant protein. Especially, it is noted that the five resonance lines at very low field are conserved as well as the high field shifted protons from Leu46, indicating that the overall structure of the kringle is highly conserved in all derivatives and the recombinant wild type (rPKr4$_{wt}$). Furthermore, the binding constants of the two ligands 6-AHA and AMCHA to rPKr4$_{wt}$ are very similar, respectively, to those obtained in previous studies of the non-recombinant protein (De Marco et al., 1987; Novokhatny et al., 1989; Sehl & Castellino, 1990; Menhart et al., 1991). These results indicate that the three-dimensional scaffold of the recombinant kringle domains and their derivatives studied here is closely similar to that of the wild-type kringle domains.

Table I. Molecular Mass of the rPKr4 Variants[a]

variant	molecular mass (Da) theoretical	experimental
rPKr4$_{wt}$	9336.30[b]	9336.36
rPKr4$_{K35I}$	9679.64	9681.32
rPKr4$_{K35M}$	9697.68	9698.46
rPKr4$_{R71Q}$	9666.59	9668.25
rPKr4$_{K35MR71Q}$	9669.62	9671.46

[a] Molecular masses were determined by plasma desorbtion mass spectrometry. [b] The rPKr4$_{wt}$ does not contain the tail piece SGTEA as do the rest of the variants of rPKr4.

Titration experiments with AMCHA were performed for all mutants except for rPKr4$_{D57N}$ using ^{1}H NMR spectroscopy. Similarly, the binding of 6-AHA to rPKr4$_{wt}$, rPKr4$_{K35M}$, and rPKr4$_{R71Q}$ was measured. The close similarity enabled the identification of the nine resonances of interest in the aromatic region of the ^{1}H NMR spectra from assignments of DeMarco et al. (1985) and Ramesh et al. (1986); these are shown in Figure 1. Overall the ^{1}H NMR spectra show that the six derivatives of PKr4 are quite similar with regard to the chemical shifts of the resolved resonances. It is clear though that a number of the resonances in the spectra have changed chemical shift as a consequence of the replacements of amino acid residues. The double mutants rPKr4$_{K35M-R71Q}$ and rPKr4$_{K35I-R71Q}$ have ^{1}H NMR spectra with rather broad resonance lines suggesting that two simultaneous modifications in the binding site have caused a time-dependent conformational averaging in the residues around the binding site. For all recombinant kringles a set of these signals shifted as a function of the ligand concentration. In most of the titrations the resonances 1, 4, and 7 were followed and used in the calculation since they are readily identified (Figure 1), they display large changes in chemical shift (Table II), and they are rather insensitive to small changes in pH (De Marco et al., 1985, 1986). In addition, the resonances 1 and 7 from $H^{\delta 1}$ of Trp72 and Trp62, respectively, are both located directly in the binding cleft (Petros et al., 1989; Wu et al., 1991). The chemical shift changes followed smooth binding curves as shown in Figure 2, where the titration of rPKr4$_{wt}$ with AMCHA is shown. All the signals 1, 2, 4, 7, 8, and 9 behave alike giving the same binding constant when analyzed. Table II summarizes the ligand-induced changes in chemical shifts of all the investigated kringle derivatives. In Figure 3 are shown the normalized changes of peak 7 from rPKr4$_{wt}$ and of rPKr4$_{R71Q}$ titrated with AMCHA and 6-AHA to illustrate the binding curves obtained from strong and weak binding, respectively. Table III summarizes the results of all titrations.

For a protein concentration of 0.2 mM binding constants in the range 10^3 to 5×10^4 M^{-1} can be determined accurately whereas binding constants larger than this are measured less accurately (Table III). Measurement of the stronger binding

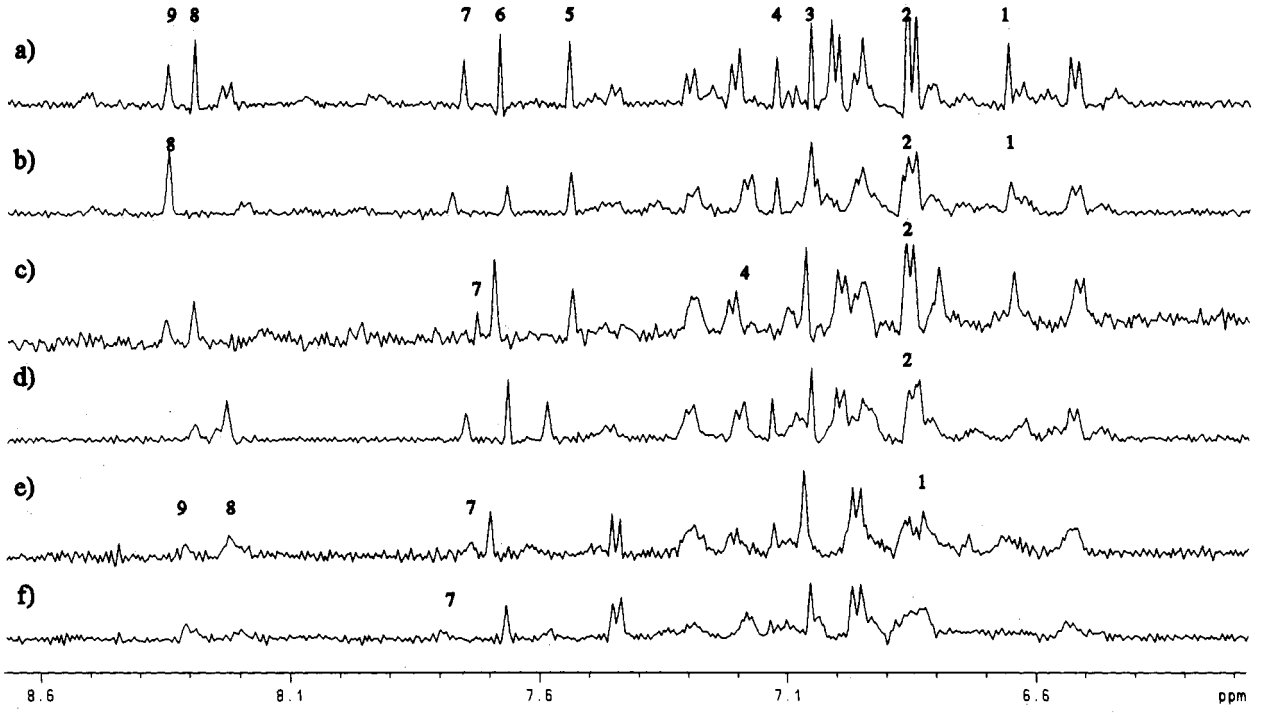

FIGURE 1: ^{1}H NMR spectra of each of kringles investigated. No ligand was added. The numbers above spectrum a identify the different resonances mentioned in Table II. In the spectra of mutant proteins the resonances that are difficult to identify from spectrum a are marked by the appropriate number. The spectra are of (a) rPKr4$_{wt}$, (b) rPKr4$_{K35M}$, (c) rPKr4$_{K35I}$, (d) rPKr4$_{R71Q}$, (e) rPKr4$_{K35IR71Q}$, and (f) rPKr4$_{K35MR71Q}$. All spectra were recorded at 300 K and pH 7.2. The protein samples were dissolved in ^2H_2O.

Table II: Ligand-Induced Shifts on Aromatic ^{1}H Resonances in 10^3 ppm[a]

kringle	signal residue proton	1 W72 H2	2 H33 H4	3 H3 H4	4 W25 H2	5 H31 H2	6 H3 H2	7 W62 H2	8 H33 H2	9 H31 H4
rPKr4$_{wt}$	AMCHA	−21	−39	∼1	39	∼−1	∼−1	55	−59	−16
rPKr4$_{K35M}$		41	∼2	∼3	20	16	∼7	57	−101[b]	−10
rPKr4$_{K35I}$		40	∼1	∼6	21	16	∼11	70	−67	∼−9
rPKr4$_{R71Q}$		nd	4	∼2	21	30	∼5	69	∼−3	∼0
rPKr4$_{K35I,R71Q}$[c]		−36	∼1	∼3	∼3	nd	∼4	72	nd	∼0
rPKr4$_{K35M,R71Q}$[c]		nd	nd	∼−3	nd	nd	∼−8	58	−50	nd
rPKr4$_{wt}$	6-AHA	∼0	∼−6	∼−6	36	−20	−21	34	−7	−38
rPKr4$_{K35M}$		26	−35	∼1	22	nd	∼3	28	−84	−29
rPKr4$_{R71Q}$		nd	∼3	∼3	21	23	∼10	65	15	∼4

[a] The values in the table are the induced chemical shift changes in the fully bound state. The values were determined from the fit of the titration data to eq 1. Positions marked with a "∼" indicate that the value is not derived from the fit but is the difference between the first and last measurement due to small changes in δ. [b] The signal was not observable before addition of ligand. This value is therefore estimated from the titration curve. [c] The signals were difficult to identify because of low intensity.

Table III: Binding Constants for Binding of AMCHA and 6-AHA[a]

	by NMR spectroscopy[b]		by intrinsic fluorescence spectroscopy[c]	
	ligand		ligand	
kringle	AMCHA (no buffer, pH* 7.2)	6-AHA (no buffer, pH* 7.2)	6-AHA (10 mM Tris-HCl, pH 8.0)	6-AHA (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl)
rPKr4$_{wt}$	1.50 ± 0.20	0.30 ± 0.02	0.49 ± 0.01	0.127 ± 0.004
rPKr4$_{K35M}$	1.60 ± 0.25	0.42 ± 0.05		0.115 ± 0.004
rPKr4$_{K35I}$	1.67 ± 0.30			0.118 ± 0.004
rPKr4$_{R71Q}$	0.085 ± 0.002	0.025 ± 0.002		0.022 ± 0.001
rPKr4$_{K35I,R71Q}$	0.078 ± 0.003			0.020 ± 0.001
rPKr4$_{K35M,R71Q}$	0.049 ± 0.003			0.027 ± 0.001

[a] The association constants are given in units of 10^5 M^{-1}. [b] The association constants were obtained by fitting the change in chemical shift accompanying addition of the respective ligand to the kringle given in the column to eq 1. [c] The association constants were obtained from eq 1 by fitting the change in intrinsic fluorescence accompanying addition of the ligand.

between AMCHA and rPKr$_{wt}$ for which K_a is 1.5×10^5 M^{-1} would ideally require a lower protein concentration. The lower concentration required to measure a stronger binding accurately, however, is not feasible for the NMR method, and therefore in the present series of measurements these were maintained at 0.2 mM. Nevertheless, the accuracy of the resulting binding constants is more than sufficient to imply that the modification at amino acid sequence position 35 has no measurable effect on the binding of ω-amino acids. In contrast, the binding of ligands to rPKr4$_{R71Q}$ is reduced by

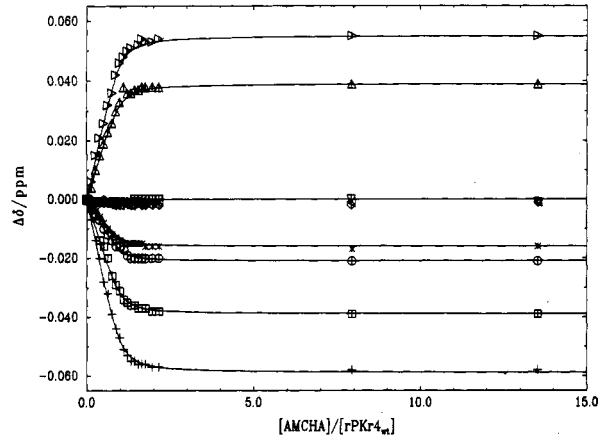

FIGURE 2: Titration of $rPKr4_{wt}$ with AMCHA at 300 K, pH* 7.2, and $[rPKr4_{wt}] = 0.17$ mM in ^2H_2O. The symbols represent the titration curves of the nine resonances: 1 (○), 2 (□), 3 (◇), 4 (△), 5 (△), 6 (▽), 7 (▽), 8 (+), 9 (×). The solid lines were obtained by fitting to eq 1 of the titration data for the respective resonances.

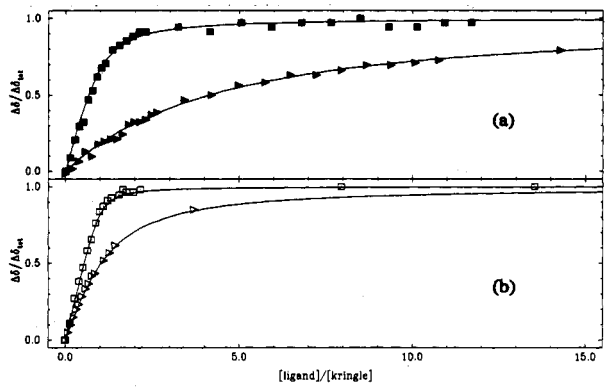

FIGURE 3: Titration curves obtained by ^1H NMR spectroscopy for titration with 6-AHA (a) and AMCHA (b). In both diagrams the upper line represents the stronger binding of $rPKr4_{wt}$ and the lower line the weaker binding of $rPKr4_{R71Q}$. The solid lines were obtained by fitting the data to eq 1. The monitored signal is peak 7. The experimental conditions are the same as for Figure 2.

a factor of 12 and 19, respectively, for 6-AHA and AMCHA, to a much weaker binding which is, however, still measurable. The results obtained for the two double-mutant rPKr4s are in agreement with this as the binding constants measured are essentially the same as for $rPKr4_{R71Q}$. The substitution of lysine at position 35 with either methionine or isoleucine clearly has no effect on the binding of ligand when the guanidino group of Arg71 is present. Neither do modifications of the 35 position have any noticeable effect on the residual ligand-binding capacity of the $rPKr4_{R71Q}$ derivative. The mutation of Asp57 seem to completely abolish the ligand binding since the derivative does not bind to the lysine-Sepharose affinity column used for the purification of proteins. These results suggest that the carboxylate group of Asp57 is essential for binding and interacting with the ϵ-amino group equivalent of the ligands. Furthermore, it seems clear that the guanidino-group of Arg71 contributes significantly to the binding of the α-carboxylate group equivalent of the ligands. The significant residual binding capacity of the $rPKr4_{R71Q}$ derivative suggests that the binding of the ϵ-amino group as well as the nonpolar interactions between the aromatic residues in the binding site and the aliphatic part of the lysine residue are providing sufficient binding energy to keep the ligand and the protein weakly complexed.

In order to examine this further, the effect of ionic strength on the binding of 6-AHA to the $rPKr4_{wt}$ and five derivatives was measured using fluorescence spectroscopy. For the unmodified kringle domain as well as for the two derivatives modified only at the position of Lys35, an ionic strength of 0.5 M reduced the binding constant by a factor of 3, to $0.12 \times 10^5\ M^{-1}$. The derivatives modified at the position of Arg71 were not affected by ionic strength and maintained a rather low binding constant of ca. $0.02 \times 10^5\ M^{-1}$. This observation suggests that the ligand binding to $rPKr_{R71Q}$ and the double mutants may not involve even the electrostatic interaction between the 6-amino group and the carboxylate group of Asp57. For the binding of the unmodified recombinant $rPKr_{wt}$ to the ligands, these observations suggest that the carboxylate of Asp57 and the guanidino group of Arg71 provide, respectively, the all important electrostatic interaction in the binding site for the 6-amino group and the carboxylate group of 6-AHA.

A comparison of these results with the primary structures of all the known kringles supports the picture outlined above. PKr1, PKr4, and t-PAKr2 are all known to bind lysine analogues. These kringles all have an aromatic residue at position 72 and aspartic acid at position 57, confirming the essential role of this residue in defining the binding. The two plasminogen kringles preferably bind ligands with the distance between the amino group and the carboxylate of 0.68 nm. PKr1 and PKr4 both have arginine at position 71. From the structure of PKr4 and the modeled structure of PKr1, it can be seen that these residues define one end of the binding site (Wu et al., 1991; Tulinsky et al., 1988). In contrast to the plasminogen kringles, t-PAKr2 has higher affinity for 7-AHA (DeSerrano et al., 1991). In this kringle the residue at position 71 is a threonine and therefore it does not contribute to the binding. From the NMR and the X-ray structure of t-PAKr2 it can be seen that Lys 33 takes the position of the arginine in PKr4 (Byeon & Llinás, 1991; de Vos et al., 1992). The distance between the cationic and the anionic locus is therefore larger, suggesting an explanation for the preference for the longer 7-AHA. From the measurements done by Castellino and co-workers, it is clear that Lys33 is responsible for interaction with the carboxylate group of the ligand (De-Serrano et al., 1991; DeSerrano & Castellino, 1992) similar to Arg71 of PKr4. These observations indicate strongly that the anionic locus of the binding site together with the aromatic residues at position 72 give the conditions that enable the binding of aliphatic amino acids.

From the results presented above it is clear that Lys35 has practically no influence on binding affinity toward ω-amino acids. This finding is supported by the fact that PKr4 is the only plasminogen kringle having lysine at this position and that PKr1 and PKr4 have the same preference for ligands with a distance of 0.68 nm between the anionic and cationic groups.

Previous studies of the binding site of kringle domains have shown that the hydrophobic cleft defined by Trp72 is essential for binding as is Asp57 in the lysine binding site (Trexler et al., 1982, 1983). The crystal structure of the complex has confirmed the importance of these residues but also suggested that the ϵ-amino group of Lys35 was an important partner for the ligand (Mulichak et al., 1991; Wu et al., 1991). In the crystal of the complex of PKr4 and 6-AHA Lys35, N^{ζ} is seen to be only 0.272 nm from one of the carboxylate oxygens of 6-AHA (Wu et al., 1991). This suggests that the binding together with other interactions is established by a hydrogen bond and otherwise by the electrostatic interaction between the two oppositely charged groups. The presence of the binding forces from the hydrogen bond and the electrostatic effects

implied by the short distances between the protein and the ligand atoms is, however, not in agreement with the finding that replacement of the lysine residue with an uncharged residue has no effect on binding of neither 6-AHA nor of AMCHA. The results of this study show that this position has no influence on the affinity toward analogues of C-terminal lysine. There is full agreement between the structure determined in crystal phase of the complex and the present studies of the influence of Arg71 in the binding of 6-AHA. In the crystallized complex both the N^{ϵ} and $N^{\eta 2}$ are close to the other oxygen of the carboxylate of 6-AHA, 0.271 and 0.289 nm, respectively (Wu et al., 1991). It can be rationalized from the decrease in affinity for binding of AMCHA and 6-AHA, respectively, when Arg71 is replaced, that this residue is essential for the specific recognition of analogues of the C-terminal lysine and this is fully compatible with the interactions observed in the crystal of the complex.

The work presented here is relevant not only because it specifically implies that one significant interaction in the binding site in plasminogen kringle 4 as determined in the crystal state is seen not to be important for binding in the solution state. It is also highly relevant because it serves as a reminder in general that solution and crystal structures may not always be fully identical and that only a comparison of similar interactions in the two phases can reveal this.

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