Involvement of Tyrosine-76 of the Kringle 2 Domain of Tissue-Type Plasminogen Activator in Its Thermal Stability and Its ω-Amino Acid Ligand Binding Site†

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ABSTRACT: A series of conservative and radical mutations have been made at an aromatic residue, Y^{76}, of the isolated kringle 2 domain of tissue-type plasminogen activator ([$K2_{tPA}$]) in order to assess the importance of this residue in the ligand binding properties and structural stability of this protein domain. We have successfully expressed in *Escherichia coli* r-[$K2_{tPA}$] variants with the following amino acid mutations at Y^{76}: $Y^{76} \rightarrow A$, $Y^{76} \rightarrow E$, $Y^{76} \rightarrow F$, $Y^{76} \rightarrow K$, $Y^{76} \rightarrow L$, $Y^{76} \rightarrow Q$, and $Y^{76} \rightarrow W$. The binding constants of 6-aminohexanoic acid (EACA) and 7-aminoheptanoic acid (7-AHpA) to each of these mutants were investigated by titration of the alterations in intrinsic fluorescence of the mutant kringles with these amino acid ligands. Compared to the wild-type kringle (r-[$K2_{tPA}$]), which possessed dissociation constants (K_d) of 43 and 6 μM, respectively, for EACA and 7-AHpA, only the $Y^{76} \rightarrow E$ mutant displayed a substantially increased K_d value for these amino acids, *viz.*, 117 μM for 7-AHpA. More moderate increases in this parameter were observed for the $Y^{76} \rightarrow A$ and $Y^{76} \rightarrow K$ variants (2–3-fold increases in the K_d), with no significant differences noted in the cases of $Y^{76} \rightarrow L$, $Y^{76} \rightarrow Q$, and $Y^{76} \rightarrow W$. A most interesting observation was made with the $Y^{76} \rightarrow F$ mutant, which showed a 4–6-fold reduction in the K_d for these amino acid ligands. The conformations of all of the mutants were less stable than that of wtr-[$K2_{tPA}$], as revealed by thermal denaturation studies, suggesting that a Y at sequence position 76 is of importance to the conformational stability of this kringle domain. The temperature of maximum heat capacity (T_m) of the thermal denaturation of wtr-[$K2_{tPA}$], of 75.6 °C, was destabilized by 10 °C ($Y^{76} \rightarrow W$) to 16 °C ($Y^{76} \rightarrow E$) for these mutants. In all cases, at a concentration of EACA that saturated its binding site on each of the mutants, a shift in T_m of approximately 8 °C ($Y^{76} \rightarrow L$) to 21 °C ($Y^{76} \rightarrow Q$) occurred, demonstrating the stabilization of the native structure of the wild-type and mutant kringles by binding of this ligand. ^{1}H-NMR analysis of the methyl proton region of each of the mutant kringles was employed as another method to assess their overall folding properties. Of special significance is the high upfield chemical shifts of the $CH_3^{\delta'}$ protons, which result from their close proximity to the aromatic ring currents of W^{25}. This location of this doublet signal was nearly fully preserved in all of the mutants, further demonstrating that none of the mutations at Y^{76} resulted in significantly misfolded polypeptides. We conclude that Y^{76} does influence the ligand binding properties of r-[$K2_{tPA}$], likely through long-range effects on binding site residues, and also plays an important role in maintenance of the structural stability of the native structure of this domain.

Kringles are structural motifs that exist in a variety of proteins. These units contain approximately 80 amino acids, within which are present three disulfide bonds. In genes encoding the proteins in which they reside, kringles normally occur as one or two exons (Patthy, 1985), and the information for proper kringle folding appears to exist entirely within the kringles themselves (Castellino et al., 1981). A variable number of kringles have been found in their resident proteins, from a single copy in urokinase (Gunzler et al., 1982) up to 38 in human apolipoprotein(*a*) (McLean et al., 1987). Two kringles exist in tPA[1] (Pennica et al., 1983).

Kringle domains are present in noncatalytic portions of proteins, and their roles are believed to center on stabilization of protein–protein interactions. The binding of HPg (Thorsen, 1975; Wiman & Wallen, 1977; Thorsen et al., 1981) and tPA (van Zonneveld et al., 1986a–c) to fibrin, the initial interaction of HPm and α_2-antiplasmin (Wiman et al., 1978), and the interaction of HPg with peripheral blood cells (Miles & Plow, 1987; Miles et al., 1988) are examples of these types of interactions. Such protein–protein binding is inhibited by ω-amino acids, a fact that lends importance to understanding of the interactions of these low-molecular-weight ligands to kringles. Kringles that have been shown to interact with ω-amino acid ligands are [$K1_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991), [$K4_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; Sehl & Castellino, 1990), [$K5_{HPg}$] (Castellino et al., 1981; Novokhatny et al., 1989; Menhart et al., 1993), and [$K2_{tPA}$] (Cleary et al., 1989; De Serrano & Castellino, 1992b).

Investigation of specific residues of kringles that stabilize the binding of ω-amino acid ligands to these structural units

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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; [$K2_{HPg}$], the kringle 2 region (residues C^{166}–C^{243}) of human plasminogen; [$K3_{HPg}$], the kringle 3 region (residues C^{256}–C^{333}) 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; [K_{uPA}], the kringle region (residues C^{50}–C^{131}) of human urokinase; [$K2_{HPT}$], the kringle 2 region (residues C^{170}–C^{248}) of human prothrombin; EACA, 6-aminohexanoic acid; 7-AHpA, 7-aminoheptanoic acid; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; DSC, differential scanning calorimetry; ES/MS, electrospray mass spectrometry; K_d, dissociation constant.

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has been performed using site-directed mutagenesis and ^{1}H-NMR methodologies and has been predicted by analysis of X-ray crystallographic patterns of isolated kringle domains. In the case of [$K2_{tPA}$], the principal cationic center for stabilization of binding of these ligands is K^{33} (De Serrano & Castellino, 1992a; De Serrano et al., 1992b), and the anionic binding loci are provided by both D^{57} and D^{59} (De Serrano & Castellino, 1993). Residue W^{74} plays an essential role in stabilizing these same complexes through interactions with the methylene backbones of the ligands (De Serrano & Castellino, 1992b). These conclusions are in full agreement with those obtained by modeling of the ligand binding pocket on the basis of results from X-ray crystallography with a pseudoligand complexed to r-[$K2_{tPA}$] (the side chain of K^{49} of one wtr-[$K2_{tPA}$] molecule was found to be inserted in a putative ligand binding site of a second wtr-[$K2_{tPA}$] molecule in the unit cell used for X-ray crystallographic structural studies) (de Vos et al., 1992), as well as those from investigations of the structure of r-[$K2_{tPA}$] by ^{1}H-NMR methodology (Byeon et al., 1989, 1991).

The aromatic residue Y^{76} is located in the vicinity of the binding pocket in the pseudoligand/r-[$K2_{tPA}$] complex (de Vos et al., 1992), and its proton resonances are perturbed upon ligand binding (Byeon et al., 1989, 1991). Thus, this residue may have direct or long-range effects on ligand binding. The importance of this residue in maintenance of the structure and ligand binding integrity of [$K2_{tPA}$] cannot be assessed with certitude from the crystal structure—its relevance can only be predicted from assessment of its proximity to other atoms. Likewise, from ^{1}H-NMR experiments, it is not known whether perturbations of the proton resonances originating from this residue by ligands are related to ligand binding through short-range direct interactions of the ligand with Y^{76} or long-range effects of ligand binding on polypeptide structural interactions. We believed that investigations with site-directed mutants at Y^{76} would contribute to clarification of the role of this amino acid residue in ligand binding and in maintenance of the structural integrity of the kringle. This paper is a report of the results of such a study.

MATERIALS AND METHODS

Proteins. Restriction endonucleases were obtained from the Fisher Scientific Co. (Springfield, NJ). Recombinant Taq DNA polymerase (AmpliTaq) was purchased from Perkin-Elmer Cetus (Norwalk, CT).

Construction of Expression Plasmids. The expression plasmid for r-[$K2_{tPA}$], pSTII/[$K2_{tPA}$], has been described earlier (De Serrano & Castellino, 1992b). The translation product contains residues C^{180}–C^{261} of tPA (in kringle numbering this represents C^1–C^{81}) with a dipeptide, SD, amino terminal to C^{180}, and a single residue, S, carboxy terminal to C^{261}. This same plasmid was the template for production of the mutants.

For construction of r-[$K2_{tPA}/Y^{76}F$], the following oligonucleotide was used (the mutagenic bases are represented by lower-case lettering):

5′-CTG ACG TGG GAa TtC TGT GAT GTG CCC

Screening of positive transformants was accomplished by the incorporation of the *Eco*RI site that accompanies the designed mutation. All other mutants were prepared from the plasmid carrying the $Y^{76}F$ mutation and were screened by loss of the *Eco*RI site that was inserted into this cDNA.

Expression and Purification of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] Variants. Expression of all cDNAs was accomplished in *Escherichia coli* DH5α cells, as described earlier for a series of other mutants of this kringle domain (De Serrano & Castellino, 1992a,b, 1993).

In order to purify the mutant kringles, the transformed *E. coli* cells were first separated into periplasmic and oxidatively refolded fractions (Menhart et al., 1991). The resulting samples were subjected to FPLC on a lysine–Sepharose column equilibrated with 25 mM Tris-HCl, pH 8.0. The major fraction that was eluted with the EACA gradient was then purified to apparent homogeneity by FPLC using a Mono S column (De Serrano & Castellino, 1992b).

Intrinsic Fluorescence Titrations. The binding of ω-amino acid ligands to the mutant kringle domains was measured by taking advantage of the intrinsic fluorescence change in the kringle that accompanies this binding. The procedures used have been described in earlier reports (De Serrano & Castellino, 1992a,b, 1993). These experiments were conducted at 25 °C in a buffer containing 50 mM Tris–OAc/150 mM NaOAc, pH 8.0 (Menhart et al., 1991). The K_d values that characterize the r-[$K2_{tPA}$]/ω-amino acid interactions were calculated from the fluorescence titrations by nonlinear least squares iterative curve fitting of the titration data (Menhart et al., 1991).

^{1}H-NMR. The samples were dissolved in 0.05 M sodium phosphate, pH 7.4, that was fully preexchanged with ^2H_2O. These solutions were lyophilized and then redissolved in the same volume of ^2H_2O. This procedure was repeated two additional times.

The 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. The detailed procedures have been described previously (De Serrano & Castellino, 1992b).

Differential Scanning Calorimetry. The samples were 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 EACA, pH 8.0. Thermograms were obtained with use of a Microcal (Northampton, MA) MC-2 scanning calorimeter. Thermal denaturation scans were conducted between the temperature range of 25 and 100 °C, at scan rates of 30 °C/h. Under these conditions, the T_m was independent of the temperature scan rate. The baseline for each run was obtained in an identical experiment with the sample buffer in each cell.

The temperature of maximum heat capacity (T_m) was obtained from the thermograms as previously described (Radek & Castellino, 1988; Sehl & Castellino, 1990).

DNA Methodology. All methods that we employ for manipulations of the DNA samples have been published earlier (Menhart et al., 1991; De Serrano & Castellino, 1992b; De Serrano et al., 1992a).

Analytical Methods. ES/MS was conducted using a JOEL (Peabody, MA) AX505 HA mass spectrometer equipped with the JOEL electrospray ionization source as described previously (De Serrano & Castellino, 1992a,b). $NaDodSO_4$–PAGE was conducted as described (Laemmli, 1970).

RESULTS

A series of mutations of Y^{76} of r-[$K2_{tPA}$] (its amino acid sequence is illustrated in Figure 1) were constructed and the resulting cDNAs expressed in *E. coli* cells. The primers employed for the mutagenesis and the screening strategies used are summarized in Table 1. After expression and disruption of the cells, the desired recombinant materials were found to be present predominantly in the cell-associated

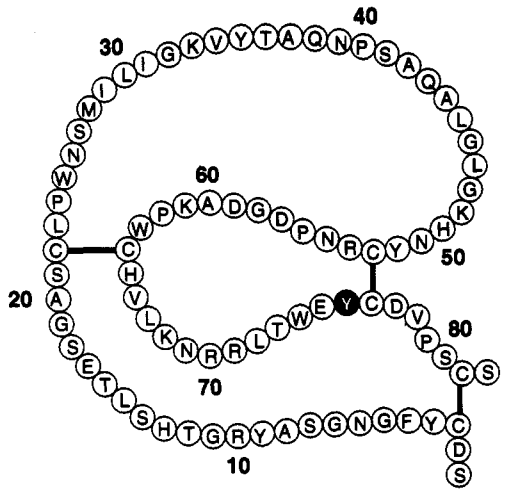

FIGURE 1: Amino acid sequence of the particular construction of r-[$K2_{tPA}$]. The position of the Y^{76} residue is indicated by a color reversal.

Table 1: Construction of Y^{76} Variants of r-[$K2_{tPA}$]

mutation[a]	primer[b]	screen
wild-type[b]	5'-CTG ACG TGG GAA TAC TGT GAT GTG CCC	
$Y^{76}\rightarrow A^c$	5'-CTG ACG TGG GAA gca TGT GAT GTG CCC	- *Eco* RI
$Y^{76}\rightarrow E^c$	5'-CTG ACG TGG GAA gaa TGT GAT GTG CCC	- *Eco* RI
$Y^{76}\rightarrow F$	5'-CTG ACG TGG GAa TtC TGT GAT GTG CCC	+ *Eco* RI
$Y^{76}\rightarrow K^c$	5'-CTG ACG TGG GAA aag TGT GAT GTG CCC	- *Eco* RI
$Y^{76}\rightarrow L^c$	5'-CTG ACG TGG GAA cTg TGT GAT GTG CCC	- *Eco* RI
$Y^{76}\rightarrow Q^c$	5'-CTG ACG TGG GAA cag TGT GAT GTG CCC	- *Eco* RI
$Y^{76}\rightarrow W^c$	5'-CTG ACG TGG GAA Tgg TGT GAT GTG CCC	- *Eco* RI

[a] The column below indicates the wild-type amino acid in r-[$K2_{tPA}$] and its sequence position beginning from C^1 of the kringle, followed by the amino acid replacement at that position. [b] The bases that represent mutations from the wild-type cDNA are represented by lower-case letters. The wild-type sequence in the vicinity of Y^{76} is shown for reference. [c] These mutants were constructed from $Y^{76}\rightarrow$ F and screened by loss of the *Eco*RI restriction site previously cloned into this latter cDNA with the $Y^{76}\rightarrow$ F mutation. The bases that represent mutations from the $Y^{76}\rightarrow$ F cDNA are represented by lower-case letters.

fractions and were successfully oxidatively refolded. The mutant kringles have been purified in successive steps by virtue of their specific affinities for lysine–Sepharose, followed by a HPLC step using Mono S resin. Despite weakened ω-amino acid binding by some of the mutants, the recombinant variants were nonetheless effectively adsorbed to the affinity chromatography column. Final yields of purified r-polypeptides ranged from 0.4 to 3 mg/100 g of cells (wet weight).

The variant polypeptides were examined for the incorporation of the mutation by determination of the molecular weights of the resulting product using ES/MS. In all cases, the dominant charged species were +6 and +7, and the final molecular weights are listed in Table 2. The close agreement between the calculated and experimental molecular weights provides very strong evidence that the mutants were those that were expected and that proper signal polypeptide processing occurred.

All mutant kringles underwent saturable alterations in intrinsic fluorescence as a result of addition of the ligands employed in this work. The maximal changes ranged from approximately 12% to 38% for the different muteins and ligands. The titration data, an example of which is illustrated in Figure 2, were best fit to K_d values and maximal fluorescence changes for the ligand/kringle interaction by routine iterative

Table 2: Molecular Weights of Y^{76} Variants of r-[$K2_{tPA}$]

variant	molecular weight: calculated	molecular weight: experimental
r-SD[$K2_{tPA}$]S[a]	9365.69	9363.4
r-SD[$K2_{tPA}/Y^{76}A$]S[b]	9273.42	9272.5
r-SD[$K2_{tPA}/Y^{76}E$]S[b]	9331.46	9332.5
r-SD[$K2_{tPA}/Y^{76}F$]S[b]	9349.52	9349.5
r-SD[$K2_{tPA}/Y^{76}K$]S[b]	9330.52	9329.4
r-SD[$K2_{tPA}/Y^{76}L$]S[b]	9315.50	9318.6
r-SD[$K2_{tPA}/Y^{76}Q$]S[b]	9330.48	9329.0
r-SD[$K2_{tPA}/Y^{76}W$]S[b]	9388.56	9390.0

[a] Refers to the wild-type recombinant molecule. The bracketed portion is the amino acid sequence of $K2_{tPA}$ (from C^1–C^{81}). Flanking the amino terminus of C^1 is the dipeptide SD, and the flanking the carboxy 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 and its sequence position beginning from C^1 of the kringle, followed by 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.

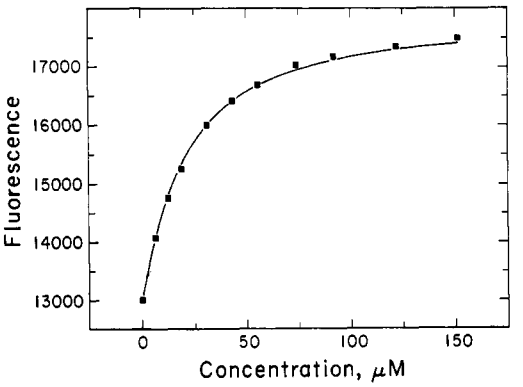

FIGURE 2: Titration of the increase in intrinsic fluorescence (arbitrary units) of r-[$K2_{tPA}/Y^{76}K$] (4.6 μM) with 7-AHpA. The experimental points (■) are best fit to a line generated by employing values of K_d = 17.4 μM and a maximal relative fluorescence change of +37.89%. The stoichiometry was set at 1.0 for these iterations.

Table 3: Dissociation Constants for ω-Amino Acids to Y^{76} Variants of r-[$K2_{tPA}$] As Determined by Intrinsic Fluorescence Titrations at 25 °C

variant	dissociation constant (μM) for: EACA	dissociation constant (μM) for: 7-AHpA
wt[a]	43	6
$Y^{76}\rightarrow A^b$	102	18
$Y^{76}\rightarrow E^b$	nd[c]	117
$Y^{76}\rightarrow F^b$	12	1
$Y^{76}\rightarrow K^b$	104	17
$Y^{76}\rightarrow L^b$	66	12
$Y^{76}\rightarrow Q^b$	37	18
$Y^{76}\rightarrow W^b$	51	9

[a] Refers to the wild-type recombinant molecule. [b] The mutations made in wtr-[$K2_{tPA}$]: the amino acid in the wt molecule and its sequence position beginning from C^1 of the kringle, followed by the amino acid replacement at that position. [c] Not determined.

nonlinear least squares analysis (Menhart et al., 1991) with the assumption of a stoichiometry of 1.0. The K_d values obtained for the kringle/ligand interactions are summarized in Table 3.

The role of Y^{76} in the elucidation and/or stabilization of the native r-[$K2_{tPA}$] structure was assessed by investigation of the thermal denaturation properties of the kringle of interest. The DSC thermograms of each of the mutants displayed a single two-state transition from which the T_m values were obtained. These parameters are summarized in Table 4. In

Table 4: Thermal Stabilities of Y^{76} Variants of r-[$K2_{tPA}$]

variant	T_m (°C)[a] -EACA[b]	T_m (°C)[a] +EACA[c]
wt[d]	75.6	86.1
$Y^{76} \rightarrow A$[e]	64.5	74.8
$Y^{76} \rightarrow E$[e]	59.5	70.1
$Y^{76} \rightarrow F$[e]	66.7	81.5
$Y^{76} \rightarrow K$[e]	61.7	71.2
$Y^{76} \rightarrow L$[e]	65.3	73.5
$Y^{76} \rightarrow Q$[e]	64.3	85.2
$Y^{76} \rightarrow W$[e]	64.8	79.5

[a] The temperature of maximum heat capacity. [b] The buffer was 50 mM Tris–OAc/100 mM NaOAc, pH 8.0. [c] The buffer was 50 mM Tris–OAc/100 mM NaOAc/50 mM EACA, pH 8.0. [d] Refers to the wild-type recombinant molecule. [e] The mutation made in wtr-[$K2_{tPA}$]: the amino acid in the wt molecule and its sequence position beginning from C^1 of the kringle, followed by the amino acid replacement at that position.

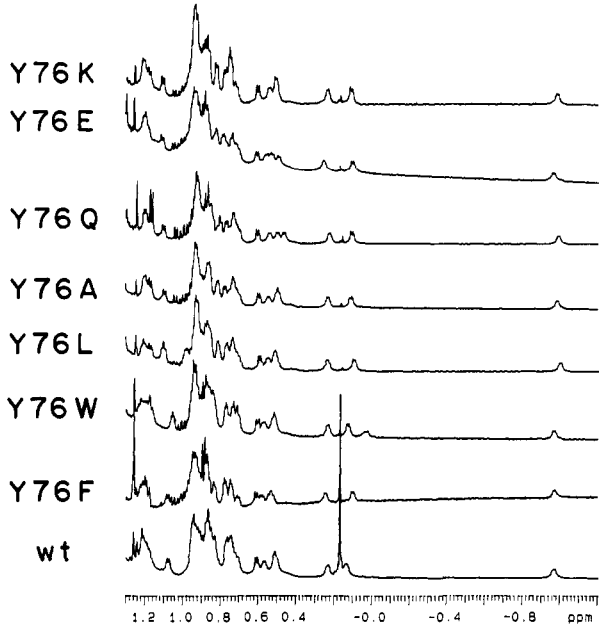

FIGURE 3: Methyl proton spectral region of the ^{1}H-NMR spectra of wtr-[$K2_{tPA}$] and Y^{76} recombinant mutants of r-[$K2_{tPA}$]. The spectrum representing each of the mutants is labeled in the illustration. The temperature was 37 °C and the pH* was 7.4. The spectral width was 6000 Hz and the number 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 at 3.77 ppm downfield of tetramethylsilane. Enhancement of the resolution was achieved by Gaussian convolution.

no case was the T_m value dependent upon the scan rate employed.

An important conformational determinant of all native kringle domains thus far examined is the close interaction with the $CH_3^{\delta'}$ protons of L^{47} and the aromatic ring of W^{25} (Byeon et al., 1989l De Serrano & Castellino, 1994). This interaction restricts the mobility of the $CH_3^{\delta'}$ protons and leads to their resolution from the apparently equivalent CH_3^{δ} protons from this same L^{47}. A large upfield shift in these $CH_3^{\delta'}$ protons of L^{47} methyl protons (*ca.* −1 ppm) is thus found because of the resulting ring current effects from their proximity to W^{25}. One-dimensional ^{1}H-NMR spectra of the methyl proton regions of all of the Y^{76} mutants of r-[$K2_{tPA}$] are provided in Figure 3. In all cases, the $CH_3^{\delta'}$ protons of L^{47} are found at approximately −1 ppm. A summary of the resonance positions of the CH_3^{γ}, CH_3^{δ}, and $CH_3^{\delta'}$ protons from L^{47} in these mutants obtained from two-dimensional measurements is provided in Table 5.

Table 5: Chemical Shifts of ^{1}H Resonances from L^{47} in Y^{76} Variants of r-[$K2_{tPA}$] at pH* 7.4 and 37 °C

variant	chemical shifts (ppm) CH_3^{γ}	CH_3^{δ}	$CH_3^{\delta'}$[a]
wt[b]	1.120	0.569	−0.967
$Y^{76} \rightarrow A$[c]	1.098	0.598	−0.985
$Y^{76} \rightarrow E$[c]	1.109	0.531	−0.960
$Y^{76} \rightarrow F$[c]	1.084	0.540	−0.967
$Y^{76} \rightarrow K$[c]	1.107	0.502	−0.984
$Y^{76} \rightarrow L$[c]	1.092	0.512	−0.992
$Y^{76} \rightarrow Q$[c]	1.100	0.504	−0.984
$Y^{76} \rightarrow W$[c]	1.054	0.527	−0.968

[a] The average of a doublet observed for this set of protons. [b] Refers to the wild-type recombinant molecule. [c] The mutations made in wtr-[$K2_{tPA}$]: the amino acid in the wt molecule and its sequence position beginning from C^1 of the kringle, followed by the amino acid replacement at that position.

DISCUSSION

The amino acid residue Y^{76} of [$K2_{tPA}$] is conserved at homologous locations in other kringles that interact with ω-amino acids, *viz.*, [$K1_{HPg}$], [$K4_{HPg}$], and [$K5_{HPg}$], while not being rigidly conserved in some of kringles that do not interact with ligands of this type, *e.g.*, [$K2_{HPg}$], [K_{uPA}], and [$K1_{hPT}$]. This, coupled with observations from ^{1}H-NMR studies that the protons of Y^{76} are perturbed by ligand binding (Byeon et al., 1989), suggests that Y^{76} is a candidate residue for more rigorous examination of its role in the ω-amino acid ligand binding properties of [$K2_{tPA}$]. The X-ray structure of wtr-[$K2_{tPA}$] (de Vos et al., 1992) places the side chain of Y^{76} in a hydrophobic cluster of residues that have been shown or predicted to be of importance in the binding of ligands or in the structural integrity of this domain. The side-chain residues involved in this cluster are W^{25} (Byeon et al., 1991; De Serrano & Castellino, 1994), L^{30} (Byeon et al., 1991), Y^{35} (Byeon et al., 1991), L^{47} (Byeon et al., 1989), W^{63} (Tulinsky et al., 1988; Byeon et al., 1991), H^{65} (Kelley & Cleary, 1989; Byeon et al., 1991), L^{72} (Byeon et al., 1991), W^{74} (Byeon et al., 1991; De Serrano & Castellino, 1992b), and Y^{76} (Byeon et al., 1991). A representation of the X-ray structure of wtr-[$K2_{tPA}$] emphasizing the relationships of these amino acids is provided in Figure 4. While X-ray and NMR investigations are predictive of the involvement of Y^{76} in the structural stabilization and/or ligand binding characteristics of [$K2_{tPA}$], the extent of its participation in these properties cannot be evaluated by those types of studies. Thus, we undertook an investigation wherein we subjected this residue to site-directed mutagenesis, both conservatively and nonconservatively, in order to address these questions more directly.

A series of r-[$K2_{tPA}$] mutants have been successfully expressed and purified which contain both conservative and nonconservative substitutions for Y^{76}. Radical alterations of Y^{76} to A, L, or Q did not greatly affect ligand binding, suggesting that Y^{76} does not play a direct role in stabilizing interactions with ligands. Regarding aromatic mutations (Table 3), substitution of a W for Y at this position had no dramatic effects on the K_d values for two representative ligands. However, a similar replacement of an F for Y at position 76 resulted in an upregulation of binding of these same two ligands, with K_d values 4–6-fold lower than with similar binding to wtr-[$K2_{tPA}$]. This is the first case of an amino acid replacement leading to increased ligand binding in this particular kringle module. This effect could be due to long-

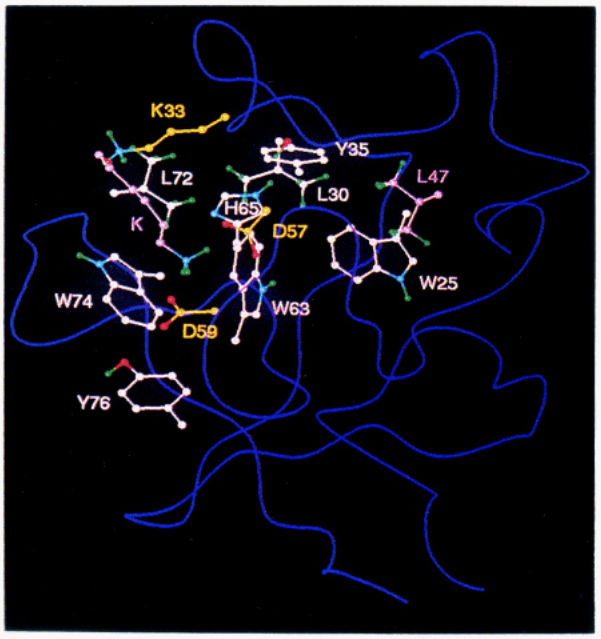

FIGURE 4: Representation of the X-ray structure of wtr-[$K2_{tPA}$], emphasizing the steric relationships of Y^{76} to the previously identified ω-amino acid ligand binding site residues K^{33}, D^{57}, D^{59}, W^{63}, H^{65}, and W^{74} and to the conformational determinants of L^{47}, W^{25}, and possibly Y^{35}. Selected amino acid side chains are displayed from their β-carbons on the blue ribbon, as indicated. The sequence position of amino acids begins at C^1 of the r-[$K2_{tPA}$] sequence and continues consecutively to the last C (C^{82}) of this domain. Most hydrogen atoms are excluded to minimize overcrowding. Aromatic and hydrophobic amino acid (W^{25}, L^{30}, Y^{35}, W^{63}, H^{65}, L^{72}, W^{74}, and Y^{76}) side-chain carbon atoms are in white, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms are in green. Amino acid side-chain carbon atoms from charged residues (K^{33}, D^{57}, and D^{59}) are in yellow. The side-chain carbon atoms of L^{47} are in pink for emphasis, as are the side-chain carbon atoms for a pseudoligand lysine residue (K is K^{49} from another [$K2_{tPA}$] molecule in the unit cell that is possibly inserted into the ω-amino acid binding pocket).

range influences of this residue on the ligand, perhaps *via* reorientation of W^{74}, given the proximity of Y^{76} and W^{74}. For example, calculations from the X-ray structure (represented in Figure 4), indicate that the distance from the CE1 carbon atom of Y^{76} is only 4.0 Å from the CH2 carbon atom of W^{74}, and the corresponding protons are 3.7 Å apart. Thus, it is possible that some changes at Y^{76} could influence orientation of W^{74} in the binding pocket, with consequent enhancement or diminutation of ligand binding. That the W at position 74 (and its possible orientation) may be crucial to ligand binding has been demonstrated from mutagenesis studies at this location (De Serrano & Castellino, 1992b) and from investigations with another kringle module. In this latter situation, greatly enhanced (>10-fold) ligand binding to r-[$K1_{HPg}$] occurred with alteration of a Y to a W at sequence position 71, which is a homologous location to W^{74} in [$K2_{tPA}$] (Hoover et al., 1993). Thus, indirect influences on W^{74}, as may be the case with aromatic substitutions at Y^{76}, could dramatically influence ligand binding. In addition to the above, from the X-ray structure of r-[$K2_{tPA}$], it appears as though the aromatic residue, W^{63}, is potentially located in the binding pocket of the ligand, although its exact role in binding has not as yet been evaluated. It has been demonstrated that, as a result of irradiation of the indole CH2 or W^{63}, a NOE cross-peak is produced on the doublet protons from Y^{76} (Byeon et al., 1989), suggesting distances between these groups of <5 Å. Thus, it would appear that mutagenesis of Y^{76} could affect ligand binding through influences on W^{63}. However, measurements from the X-ray structure indicate that the distances from the CH2 proton of W^{63} to the HD1 or HE1 protons of Y^{76} are 9–12 Å apart, a distance too far to show a NOE cross-peak. This could be indicative of a difference between the crystal structure and the solution structure. However, in this same X-ray structure, there are protons in W^{63} and Y^{76} that are sufficiently close to indeed show that such contacts are possible. For example, measurements from the X-ray structure of r-[$K2_{tPA}$] show that the HD1 proton of W^{63} and the HD1 or HE1 protons of Y^{76} are only approximately 3.6 Å apart. Further, the HB2 proton of W^{63} and the HD1 proton of Y^{76} are only 2.6 Å apart. This suggests that Y^{76} and W^{63} are also sufficiently proximal such that mutations in Y^{76} could in fact also influence the orientation of W^{63} and possibly exert indirect effects on ligand binding through this residue.

The largest decreases in ligand binding energy resulting from alterations at Y^{76} result from mutations to E or K. Here, it is possible that indirect effects on the orientation of the essential D^{59} (De Serrano & Castellino, 1993) can occur by replacement of Y^{76}. In this case, the distance from the OH oxygen of Y^{76} is only 3.5 Å from the OD1 oxygen of D^{59} (see Figure 4). Thus, replacement of Y^{76} to E could lead to movement of D^{59} due to charge repulsion, and similar replacement of Y^{76} to K could also influence D^{59} due to charge attraction. In either case, such reorientation could result in influences on the interaction of D^{59} with the amino group of the ligand.

It appears as though Y^{76} contributes significantly to the stability of the native structure of [$K2_{tPA}$]. Substantial destabilizations of the native kringle structure resulted from mutations of Y^{76} to the charged amino acids E and K (–16 and –14 °C, respectively), whereas more modest, but very significant, destabilizations were observed with more conservative mutations (Table 4). However, all substitutions at Y^{76} resulted in a lowering of the T_m of wtr-[$K2_{tPA}$], with the smallest change observed with the $Y^{76} \rightarrow$ F mutant (–9 °C). Thus, of all amino acids so far mutated in r-[$K2_{tPA}$] (De Serrano & Castellino, 1992a,b, 1993, 1994), the largest contributors to conformational stability of this kringle domain have been identified to be W^{25} (De Serrano & Castellino, 1994) and Y^{76} (this study).

Finally, some comment can be made regarding the effects of substitutions at Y^{76} on the conformation of wtr-[$K2_{tPA}$]. It has been shown in numerous investigations that a signal for proper folding of kringle domains is the high-field doublet proton resonance of the $CH_3^{\delta'}$ protons that results from the ring current shifts that are due to the proximity of W^{25} to this particular methyl group of L^{47}. Both of these amino acid residues are conserved in all kringle structures identified to date. Given the expectation that the chemical shifts of these methyl protons would be strongly dependent on distance relationships of L^{47} and W^{25}, a very sensitive probe of kringle domain conformation is available. The data of Figure 3 and Table 5 demonstrate that only very small differences in the chemical shifts of the protons that originate from the CH^{γ}, CH_3^{δ}, and $CH_3^{\delta'}$ of L^{47} occur in all of the Y^{76} mutants. This demonstrates that the orientation of L^{47}, and all amino acid side chains that contribute to this orientation, is essentially unchanged in each of the Y^{76} mutant forms of this kringle module.

In conclusion, a series of mutagenesis investigations by this laboratory have resulted in evaluation of the importance of amino acid side chains of [$K2_{tPA}$] that stabilize its interaction with ω-amino acids. We find that the essential components of the binding pocket are K^{33} (De Serrano et al., 1992b; De Serrano & Castellino, 1992b), D^{57} (de Serrano & Castellino,

1993), D^{59} (De Serrano & Castellino, 1993), and W^{74} (De Serrano & Castellino, 1992b), and from X-ray (de Vos et al., 1992) and NMR (Byeon et al., 1989, 1991) analyses, W^{63} also is likely to be of considerable importance. H^{65} has been shown to contribute to the specificity of the binding site for these ligands (Kelley & Cleary, 1989; De Serrano & Castellino, 1992b). Of the aromatic residues so far investigated, it has been directly demonstrated that the conformational stability of this kringle is greatly influenced by the presence of W^{25} (De Serrano & Castellino, 1994) and Y^{76} (this study), with W^{74} playing a more minor role therein. H^{65} (Kelley & Cleary, 1989), D^{57} (De Serrano & Castellino, 1993), and E^{75} (De Serrano & Castellino, 1993) also make a significant contribution to the stability of r-[$K2_{tPA}$]. With the continuing development of knowledge in this area, we will come to a detailed understanding of the nature of ligand interactions with their binding regions along with a rigorous comparison of crystallographic and solution structures of these domains. These results are of importance not only to the specific fields which are addressed but also to fundamental questions of protein structure–function relationships.

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