Specific Anionic Residues of the Recombinant Kringle 2 Domain of Tissue-Type Plasminogen Activator That Are Responsible for Stabilization of Its Interaction with ω-Amino Acid Ligands†

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ABSTRACT: The involvement of specific aspartic acid (D) and glutamic acid (E) residues of the recombinant (r) kringle 2 (K2) domain of tissue-type plasminogen activator (tPA) in stabilizing its interaction with ω-amino acid ligands has been assessed by examination of these binding events subsequent to site-directed mutagenesis of the relevant amino acid residues. We have expressed and purified nonconservative alanine (A) replacement mutants at the following amino acid sequence locations in r-$K2_{tPA}$: E^{17} (r-[$K2_{tPA}/E^{17}A$]), E^{75} (r-[$K2_{tPA}/E^{75}A$]), and D^{78} (r-[$K2_{tPA}/D^{78}A$]). More conservative E for D replacements were generated at the only other anionic (at neutral pH) amino acids of r-[$K2_{tPA}$], viz., D^{57} (r-[$K2_{tPA}/D^{57}E$]) and D^{59} (r-[$K2_{tPA}/D^{59}E$]). Each of these variant polypeptides was then utilized for binding investigations with a series of ω-amino acids. No substantial differences were found in the binding constants (pH 8.0, 25 °C) for the ligands, 6-aminohexanoic acid (6-AHxA), 7-aminoheptanoic acid (7-AHpA), L-lysine, and *trans*-(aminomethyl)cyclohexane-1-carboxylic acid (AMCHA), among wild-type (wt) r-$K2_{tPA}$, r-[$K2_{tPA}/E^{17}A$], r-[$K2_{tPA}/E^{75}A$], and r-[$K2_{tPA}/D^{78}A$]. On the other hand, dramatic effects on this same binding were observed in recombinant mutants with alterations at D^{57} and D^{59}. In these cases, even with the most conservative replacements, i.e., r-[$K2_{tPA}/D^{57}E$] and r-[$K2_{tPA}/D^{59}E$], the K_d values for these ligands were increased approximately 3–6-fold and 18–85-fold, respectively. NMR analysis of these variants suggested that no substantial gross conformational changes occurred as a result of the mutations made, but some localized alterations in amino acid microenvironments did take place. However, since similar perturbations were also present in variants that interacted normally with the ω-amino acid ligands, the changes were most likely unimportant to the steric features that define the integrity of the [$K2_{tPA}$] binding pocket. The results of this study suggest that E^{17}, E^{75}, and D^{78} do not play important roles in stabilizing binding of ω-amino acid ligands to r-[$K2_{tPA}$]. Both D^{57} and D^{59} have great influence on this binding energy, with D^{59} being the most dominant anionic center.

A variety of proteins involved in blood coagulation and blood clot dissolution, as well as proteins as diverse as apolipoprotein(*a*) and HGF[1]-like proteins, contain defined peptide regions classified as kringles (Magnusson et al., 1975). These highly homologous triple disulfide-bonded regions contain approximately 80 amino acids, are all flanked by one or two introns (Patthy, 1985), and appear to contain all information needed for their proper folding (Castellino et al., 1981; Tulinsky et al., 1988; Novokhatny et al., 1991). It is therefore believed that they function as independent domains in some of the proteins in which they reside.

The number of kringles contained in the relevant proteins vary considerably. As examples, one kringle is present in blood coagulation factor XII (McMullen & Fujikawa, 1985) and in urokinase-type plasminogen activator (Steffens et al., 1982), two kringles have been identified in tPA (Pennica et al., 1983) and prothrombin (Magnusson et al., 1975), four kringles have been found in HGF (Nakamura et al., 1989) and in a human liver protein that shows sequence homology to HGF (Han et al., 1991), five kringles exist in HPg (Sottrup-Jensen et al., 1978), and 38 kringles are present in the apolipoprotein(*a*) form that has been cloned (McLean et al., 1987).

The major functions of kringle domains lie in their capacities to interact with ω-amino acids, the latter of which compete with some of the physiologically important interactions of kringle-containing proteins. In this regard, it has been shown that complexes of HPg (Thorsen, 1975; Wiman & Wallen, 1977; Thorsen et al., 1981) and tPA (van Zonneveld et al., 1986a,b) with fibrin are displaced by ω-amino acids. This led to the proposal that these interactions stabilize the binding of tPA and HPg to the carboxyl-terminal lysine residues that appear in HPm-degraded fibrin clots. Kringle regions of HPm also mediate initial binding of HPm to its fast-acting inhibitor, α_2-antiplasmin (Wiman et al., 1978), and a recognition site has been discovered within the first three HPg kringles for binding of HPg to thrombin-stimulated and nonstimulated platelets (Miles et al., 1988). These interactions are also inhibited by ω-amino acids. Additionally, the binding site for the activation regulator Cl^- has been located within the first three kringles of HPg (Urano et al., 1987), and an anion binding site has also been discovered in the r-[$K2_{tPA}$] domain (de Vos et al., 1992). Finally, the interactions of Cl^- and

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[1] Abbreviations: HGF, hepatocyte growth factor; tPA, tissue-type plasminogen activator; HPg, human plasminogen; HPm, human plasmin; [$K1_{HPg}$], the kringle 1 region (residues C^{84}–C^{162}) of human plasminogen; [$K4_{HPg}$], the kringle 4 region (residues C^{358}–C^{435}) of human plasminogen; [$K5_{HPg}$], the kringle 5 region (residues C^{462}–C^{541}) of human plasminogen; [$K2_{tPA}$], the kringle 2 region (residues C^{180}–C^{261}) of human tissue-type plasminogen activator; 6-AHxA, 6-aminohexanoic acid; 7-AHpA, 7-aminoheptanoic acid; AMCHA, *trans*-(aminomethyl)cyclohexane-1-carboxylic acid; r, recombinant; wt, wild-type; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; ES/MS, electrospray/mass spectrometry; DTC, differential titration calorimetry; DSC, differential scanning calorimetry.

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ω-amino acids with HPg kringles have been shown to have dramatic consequences on the structure of the native molecule [Glu^1]Pg. In this regard, binding of Cl^- results in a more compact form of [Glu^1]Pg (Urano et al., 1987), which has a greatly reduced capacity for activation. The structure of [Glu^1]Pg becomes less compact in the presence of ω-amino acids (Violand et al., 1975), and activation becomes much more facile (Urano et al., 1987).

Despite the fact that the kringle regions of these proteins are highly homologous, they do not all interact in a similar manner with ω-amino acids. The tightest ω-amino acid binding kringle is [$K1_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991), with [$K4_{HPg}$] (Lerch et al., 1980; De Marco et al., 1987, 1989; Sehl & Castellino, 1990), [$K5_{HPg}$] (Castellino et al., 1981; Novokhatny et al., 1989; Thewes et al., 1990), and [$K2_{tPA}$] (Cleary et al., 1989; De Serrano et al., 1992b) also exhibiting such binding properties. In an effort to investigate the roles of specific amino acid residues of [$K2_{tPA}$] in stabilizing its interaction with ω-amino acid ligands, we have demonstrated by oligonucleotide-directed mutagenesis of r-[$K2_{tPA}$] that K^{33} is the principal cationic center for stabilization of binding of ω-amino acid ligands (De Serrano & Castellino, 1992a,b) and that W^{74} also plays an essential role in stabilizing these same complexes through interactions with the methylene backbone of the ligands (De Serrano & Castellino, 1992b). The relative importance of the anionic centers of [$K2_{tPA}$] responsible for stabilizing its interaction with ω-amino acid ligands has not been directly identified. With our previous success in the use of site-directed mutagenesis to identify other amino acid residues of importance in this regard, we have extended these investigations to all acidic amino acid residues in r-[$K2_{tPA}$]. The current report is a summary of the results obtained.

MATERIALS AND METHODS

Proteins. Restriction endonucleases were obtained from Fisher Scientific Co. (Springfield, NJ) and BRL (Gaithersberg, MD). Recombinant Taq DNA polymerase (AmpliTaq) was a product of Perkin-Elmer Cetus (Norwalk, CT).

DNA Analytical Methods. All methods used for DNA manipulations, including oligonucleotide synthesis, cDNA sequencing, cell transformations, plasmid minipreparations, generation of single-stranded DNAs, and purification of DNA fragments, were performed as described in previous publications from this laboratory (Menhart et al., 1991; De Serrano & Castellino, 1992b; De Serrano et al., 1992b).

Construction of Expression Plasmids. The vector employed for expression of r-[$K2_{tPA}$], pSTII/[$K2_{tPA}$], has been described in detail (De Serrano & Castellino, 1992a; De Serrano et al., 1992b). The mature polypeptide expressed with this system consists of residues C^{180}–C^{261} of tPA (in kringle numbering this represents C^1–C^{81}), flanked on the amino terminus by the dipeptide SD and on the carboxyl terminus by the amino acid S. This plasmid was the starting point for the mutants described herein, which were obtained by site-directed mutagenesis with synthetic oligonucleotide primers.

Expression and Purification of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] Variants. Expressions were conducted in *Escherichia coli* DH5α cells, as previously described (De Serrano & Castellino, 1992a,b).

Purification of the recombinant kringles was accomplished after separation of the conditioned *E. coli* cells into periplasmic and oxidatively refolded fractions (Menhart et al., 1991; De Serrano & Castellino, 1992a). These materials were passed over a column of lysine–Sepharose equilibrated with low salt buffer (25 mM Tris-HCl, pH 8.0). The fraction eluted with the 6-AHxA gradient was then further resolved on a column of MonoS (De Serrano & Castellino, 1992a). In all cases, highly purified r-[$K2_{tPA}$] mutants resulted from this purification method.

Intrinsic Fluorescence Titrations. Titrations of the effect of ω-amino acids on the intrinsic fluorescence of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] mutants were accomplished at 25 °C in a buffer containing 50 mM Tris–OAc/150 mM NaOAc, pH 8.0, as previously described (Menhart et al., 1991). Dissociation constants (K_d) characterizing the binding of the r-[$K2_{tPA}$] mutants to the relevant ω-amino acids were calculated from the fluorescence titrations by nonlinear least squares iterative curve fitting, as described in detail earlier (Menhart et al., 1991).

Titration Calorimetry. DTC measurements of the heat changes resulting from titration of ligand into solutions of the r-[$K2_{tPA}$] variants of choice were performed as described previously (Sehl & Castellino, 1990; Menhart et al., 1991). A Microcal (Northampton, MA) OMEGA titration calorimeter was employed for these experiments. The data were deconvoluted as described earlier (Sehl & Castellino, 1990; Menhart et al., 1991). The calorimetric titrations were performed in a buffer of 50 mM Tris–OAc/150 mM NaOAc, pH 8.0, at 25 °C.

^{1}H-NMR. For NMR analysis, the lyophilized r-[$K2_{tPA}$] samples were dissolved in 0.05 M sodium phosphate, pH 7.4 (fully preexchanged with ^2H_2O), then relyophilized, and redissolved in the same volume of ^2H_2O. Two additional ^{1}H–^{2}H exchanges were then accomplished in the same manner. One-dimensional ^{1}H-NMR spectra and two-dimensional RELAYED-COSY spectra were obtained at 37 °C on a Varian (Palo Alto, CA) VXR 500S spectrometer in the Fourier mode at 500 MHz with quadrature detection. Our complete methodology was as described previously (De Serrano & Castellino, 1992b).

Differential Scanning Calorimetry. DSC experiments were conducted with use of a Microcal (Northampton, MA) MC-2 scanning calorimeter, with samples dialyzed against a solution of 50 mM Tris–OAc/150 mM NaOAc, pH 8.0, or 50 mM Tris–OAc/100 mM NaOAc/50 mM 6-AHxA, pH 8.0. Thermal denaturation scans were conducted in the temperature range of 25–100 °C, at scan rates of 30°/h. The baseline for each run was determined by an identical experiment with the sample buffer placed in each chamber. The data were recorded on an IBM-PC computer for further data reduction.

We have previously described the methods that we employ for deconvolution of the traces and for obtaining the temperature (T_m) of maximum heat capacity (Radek & Castellino, 1988; Sehl & Castellino, 1990).

Analytical Methods. ES/MS analysis for determination of the molecular weight of the samples was conducted using a JOEL (Peabody, MA) AX505 HA mass spectrometer equipped with the JEOL electrospray ionization source. The handling of samples was as described previously (De Serrano & Castellino, 1992a). $NaDodSO_4$/PAGE was performed in the usual manner (Laemmli, 1970). FPLC was conducted with a Pharmacia (Piscataway, NJ) system.

RESULTS

In order to identify the individual contributions of the anionic (at neutral pH) residues of r-[$K2_{tPA}$] that contribute energy for ω-amino acid binding, we have constructed single-site conservative and nonconservative recombinant mutants of this domain at all D and E residues, viz., E^{17}, D^{57}, D^{59}, E^{75}, and

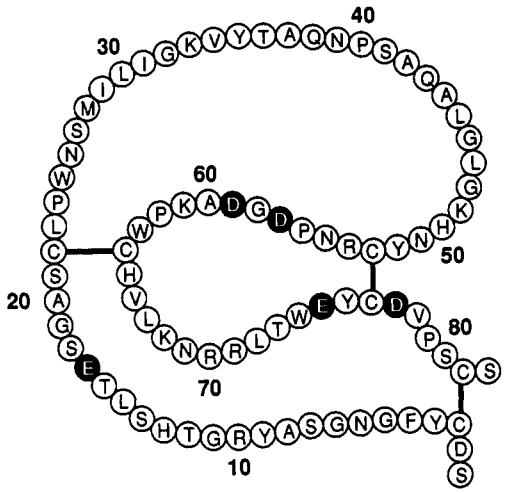

FIGURE 1: Amino acid sequence of the recombinant wild-type [$K2_{tPA}$] construct expressed in *E. coli* cells. Single-letter codes for amino acids are used. The sequence locations of the negatively charged (at pH 8.0) amino acids are indicated by black-white color reversals.

Table I: Construction of r-[$K2_{tPA}$] Variants

mutation[a]	primer[b]	screen
$E^{17}A$	5'-G CAC AGC CTC ACC Gcc TCa GGT GCC TCC TGC CT	+ *Sau* I
$D^{57}E$[c]	5'-C TGC CGG AAT CCa GcT GGG GAT GCC AA	+ *Pvu* II
	5'-C TGC CGG AAT CCt Gaa GGG GAT GCC AAG	- *Pvu* II
$D^{59}E$[c]	5'-GG AAT CCT GAT GGc Gcc GCC AAG CCC TGG	+ *Nar* I
	5'-AAT CCT GAT GGc Gag GCC AAG CCC TGG	- *Nar* I
$E^{75}A$	5'-CTG ACG TGG Gcc TAC TGT GAT G	+ *Sau96* I
$D^{78}A$	5'-G TGG GAG TAC TGc Gca GTG CCC TCC TGC	+ *Fsp* I

[a] The column below indicates the wild-type amino acid in r-[$K2_{tPA}$], its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position. [b] The mutated bases are represented by lower-case lettering. [c] Two separate stages were employed for the mutagenesis. In the first, a restriction site was cloned into the desired location. In the second, this site was cloned out concomitant to placement of the desired amino acid substitution.

D^{78}. The sequential relationships of these anionic residues to other amino acids in [$K2_{tPA}$] are shown in Figure 1. Engineering the mutations into the cDNA for wtr-[$K2_{tPA}$] was accomplished by site-directed mutagenesis with the synthetic oligonucleotides listed in Table I, which also provided the restriction endonuclease sites used for screening the transformed *E. coli* cells. For the mutations $E^{17}A$, $E^{75}A$, and $D^{78}A$, a new restriction endonuclease site was embodied into the cDNA along with the desired mutation, but for the $D^{57}E$ and $D^{59}E$ mutations, this was difficult to accomplish in one step. Therefore, in these latter cases, two stages were employed for the mutagenesis. First, a mutagenic oligonucleotide was designed which allowed a new and easily screened restriction site to be incorporated into the desired region of the cDNA. This was followed by design and transformation with a second mutagenic oligonucleotide, in which the first restriction site was eliminated and the desired final mutation incorporated into the mutated cDNA.

The mutated cDNAs were expressed in *E. coli* DH5α cells, as previously described (Menhart et al., 1991; De Serrano & Castellino, 1992a,b; De Serrano et al., 1992b). The desired recombinant materials were found in both periplasmic and oxidatively refolded cell fractions and were purified by a combination of affinity chromatography on lysine–Sepharose and a MonoS FPLC column (De Serrano & Castellino, 1992a,b; De Serrano et al., 1992b). Affinity chromatography was usually successful even with the poorest ω-amino acid

Table II: Molecular Weights of r-[$K2_{tPA}$] Variants

variant	molecular weight calcd	molecular weight exptl
r-SD[$K2_{tPA}$]S[a]	9365.69	9363.4
r-SD[$K2_{tPA}/E^{17}A$]S[b]	9307.65	9307.1
r-SD[$K2_{tPA}/D^{57}E$]S[b]	9379.72	9379.1
r-SD[$K2_{tPA}/D^{59}E$]S[b]	9379.72	9376.0
r-SD[$K2_{tPA}/E^{75}A$]S[b]	9307.65	9310.0
r-SD[$K2_{tPA}/D^{78}A$]S[b]	9321.68	9321.1

[a] Refers to the wild-type recombinant molecule. The bracketed portion is the amino acid sequence of $K2_{tPA}$ (from C^1 to C^{81}). Flanking the amino terminus of C^1 is the dipeptide SD, and flanking the carboxyl terminus of C^{81} is the single amino acid S. [b] The bracketed portion is the amino acid sequence of $K2_{tPA}$/the wild-type amino acid, its sequence beginning from C^1 of the kringle, and the amino acid replacement at that position. Flanking the amino terminus of C^1 of the kringle is the dipeptide SD, and flanking the carboxyl terminus of C^{81} of the kringle is the single amino acid S.

binding mutants, as long as this step was performed at low ionic strength (De Serrano & Castellino, 1992b). With the periplasmic cell fractions, a major impurity found after affinity chromatography under these low ionic strength conditions was the lysozyme employed to lyse the cells. However, this impurity was easily resolved by the MonoS step. Highly purified r-[$K2_{tPA}$] variants were obtained using this purification sequence. The amounts of materials ultimately obtained varied considerably, from a high of 10–15 mg/100 g of cells for r-[$K2_{tPA}/E^{17}A$] to a low of approximately 0.2 mg/100 g of cells for r-[$K2_{tPA}/D^{59}E$]. The small amounts of this latter variant made it impractical to perform some of the NMR and DSC investigations to be described below at high signal-to-noise ratios. All of the mutant gene products were analyzed by ES/MS analysis (Table II), which demonstrated that they possessed the expected molecular weights. This showed very clearly that the desired mutations were incorporated into the recombinant polypeptides.

Intrinsic fluorescence titrations were employed to calculate binding constants of the r-[$K2_{tPA}$] variants to several ω-amino acids in a fashion similar to that described in previous publications (Menhart et al., 1991; De Serrano & Castellino, 1992a,b; De Serrano et al., 1992b). Very large maximum intrinsic fluorescence changes accompanied ligand binding to r-[$K2_{tPA}/E^{17}A$], r-[$K2_{tPA}/D^{57}E$], r-[$K2_{tPA}/D^{59}E$], and r-[$K2_{tPA}/D^{78}E$], ranging from 11.1% for the AMCHA/r-[$K2_{tPA}/D^{59}E$] interaction to 48.3% for the interaction of lysine with r-[$K2_{tPA}/D^{57}E$]. On the other hand, smaller maximum intrinsic fluorescence alterations accompanied the binding of 6-AHxA (5.3%) and 7-AHpA (7.7%) to r-[$K2_{tPA}/E^{75}A$]. An example of the type of fluorescence titration data observed for these kringle/ligand interactions is illustrated in Figure 2 for the 7-AHpA/r-[$K2_{tPA}/E^{75}A$] interaction. Here, we purposely chose the example of one of the lowest intrinsic fluorescence alterations observed in this work to demonstrate that, even with these small changes, excellent data were obtained.

Because of the smaller fluorescence changes observed for 6-AHxA and 7-AHpA binding to r-[$K2_{tPA}/E^{75}A$], we employed DTC to confirm the K_d values determined from these fluorescence titrations. A plot of the incremental heat changes accompanying titration of r-[$K2_{tPA}/E^{75}A$] with 7-AHpA is provided in Figure 3. The K_d value obtained, of 10.4 μM, compares very favorably to that of 9.9 μM determined by fluorescence titration. From the DTC experiment, ΔH and ΔS values are also obtained and are –6.3 kcal/mol and 1.81 eu, respectively. While both of these parameters contribute to the binding energy of the r-[$K2_{tPA}/E^{75}A$]/7-AHpA in-

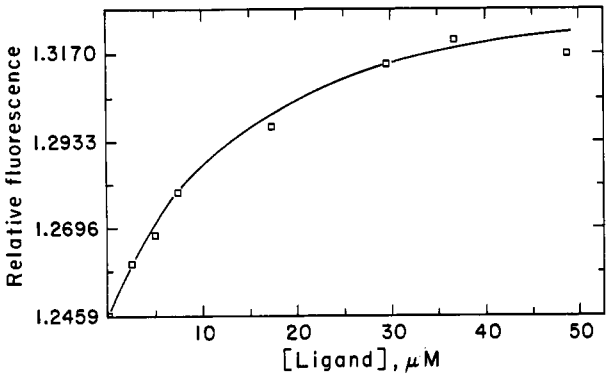

FIGURE 2: Titration of the relative change in intrinsic fluorescence of r-[$K2_{tPA}$/$E^{75}A$] (5.3 μM) with 7-AHpA. The experimental points (□) are best fit to a line generated by employing values of n = 1.0 and K_d = 9.9 μM and a maximal relative fluorescence change (ΔF_{max}) of 7.7%. The buffer was 50 mM Tris–OAc/150 mM NaOAc, pH 8.0, at 25 °C. The excitation and emission wavelengths were 285 and 340 nm, respectively.

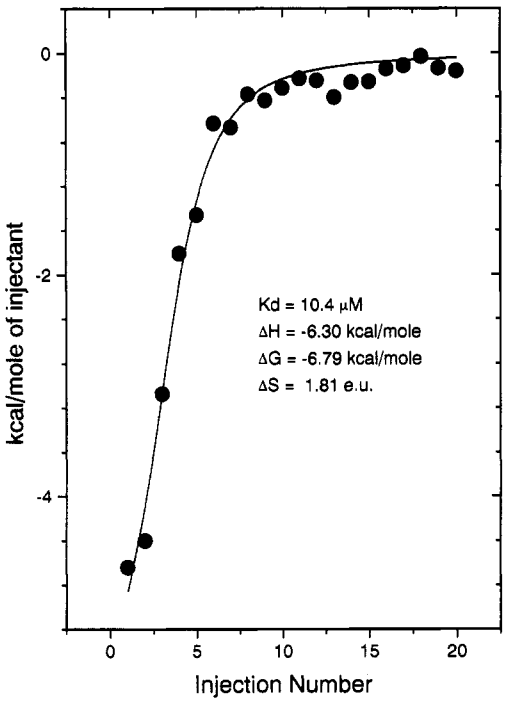

FIGURE 3: Titration of the heat of reaction of r-[$K2_{tPA}$/$E^{75}A$] with 7-AHpA. An automated sequence of aliquots (1 μL) of a stock solution (20 mM) of 7-AHpA was titrated into a 57 μM solution of r-[$K2_{tPA}$/$E^{75}A$], and the heat changes were recorded. The data (●) were deconvoluted and best fit to the line representing a binding isotherm with values of n = 1.0, K_d = 10.4 μM, ΔH = –6.3 kcal/mol, and ΔS = 1.81 eu. The buffer was 50 mM Tris–OAc/150 mM NaOAc, pH 8.0, at 25 °C.

teraction, the ΔH provides the main driving force for binding. All K_d values obtained in this study are summarized in Table III.

In order to determine whether alterations in the structure of r-[$K2_{tPA}$] accompanied the mutagenic changes made, ^{1}H-NMR spectra were obtained for each of the variants. The high field (–1.2 ppm < δ < 1.4 ppm) methyl proton region of the spectra of each of the variants is illustrated in Figure 4 and compared to the wtr-[$K2_{tPA}$]. While some differences in these spectra of the various mutants are observed, it is important to note that the COSY-connected doublets at approximately –1 and 0.55 ppm are present in their approximate proper locations (Table IV). These resonances arise from the $CH_3^{\delta'}$ and CH_3^{δ} groups of L^{47}, both of which are connected in a scalar fashion to the CH^{γ} of L^{47} that is present

Table III: Dissociation Constants for ω-Amino Acids to r-[$K2_{tPA}$] Variants As Determined by Intrinsic Fluorescence Titrations

	dissociation constant (μM) for			
variant	6-AHxA	7-AHpA	L-Lys	AMCHA
wt[a]	43	6	85	27
$E^{17}A$[b]	44	7	74	32
$D^{57}E$[b]	142	33	237	84
$D^{59}E$[b]	813	513	1609	487
$E^{75}A$[b]	39.6, 24.8[c]	9.9, 10.4[c]	74	nd[d]
$D^{78}A$[b]	25	6	55	20

[a] Refers to the wild-type recombinant molecule. [b] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule, its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position. [c] From DTC experiments. [d] No fluorescence change observed.

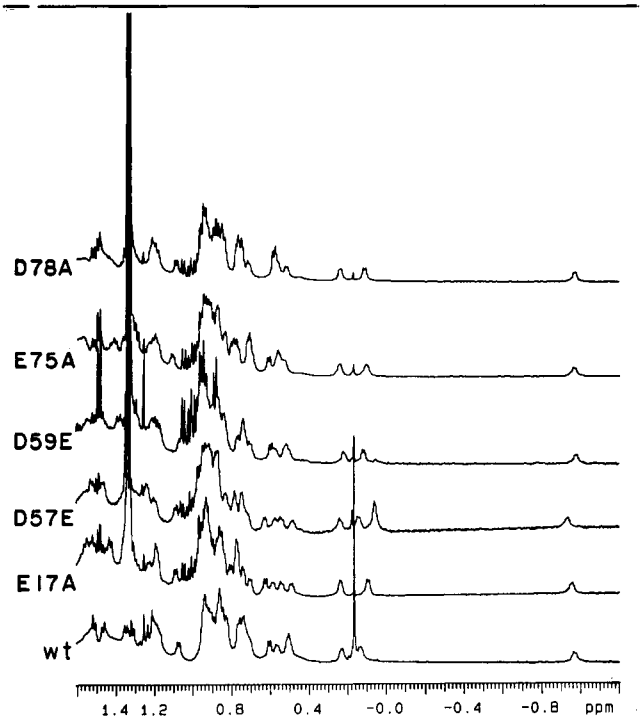

FIGURE 4: Methyl region of the ^{1}H-NMR spectra of wtr-[$K2_{tPA}$] and variants of r-[$K2_{tPA}$] containing mutations at its D and E residues: viz., wtr-[$K2_{tPA}$], r-[$K2_{tPA}$/$E^{17}A$], r-[$K2_{tPA}$/$D^{57}E$], r-[$K2_{tPA}$/$D^{59}E$], r-[$K2_{tPA}$/$E^{75}A$], and r-[$K2_{tPA}$/$D^{78}A$]. The temperature was 37 °C and the pH* was 7.4. The spectral width was 6000 Hz and the number of data points was 64 000. Suppression of the residual $^1H^2HO$ peak was accomplished by gated pulse irradiation of this resonance at low decoupling power for 1.5 s between scans. The chemical shifts (in ppm) reported are relative to an internal standard of dioxane, which resonates 3.77 ppm downfield of tetramethylsilane. Enhancement of the resolution was achieved by Gaussian convolution.

Table IV: ^{1}H-NMR Methyl Resonances for L^{47} at pH* 7.4 and 37 °C

variant	CH_3^{γ} δ (ppm)	$CH_3^{\delta'}$ δ (ppm)	CH_3^{δ} δ (ppm)
wt[a]	1.120	–0.964, –0.971	0.564, 0.574
$E^{17}A$[b]	1.105	–0.954, –0.964	0.533[c]
$D^{57}E$[b]	1.100	–0.940, –0.943	0.538
$D^{59}E$[b]	nd[d]	–0.978, –0.990	nd[d]
$E^{75}A$[b]	1.104	–0.964, –0.973	0.537[c]
$D^{78}A$[b]	1.089	–0.970, –0.979	0.510[c]

[a] Refers to the wild-type recombinant molecule. [b] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule, its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position. [c] Poorly resolved doublet. [d] Not observed due to low sample amounts.

as a multiplet at approximately 1.1 ppm. These signals have been shown to be signatures of proper gross kringle folding (DeMarco et al., 1982; Byeon et al., 1989, 1991; Atkinson &

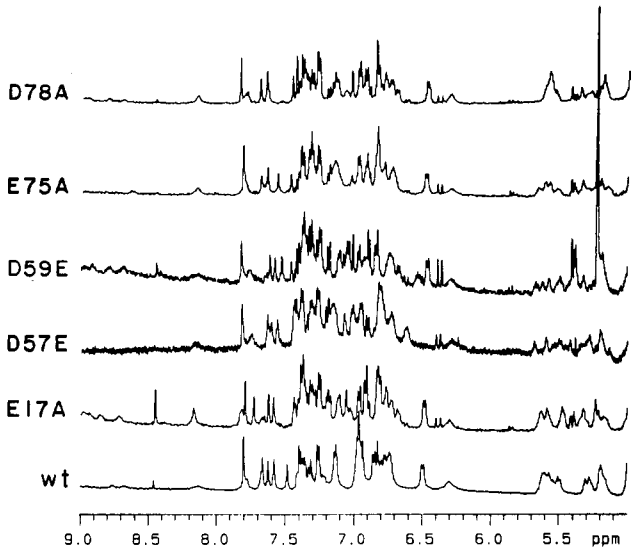

FIGURE 5: ^{1}H-NMR spectra in the aromatic region of wtr-[K2$_{tPA}$] and variants of r-[K2$_{tPA}$] containing mutations of its D and E residues: viz., wtr-[K2$_{tPA}$], r-[K2$_{tPA}$/E^{17}A], r-[K2$_{tPA}$/D^{57}E], r-[K2$_{tPA}$/D^{59}E], r-[K2$_{tPA}$/E^{75}A], and r-[K2$_{tPA}$/D^{78}A]. The temperature was 37 °C and the pH* was 7.4. The spectral collection conditions were as in Figure 4.

Table V: Changes in Chemical Shifts of Histidyl and Tryptophanyl Proton Resonances of r-[K2$_{tPA}$] Resulting from Mutations of Anionic Residues at pH* 7.4 and 37 °C

	$\Delta\delta$ (×10^3 ppm) of H2 protons for[a]					
variant	H^{13} [b]	H^{50} [b]	H^{65} [b]	W^{25} [b]	W^{63} [b]	W^{74} [b]
E^{17}A[c]	9	−3	39	106	69	−60
D^{57}E[c]	16	−18	23	−140	−36	68
D^{59}E[c]	28	−39	0	51	−45	70
E^{75}A[c]	31	19	9	84	24	25
D^{78}A[c]	43	21	19	−20	32	−53

[a] Chemical shift differences from wtr-SD[K2$_{tPA}$]S. [b] The chemical shifts (in ppm) of these H2 proton resonances in wtr-[K2$_{tPA}$]S are 7.807 (H^{13}), 7.629 (H^{50}), 8.140 (H^{65}), 7.490 (W^{25}), 7.671 (W^{63}), and 6.893 (W^{74}). [c] The mutation made in wtr-SD[K2$_{tPA}$]S: the amino acid in the wt molecule, its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position.

Williams, 1990; Thewes et al., 1990; Menhart et al., 1991; De Serrano & Castellino, 1992b; De Serrano et al., 1992a).

Figure 5 illustrates the spectral dispersion of the aromatic regions of the spectra (5.0 ppm < δ < 9.0 ppm) of the r-[K2$_{tPA}$] variants. Clearly, there are some environmental differences seen in several of the variants when compared to wtr-[K2$_{tPA}$]. Those resonances that can be clearly assigned to specific histidyl and tryptophanyl protons are listed in Table V, along with differences in their chemical shifts from those of the wtr-[K2$_{tPA}$]. Chemical shift differences, which are reliable to 0.01 ppm, are seen in many cases, with the largest observed for the H2 protons of W^{25} and W^{74}. Smaller chemical shift differences between the mutants and wtr-[K2$_{tPA}$] are noted for tyrosyl protons (Table VI), with the exception of the H2,6 protons of Y^{76} ($\Delta\delta$ = −0.105 ppm) in the E^{17} mutant, the H2,6 protons of Y^2 ($\Delta\delta$ = −0.122 ppm) and the H3,5 protons of Y^{76} ($\Delta\delta$ = 0.092 ppm) in the D^{57}E mutant, the H3,5 protons of Y^{76} in the D^{59}E mutant ($\Delta\delta$ = 0.110 ppm), and the H2,6 protons of Y^{35} ($\Delta\delta$ = 0.090 ppm) in the E^{75}A mutant. The broad resonances for the H2,6 and H3,5 protons of Y^9 were not detected under these experimental conditions, as has been observed previously (Byeon et al., 1989).

Finally, the structural stabilities of the mutant forms of r-[K2$_{tPA}$] have been examined by DSC methodology. The T_m values, i.e., midpoints of the thermal denaturation

Table VI: Chemical Shifts of Tyrosyl Proton Resonances of r-[K2$_{tPA}$] Resulting from Mutations of Anionic Residues at pH* 7.4 and 37 °C

	$\Delta\delta$ (×10^3 ppm) for[a]							
	Y^2		Y^{35}		Y^{52}		Y^{76}	
variant	H2,6[b]	H3,5[b]	H2,6[b]	H3,5[b]	H2,6[b]	H3,5[b]	H2,6[b]	H3,5[b]
E^{17}A[c]	−10	−4	20	0	−9	nd[d]	−105	−18
D^{57}E[c]	−122	2	−1	−25	−18	nd[d]	21	92
D^{59}E[c]	−20	−4	−28	−2	−10	nd[d]	10	110
E^{75}A[c]	−12	8	90	40	−10	nd[d]	27	8
D^{78}A[c]	−10	16	50	30	0	16	−13	−9

[a] Chemical shift differences from wtr-SD[K2$_{tPA}$]S. Here, the average resonance from the doublets is employed for generation of chemical shift difference measurements. [b] The chemical shifts (in ppm) of these proton resonances in wtr-[K2$_{tPA}$]S are H2,6 −6.507, H3,5 −6.974 for Y^2; H2,6 −7.150, H3,5 −6.850 for Y^{35}; H2,6 −6.310, H3,5 −6.737 for Y^{52}; and H2,6 −7.368, H3,5 −6.945 for Y^{76}. These are averages of each of the doublet resonances. [c] The mutation made in wtr-SD[K2$_{tPA}$]S: the amino acid in the wt molecule, its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position. [d] Not observed.

Table VII: Thermal Stabilities of Anionic Residue Variants of Recombinant Kringle 2 Domain Tissue-Type Plasminogen Activator

	T_m[a] (°C)	
variant	−EACA[b]	+EACA[c]
wt	75.6	86.1
E^{17}A[d]	71.1	83.7
D^{57}E[d]	64.3	73.6
D^{59}E[d]	nd[e]	nd[e]
E^{75}A[d]	64.0	76.9
D^{78}A[d]	71.5	85.3

[a] Temperature of maximum heat capacity change. [b] The buffer was 50 mM Tris–OAc/150 mM NaOAc, pH 8.0. [c] The buffer was 50 mM Tris–OAc/100 mM NaOAc/50 mM EACA, pH 8.0. [d] The mutation made in wtr-SD[K2$_{tPA}$]S: the amino acid in the wt molecule, its sequence position beginning from C^1 of the kringle, and the amino acid replacement at that position. [e] Not determined due to insufficient amounts of material.

temperatures (temperatures of maximum heat capacity changes), for each of the mutants are listed in Table VII. In the absence of 6-AHxA, significant decreases in the T_m values are found for each of these mutants. In the presence of a concentration of 6-AHxA that saturates its binding site on each mutant, elevations in the T_m are observed that are similar to the change in T_m for wtr-[K2$_{tPA}$].

DISCUSSION

Previous mutagenic investigations from this laboratory have served to define several of the amino acid residues of r-[K2$_{tPA}$] that contribute to the stabilization of the conformation of r-[K2$_{tPA}$] and to its ability to interact with ω-amino acids. These studies have been guided, and interpretations greatly assisted, by molecular modeling based on knowledge of the X-ray structure of the [K2$_{tPA}$/lysine complex (de Vos et al., 1992) and by the complete assignment of the chemical shifts of proton resonances in the native r-[K2$_{tPA}$] structure (Byeon et al., 1989, 1991). We have discovered that K^{33} of r-[K2$_{tPA}$] serves as the only cationic locus of the kringle domain for interaction with the carboxylate group of the ligand (De Serrano & Castellino, 1992a; De Serrano et al., 1992b) and that W^{74} plays a critical role as one of the aromatic residues that stabilizes this same binding through interactions with the hydrophobic regions of the ω-amino acid substrates (De Serrano & Castellino, 1992b). It has also been previously found that H^{65} is of importance in influencing the substrate specificity of the ω-amino acid binding pocket of [K2$_{tPA}$] (Kelley & Cleary, 1989; De Serrano & Castellino, 1992a),

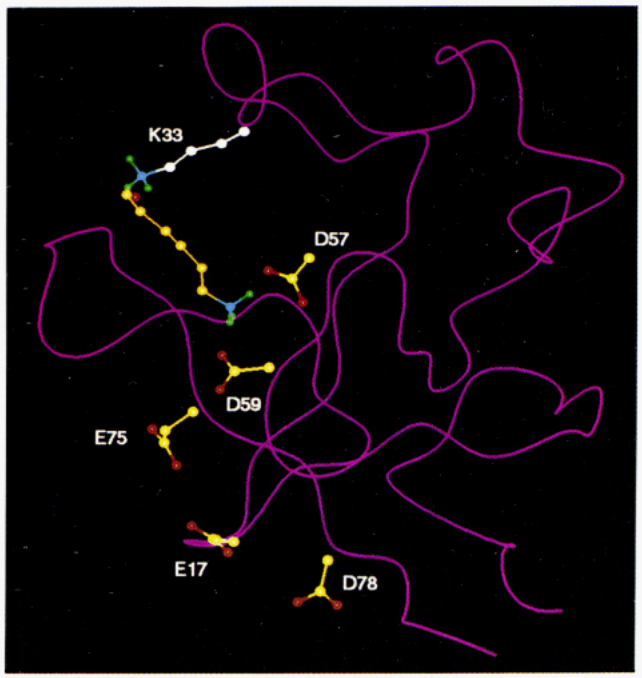

FIGURE 6: X-ray structure of wtr-[$K2_{tPA}$] from de Vos et al. (1992). Selected amino acid side chains are displayed on the backbone structure, as indicated. The numbering of amino acids begins at C^1 of the r-[$K2_{tPA}$] sequence and continues consecutively to C^{81}. Each amino acid is displayed from its β-carbon, and nonexchangeable hydrogen atoms are excluded to minimize overcrowding. The backbone conformation is in magenta. Negatively charged (at pH 8.0) amino acid side-chain carbon atoms are in yellow, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms are in green. The lysine (K) side chain (K^{49}) from another molecule of r-[$K2_{tPA}$] that is inserted into the binding pocket in the unit cell is shown with its side-chain carbon atoms in orange. The location of K^{33}, previously found to be important to ω-amino acid binding, is also displayed.

as well as for stabilization of the native structure of this kringle domain (Kelley & Cleary, 1989). Regarding the anionic sites of [r-$K2_{tPA}$] that may provide stabilization of these ligand binding interactions through the amino group of the ligand, only five candidate residues are present in the molecule (Figure 1). Of these amino acid side chains, it appears from the crystal structure (Figure 6) that only two, viz., D^{57} and D^{59}, appear to be sufficiently proximal to the ω-amino group of lysine to be potentially important in this regard. A qualitative investigation of the relevance of these latter two residues to the binding of lysine to intact tPA has been published (Weening-Verhoeff et al., 1990). As part of our program to completely define the nature of the important binding interaction of ω-amino acids with kringle domains, we decided to apply a site-directed mutagenesis strategy toward the anionic residues of r-[$K2_{tPA}$] in order to define in a quantitative fashion their role in binding to ligands of these types. We believed that such an investigation would not only serve to validate in solution these aspects of the crystal structure predictions but also allow detailed analyses of the relative contributions of the anionic residues in stabilizing binding of several such ligands through short- and long- (conformationally directed) range interactions.

Several nonconservative amino acid changes were made in r-[$K2_{tPA}$], specifically $E^{17}A$, $E^{75}A$, and $D^{78}A$. In all cases, expression of these mutants was successful. The binding studies summarized in Table III demonstrate that each of these mutants interacts with the ω-amino acid ligands with approximately the same binding energy as that of wtr-[$K2_{tPA}$]. It was shown previously that when E^{75} was altered to Q in otherwise intact r-tPA, the resulting mutant interacted less efficiently with a lysine–Sepharose column as compared to wtr-tPA (Weening-Verhoeff et al., 1990). We were not able to confirm this weaker binding with r-[$K2_{tPA}/E^{75}A$] employing this much less conservative mutation at E^{75}. It is possible that the mutation to Q at this residue led to specific changes in tPA, resulting in weaker binding, but since this previous investigation was qualitative in nature, it is not possible to assess whether the differences in the two studies are important. However, since the radical changes that we generated at each of these three sites did not lead to alterations in binding that were greater than 2-fold, we did not believe it particularly relevant to generate and investigate more conservative mutations at these particular residues.

Expression of r-[$K2_{tPA}$] variants with nonconservative mutations at D^{57} and D^{59} was difficult, but better success was obtained with the more conservative mutations of each. However, this situation nonetheless suited our purposes since the data of Table III, with $D^{57}E$ and $D^{59}E$ mutations, clearly revealed the important role of these two residues in stabilizing the binding of ω-amino acids to r-[$K2_{tPA}$]. With the various ligands employed, alteration of D^{57} to E resulted in a 3–6-fold weaker binding, and alteration of D^{59} to E led to changes of 18–85-fold in this same binding. All ligands did not respond in the same manner to the amino acid mutations in these cases. The ligand with the potentially longest spacing between its amino and carboxyl moieties, 7-AHpA, appeared to show the greatest response to these mutations. Its relatively poorer fit into the binding pocket is probably reflective of the more restricted steric relationships between binding site residues imposed by substitution of the more bulky E residues for the normal D residues in this kringle domain. Given the large changes in binding observed with these conservative mutations, and the clear definition of the importance in this regard of D^{57} and D^{59} in ligand binding, we felt that attempting to construct and express molecules with more radical alterations at these locations would not be additionally revealing.

The effect of these mutations on the gross conformation of the r-[$K2_{tPA}$] domain was examined by ^{1}H-NMR analysis of the mutants. Particularly important in this regard is the steric relationship of the L^{47} side chain with the aromatic ring of W^{25}, and to some extent with Y^{35} (de Vos et al., 1992), revealed in solution by the ring current-induced large upfield shift (doublet at ca. –1 ppm) of the protons of one of the two side-chain methyl groups of L^{47}. This signal, along with the large chemical shift difference (ca. 1.5 ppm) between the protons of the two equivalent CH_3 groups of L^{47}, signifies proper overall folding of the kringle, since subtle alterations in steric relationships between W^{25}, Y^{35}, and the $CH_3^{\delta'}$ group of L47 would be expected to affect the particular unusual chemical shifts observed for the methyl proton resonances of L^{47}.

As revealed by the data of Figure 4 and Table IV, no major alterations are observed in the gross folding of r-[$K2_{tPA}$] as a result of the mutations made. Compared to wtr-[$K2_{tPA}$], a maximum change in the chemical shift of the $CH_3^{\delta'}$ doublet is only approximately 0.026 ppm and is found in the case of the $D^{57}E$ mutant. A change of approximately –0.030 ppm is observed for the CH_3^{δ} protons of this same mutant. In the case of r-[$K2_{tPA}/D^{59}E$], the alteration in the $CH_3^{\delta'}$ protons is approximately –0.017 ppm. This reveals some variation in the environment of L^{47} in these two mutants and could result from a small conformational reorientation at this location in the molecules. It is possible that this change could be related in some measure to the loss of ligand binding capacity by r-[$K2_{tPA}/D^{57}E$] and r-[$K2_{tPA}/D^{59}E$]. However, it should be

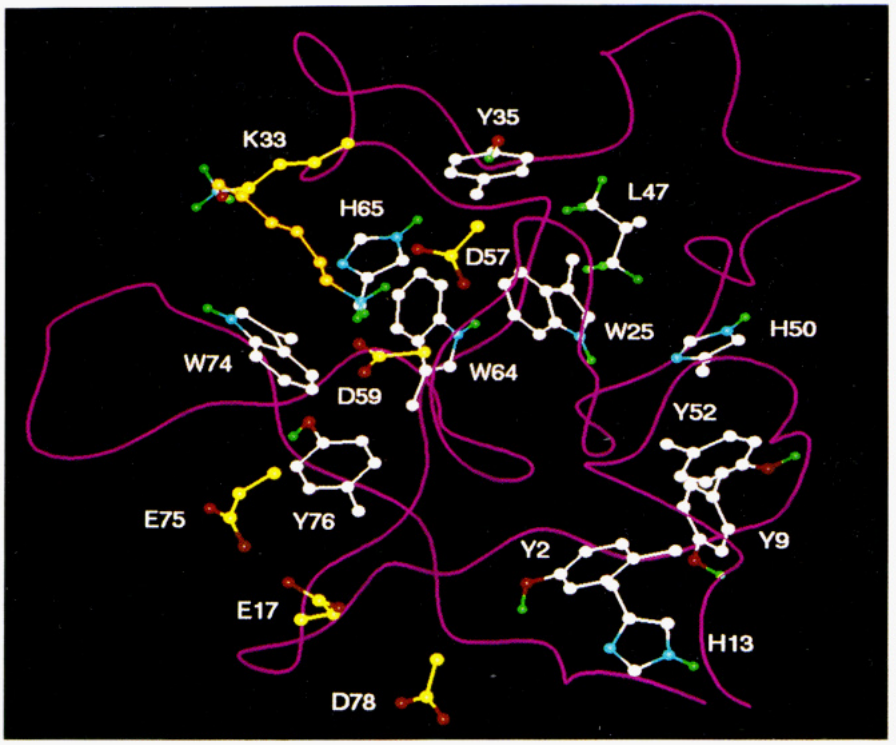

FIGURE 7: X-ray structure of wtr-[$K2_{tPA}$], emphasizing the relationships between the anionic and aromatic amino acid residues [from de Vos et al. (1992)]. Selected amino acid side chains are displayed on the backbone structure, as indicated. The numbering of amino acids begins at C^1 of the r-[$K2_{tPA}$] sequence and continues consecutively to C^{81}. Each amino acid is displayed from its β-carbon, and nonexchangeable hydrogen atoms are excluded to minimize overcrowding. The backbone conformation is in magenta. Negatively charged (at pH 8.0) amino acid side-chain carbon atoms are in yellow, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms are in green. Aromatic amino acid (including histidyl residues) residues are in white. The lysine (K) side chain (K^{49}) from another molecule of r-[$K2_{tPA}$] that is inserted into the binding pocket in the unit cell is shown with its side-chain carbon atoms in orange. The location of K^{33}, previously found to be important to ω-amino acid binding, is also displayed.

noted that an even larger chemical shift difference from wtr-[$K2_{tPA}$] (ca. –0.055 ppm) is found for the CH_3^{δ} protons of r-[$K2_{tPA}/D^{78}A$], without loss of its binding energy to these same ligands. Smaller changes are also found in these resonances for all mutants that interact normally with the ligands. Therefore, it is unlikely that these particular chemical shift differences are related in any substantial way to the binding of ligands by these proteins.

A more detailed analysis of the microenvironments of some specific amino acid residues is possible as a result of analysis of the methyl (Figure 4) and aromatic proton regions (Figure 5 and Tables V and VI) of the ^{1}H-NMR spectra of the mutants, along with the proton resonance assignments made earlier (Byeon et al., 1889, 1991). A clear assignment of the CH^{γ} resonances of V^{34} can be made at an average of 0.11 ppm. A very slight upfield shift of this resonance position is observed in the case of the $E^{17}A$ mutant, but it is essentially unaffected in all others. Similarly, the CH_3^{δ} protons of L^{30}, resonating at an average of 0.23 ppm, are also virtually unchanged in all of the mutants examined. The CH^{γ} protons of V^{79} are also clearly observed at 0.62 ppm and are only significantly altered in the r-[$K2_{tPA}/D^{78}A$] mutant. Thus, while the microenvironment of this side chain is altered in this mutant, ligand binding is normal, suggesting that the change is unimportant to this particular property of the kringle domain. Other areas of the methyl group spectra of these mutants are somewhat altered in the mutants, particularly the new average resonance that appears at ca. 0.08 ppm in the $D^{57}E$ mutant. These alterations cannot be clearly assigned to individual amino acids, and no pattern emerges relating these changes to the ability of the mutant kringle domains to interact with the ligands employed.

Several assignments of aromatic proton resonances can be easily made from the spectra of Figure 5, in particular the H2 protons of H^{13}, H^{50}, H^{65}, W^{25}, W^{63}, and W^{74}, as well as the H2,6 and H3,5 protons of four of the five Y residues, viz., Y^2, Y^{35}, Y^{52}, and Y^{76}. The steric relationship among these residues in the X-ray structure of wtr-[$K2_{tPA}$] is illustrated in Figure 7 and can be used for reference in the remainder of this discussion. With the $E^{17}A$ mutant, the H2 protons from the three W residues undergo significant changes in chemical shifts as compared to wtr-[$K2_{tPA}$] (Table V). However, since this mutant interacts with the ω-amino acid ligands in a manner similar to that of the wild-type molecule, it is clear that the microenvironment changes of the W residues are not important to the steric relationships of binding pocket residues. The influence of these particular mutations on the microenvironment of W^{25} likely explains the small changes in the chemical shifts of the $CH_3^{\delta'}$ protons observed in Table IV. Interestingly, the largest difference from wtr-[$K2_{tPA}$] in the chemical shift of the H2 protons of W^{25} is found for the $D^{57}E$ mutant, as was the situation with the $CH_3^{\delta'}$ protons. While this could represent a localized small alteration of the orientation in W^{25} in this mutant, it is also possible that this change in environment in the H2 proton of W^{25} could result from its increased shielding by the carboxyl group of E^{57}. In wtr-[$K2_{tPA}$], the distance between OD1 of D^{57} and H2 of W^{25} is 6.8 Å (de Vos et al., 1992). It is possible that insertion of E at position 57 in the case of r-[$K2_{tPA}/D^{57}E$] could result in movement of its carboxylate atoms up to 1.5 Å closer to the H2 proton of W^{25} without a localized conformational alteration, thereby increasing shielding and resulting in an upfield shift of this proton. These shielding considerations on the H2 proton of W^{25} may also apply to the E^{59} mutant (but in a less influential

manner and in the opposite direction) since the OD2 atom of D^{57} in wtr-[$K2_{tPA}$] is 8.8 Å from the H2 proton of W^{25}. Similar shielding or deshielding arguments cannot likely be made for the influence of the carboxylates of E^{17}, E^{75}, and E^{78} on the chemical shift differences in the H2 proton of W^{25} since these groups are at least 12 Å apart. Thus, a minor long-range conformational alteration in W^{25} may accompany these mutations, but this does not significantly affect the binding of ω-amino acids to these mutants. The alterations in microenvironments of the H2 proton of the two W residues in the binding pocket, viz., W^{63} and W^{74}, especially in the cases of the $D^{57}E$ and $D^{59}E$ mutants, probably also result from carboxylate shielding and deshielding changes caused by the mutations. In these cases, the distances between the OD1 and OD2 atoms of D^{59} and the H2 proton of W^{74} are <5 Å and are <7.4 Å to the H2 proton of W^{63}. The distances between the OD1 and OD2 atoms of D^{57} and the H2 proton of W^{63} are <5.4 Å, and this distance is calculated to be 7.7 Å for the D^{59}–OD2/W^{74}–H2 atoms. Thus, that shielding and deshielding mechanisms may account for the chemical shift changes in the H2 proton for these W residues in the $D^{57}E$ and $D^{59}E$ mutants is certainly possible. In an overall sense, the chemical shift differences in these aromatic proton resonances for the three mutants that interact normally with the ω-amino acid ligands are about as large as for the two mutants that show greatly altered binding. Thus, we do not believe that the microenvironmental changes observed in the H and W residues as a result of the mutations are of great importance to the ligand binding properties observed and probably reflect minor changes in solvent shielding and/or local conformations.

With a small number of exceptions, the resonance positions of the tyrosyl ring protons of the mutants are not significantly altered from those same resonances in wtr-[$K2_{tPA}$] (Table VI). The more major changes are observed for the H3,5 protons of Y^{76} in the weak ligand binding mutants, $D^{57}E$ and $D^{59}E$. Since Y^{76} is close to the binding pocket of wtr-[$K2_{tPA}$], and its ring proton chemical shifts are perturbed by the presence of the ligand (Byeon et al., 1989), it is likely that the weaker ligand binding properties of these two mutants have some basis in the effects of these mutations on the positioning of Y^{76}. Obviously, it is also possible that the local perturbations of Y^{76} are the result of chemical shielding differences of the protons by the mutations at D^{57} and D^{59}, especially in the case of the large deshielding effect on the H3,5 protons of Y^{76} resulting from the $D^{59}E$ mutation. In this case, the distance from OD2 of D^{57} to H3 of Y^{76} is only 2.7 Å, and alterations in this distance as a result of the mutation could have a pronounced direct effect on the chemical shift of this proton. While it remains possible that some perturbation of the steric relationships of Y^{76} in the binding pocket may be affected by the D^{57} and D^{59} mutations, which possibly influence the ligand binding properties of these molecules, it is pointed out that nonconservative mutation of Y^{76} (e.g., $Y^{76}L$) does not result in a kringle with substantially weaker ligand binding properties.[2] On the other hand, the relatively large perturbation of the H2,6 protons of Y^2 in the E^{57} domain must result from a localized change in the microenvironment of this residue since direct enhanced shielding effects resulting from the mutation at D^{57} are unlikely due to the relatively long distance between carboxylate oxygens of D^{57} and these protons (ca. 11 Å). Other noteworthy alterations in tyrosyl ring proton resonances occur in the H2,6 and H3,5 protons of Y^{35} in r-[$K2_{tPA}/E^{75}A$] and r-[$K2_{tPA}/D^{78}A$] and in the H2,6 protons in r-[$K2_{tPA}/E^{17}A$]. However, since these mutants interact normally with the ω-amino acid ligands examined, any amino acid microenvironmental alterations induced by the mutations do not appear to be of major importance to the geometry of the binding pocket.

As revealed by the DSC investigations, all of the anionic loci of wtr-[$K2_{tPA}$] contribute to the stability of the native conformation of this domain (except for the case of D^{59} for which we have no information). The necessity of the presence of a specific anionic residue at a given location is the case of D^{57}. Replacement of this amino acid with an E residue still results in a substantial destabilization of the native conformation of this kringle. That native conformations are indeed maintained, albeit destabilized, is indicated by the very similar increases in their stabilizations by addition of the ligand 6-AHxA. These results provide support for similar modes of binding of 6-AHxA to each of the mutants, with substantial changes in the strength of this binding in the case of $D^{57}E$ (and likely $D^{59}E$) resulting from loss of optimal fit of the ligand into the binding site.

In summary, we have clearly defined the major roles played by D^{57} and D^{59} of the kringle 2 domain of tPA in stabilization of its interaction with ω-amino acid ligands. In conjunction with earlier mutagenesis studies of other amino acid residues in this isolated domain region, a comprehensive view of the nature of the binding interaction of kringle domains with these important ligands is now emerging.

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