Role of the Strictly Conserved Tryptophan-25 Residue in the Stabilization of the Structure and in the Ligand Binding Properties of the Kringle 2 Domain of Tissue-Type Plasminogen Activator†

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ABSTRACT: The involvement of the strictly conserved tryptophan-25 (W^{25}) residue in the structural stability and ω-amino acid ligand binding properties of the recombinant (r) kringle 2 (K2) domain of tissue-type plasminogen activator (tPA) has been investigated. Two conservative mutants were constructed and expressed that contained $W^{25}\rightarrow F$ and $W^{25}\rightarrow Y$ substitutions. The binding (dissociation) constants (K_d) for three ligands, *viz.*, 6-aminohexanoic acid (EACA), 7-aminoheptanoic acid(7-AHpA), and L-lysine (Lys), to these polypeptides were determined by intrinsic fluorescence titrations. In the case of r-[$K2_{tPA}/W^{25}F$], the K_d values for these ligands were found to be 37, 16, and 89 μM for EACA, 7-AHpA, and Lys, respectively. For r-[$K2_{tPA}/W^{25}Y$], the K_d values for these same ligands were 64, 9, and 115 μM, respectively. The wild-type (wt) kringle domain possessed K_d values of 43, 6, and 85 μM for EACA, 7-AHpA, and Lys, respectively. The effect of these mutations on the stability of the r-[$K2_{tPA}$] domain has been examined by differential scanning colorimetry. The temperature of maximum heat capacity (T_m) of wt-r-[$K2_{tPA}$] (75.6 °C) was dramatically reduced to 50.8 and 58.0 °C for r-[$K2_{tPA}/W^{25}F$] and r-[$K2_{tPA}/W^{25}Y$], respectively. In the presence of EACA, the T_m values were increased to 86.1, 61.7, and 68.7 °C, respectively, indicating that EACA does interact with the r-[$K2_{tPA}$] mutants and stabilizes their native conformations, similar to the case with wt-r-[$K2_{tPA}$]. Comparison of the ^1H NMR spectra of the two variants with that of wt-r-[$K2_{tPA}$] demonstrates that the strong upfield chemical shift of the $CH_3^{\delta'}$ protons from L^{47} of r-[$K2_{tPA}$], which results from the proximity of these protons to the aromatic ring of W^{25}, is reduced, most likely because of the less intense ring currents of F^{25} and Y^{25} or a minor localized conformational perturbation that results in an increase in the distance of these protons from the aromatic ring of W^{25}. The results of this investigation indicate that W^{25} is a residue of great importance to the maintenance of the structural stability of the K2 module of tPA. On the other hand, this same residue only appears to be of minor primary importance to the determinants of the ω-amino acid ligand binding of this domain.

Kringles are triple-disulfide bond-stabilized modules of proteins discovered originally in prothrombin (Magnusson et al., 1975) and subsequently in other proteins involved in blood clot formation and dissolution, as well as in apolipoprotein(a) and HGF[1]-like proteins. These highly homologous domains contain 80 amino acid residues, all present as one or two exons in the gene (Patthy, 1985), and their folding apears to be self-directed (Castellino et al., 1981; Tulinsky et al., 1988; Novokhatny et al., 1991; Menhart et al., 1993). Thus, kringles probably function independently of the remainder of the proteins in which they reside. The number of kringles present in these proteins varies substantially, from single copies in urokinase (Gunzler et al., 1982) and coagulation factor XII (McMullen & Fujikawa, 1985) to as many as 38 in human apolipoprotein(a) (McLean et al., 1987). Two kringles are present in tPA (Pennica et al., 1983).

Kringle domains function by mediating protein–protein interactions. Relevant examples are 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 recognition of HPm by its fast-acting inhibitor, α_2-antiplasmin (Wiman et al., 1978), and the interaction of HPg with thrombin-stimulated and nonstimulated platelets (Miles et al., 1988). These binding events are inhibited by ω-amino acids, thereby imparting relevance to an understanding of the binding of these small ligands to kringle domains. Kringles that are known to possess such ligand binding sites 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; Menhart et al.), [$K5_{HPg}$] (Castellino et al., 1981; Novokhatny et al., 1989; Menhart et al., 1993), and [$K2_{tPA}$] (Cleary et al., 1989; De Serrano & Castellino, 1992b).

Studies of the participation of specific amino acid residues of kringles in stabilizing their interactions with ω-amino acid ligands have been facilitated by the ability to express properly folded recombinant kringle domains in bacterial cells. Such expression has been accomplished with r-[$K2_{tPA}$] (Cleary et al., 1989; De Serrano & Castellino, 1992b), r-[$K1_{HPg}$] (Menhart et al., 1991), r-[$K4_{HPg}$] (Menhart et al., 1993), and r-[$K5_{HPg}$] (Menhart et al., 1993). The peptide residues that participate in ω-amino acid binding to r-[$K2_{tPA}$] have been

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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; EACA, 6-aminohexanoic acid; 7-AHpA, 7-aminoheptanoic acid; $DodSO_4$–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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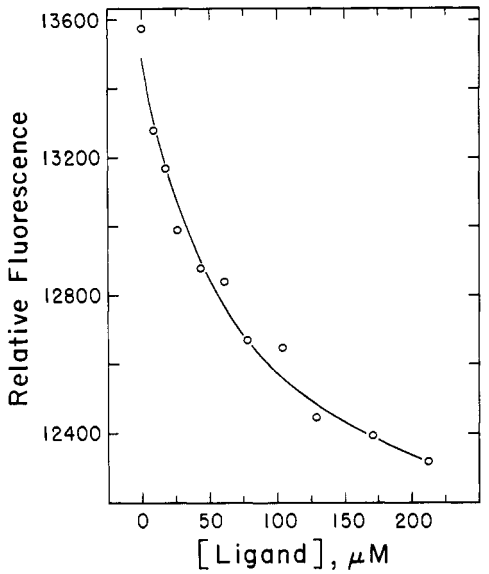

FIGURE 1: Titraion of the relative change in intrinsic fluorescence of r-[$K2_{tPA}$/$W^{25}Y$] (5.0 μM) with EACA. The experimental points (O) are the best fit to a line generated employing values of $n = 1.0$, $K_d = 68.9$ μM, and a maximal relative fluorescence change of −11.4%.

Table 1: Dissociation Constants for ω-Amino Acids to r-[$K2_{tPA}$] Variants As Determined from Intrinsic Fluorescence Titrations

	dissociation constant (μM)[a]		
variant	EACA	7-AHpA	L-Lys
wt	43	6	85
$W^{25}F$	37	16	89
$W^{25}Y$	66	9	115

[a] The error limits for K_d range from 10 to 25% of the indicated values.

implied from X-ray and NMR structural analyses of this module (de Vos et al., 1991; Byeon et al., 1989) and more thoroughly defined by site-directed mutagenesis of this isolated domain (De Serrano & Castellino, 1992a,b, 1993). The principal cationic center for the stabilization of binding of these ligands is K^{33} (De Serrano & Castellino, 1992a,b), 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 backbone of the ligands (De Serrano & Castellino, 1992b).

In addition to the direct participation of W^{74} in stabilizing ligand binding to r-[$K2_{tPA}$], ^1H NMR studies have shown that the resonances of other aromatic amino acids are perturbed by the binding of ligands to this kringle domain. These include W^{25}, W^{63}, H^{65}, W^{74}, and Y^{76} (Byeon et al., 1989). While this latter study could not distinguish between direct and long-range effects of the ligands on these amino acid side chains, the case of W^{25} is of special importance since this particular amino acid is exactly conserved at this location in all kringles identified to date. It is thus expected that this residue would play a role in the binding of ligands by relevant kringle domains and/or be an essential determinant in the folding of these modules. In order to examine these hypotheses, we have investigated the effects of point mutations at this sequence position on the conformational stability of r-[$K2_{tPA}$] and on its ability to bind ω-amino acid ligands. A summary of the results of this investigation is presented herein.

MATERIALS AND METHODS

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

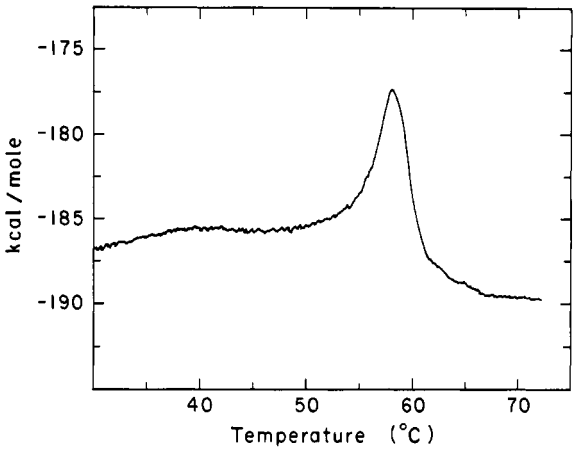

FIGURE 2: DSC thermogram of r-[$K2_{tPA}$/$W^{25}Y$]. The change in heat capacity at constant pressure (ΔC_p) is plotted against temperature. The buffer employed was 50 mM Tris–OAc/150 mM NaOAc (pH 8.0).

Table 2: Thermal Stabilites of r-[$K2_{tPA}$] Variants

	T_m (°C)[a]	
variant	−EACA	+EACA
wt	75.6	86.1
$W^{25}F$	50.8	61.7
$W^{25}Y$	58.0	68.7

[a] The temperature of maximum heat capacity.

Construction of Expression Plasmids. Plasmid pSTII[$K2_{tPA}$] (De Serrano & Castellino, 1992b) was used for the expression of r-[$K2_{tPA}$]. The gene product produced 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, which were constructed with mutagenic synthetic oligonucleotides.

For construction of the cDNA (pSTII[$K2_{tPA}$/$W^{25}F$]) encoding r-[$K2_{tPA}$/$W^{25}F$], the following oligonucleotide was used (the mutagenic bases are represented by lowercase lettering): 5′-CC TCC TGC CTC CCG Tac AAc TCC ATG ATC CTG ATA. Screening of positive transformants was accomplished by loss of the *Eco*RI site in the wild-type cDNA accompanying the designed mutation. Construction of the cDNA (pSTII[$K2_{tPA}$/$W^{25}Y$]) that codes for r-[$K2_{tPA}$/$W^{25}Y$] was accomplished using the following synthetic primer: 5′-CC TCC TGC CTC CCG Ttc AAc TCC ATG ATC CTG ATA. The screening of bacterial transformants was as above.

Expression and Purification of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] Variants. Expression of the cDNAs, pSTII[$K2_{tPA}$], pSTII[$K2_{tPA}$/$W^{25}F$], and pSTII[$K2_{tPA}$/$W^{25}Y$], was accomplished in *Escherichia coli* DH5α cells as described earlier for other mutants (De Serrano & Castellino, 1992a,b). For purification of the recombinant kringles, the conditioned *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 Mono S resin (De Serrano & Castellino, 1992a).

Intrinsic Fluorescence Titrations. The effect of ω-amino acid ligands on the intrinsic fluorescence of r-[$K2_{tPA}$] and

r-[K2$_{tPA}$] mutants was determined at 25 °C in a buffer containing 50 mM Tris–OAc/150 mM NaOAc (pH 8.0), as described (Menhart et al., 1991). K_d values characteristic of the r-[K2$_{tPA}$]/ω-amino acid interaction were calculated from the fluorescence titrations by nonlinear least-squares iterative curve-fitting (Menhart et al., 1991).

^1H NMR. The lyophilized r-[K2$_{tPA}$] or r-[K2$_{tPA}$] mutant samples were dissolved in 0.05 M sodium phosphate (pH 7.4) (fully preexchanged with ^2H_2O), relyophilized, and then redissolved in the same volume of ^2H_2O. Two additional ^1H–^2H exchanges were then accomplished in the same manner. One-dimensional ^1H NMR 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. Details on the methodology that we employ have been described previously (De Serrano & Castellino, 1992b).

Differential Scanning Calorimetry. The polypeptide 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). DSC thermograms were obtained using a Microcal (Northampton, MA) MC-2 scanning calorimeter. Thermal denaturation scans were conducted in the temperature range 25–100 °C at scan rates of 30 deg/h. Under these conditions, the T_m was independent of the temperature scan rate. The base line for each run was obtained in an identical experiment with the sample buffer in each cell.

The methods that we employ for deconvolution of the thermograms and for determination of the temperature of maximum heat capacity (T_m) have been published earlier (Radek & Castellino, 1988; Sehl & Castellino, 1990).

DNA Methodology. 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).

Analytical Methods. ES/MS was conducted using a Jeol (Peabody, MA) AX505 HA mass spectrometer equipped with the Jeol electrospray ionization source, as described previously (De Serrano & Castellino, 1992a). DodSO_4–PAGE was conducted as described (Laemmli, 1970).

RESULTS

We have successfully expressed two conservative mutants of r-[K2$_{tPA}$] in bacterial cells that contained amino acid alterations at amino acid position W^{25}. This particular residue is exactly conserved at this sequence position in all kringle structures identified to date. The only mutants that were expressed successfully contained F and Y at this position. Our attempts to express mutants with other amino acids at this location were not successful, suggesting that this residue may be of importance to the structural integrity of this kringle module.

Subcellular fractionation of the transformed *E. coli* cells demonstrated that the mutants were found in the cell-associated material. Oxidative refolding and subsequent chromatography on lysine–Sepharose columns demonstrated that lysine binding materials were obtained. The total yield of each mutant was approximately 3–5 mg/100 g of cells. The molecular weights of the purified materials were determined by ES/MS and were found to be 9325.1 (9326.66 calcd) for r-SD[K2$_{tPA}$/W^{25}F]S and 9341.9 (9342.66 calcd) for r-SD-[K2$_{tPA}$/W^{25}Y]S. This close agreement between the experimental and theoretical values clearly demonstrates that the desired mutations were translated into the polypeptides and that proper signal polypeptide cleavage occurred.

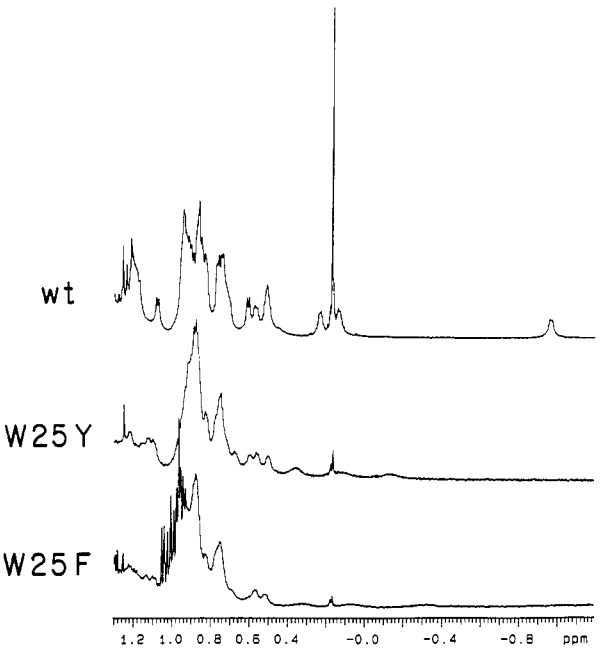

FIGURE 3: High-field spectral region of the ^1H NMR spectra of wt-r-[K2$_{tPA}$] and variants of r-[K2$_{tPA}$] containing mutations of W^{25}: top, wt-r-[K2$_{tPA}$]; middle, r-[K2$_{tPA}$/W^{25}Y]; bottom, r-[K2$_{tPA}$/W^{25}F]. The temperature was 37 °C and the pH was 7.4. The spectral width was 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.

The K_d values for the interaction of three representative ω-amino acid ligands with the mutant kringles were determined by titration of the intrinsic fluorescence changes induced in r-[K2$_{tPA}$] by these ligands. In the cases of the two mutants, fluorescence quenching (10–17%, maximum quenching depending on the particular ligand and polypeptide) occurred upon ligand binding, whereas for wt-r-[K2$_{tPA}$], an approximate maximum fluorescence enhancement of 28% was obtained (De Serrano & Castellino, 1992b). For all binding studies, the fluorescence alterations were saturable with ligand. An example of one such titration is illustrated in Figure 1 for the binding of EACA to [K2$_{tPA}$/W^{25}Y]. Iterative nonlinear least-squares minimizations of the fluorescence titrations (Menhart et al., 1991) resulted in the K_d values listed in Table 1. The data show that, for each ligand, the K_d values for the mutants were not appreciably different from those of wt-r-[K2$_{tPA}$].

The influence of the mutations at W^{25} on the stability of the native structure of wt-r-[K2$_{tPA}$] has been examined by DSC. An example of a thermogram obtained in the case of r-[K2$_{tPA}$/W^{25}Y] is shown in Figure 2. The temperature of maximum heat capacity (T_m) obtained for this scan was 58.2 °C. A summary of the data obtained for the two mutants in the absence and presence of sufficient EACA to saturate its binding site on the polypeptides is provided in Table 2. In all cases, the T_m values were independent of the thermal scan rates. Substantial reductions from wt-r-[K2$_{tPA}$] in the T_m values for each of the mutants were observed in the absence of EACA. In the presence of EACA, increases in the T_m values of approximately 11 °C were found in each case, indicating that stabilization of the native conformations by this ligand was obtained for wt-r-[K2$_{tPA}$], as well as for both mutants.

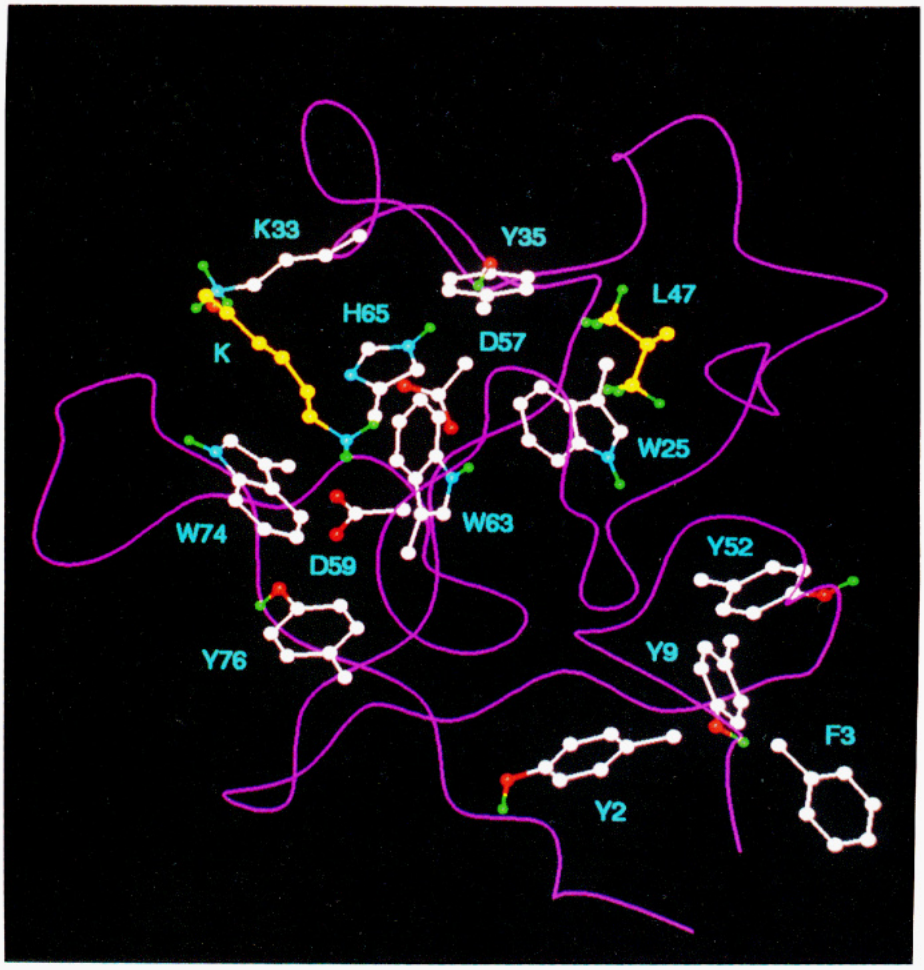

FIGURE 4: Representation of the X-ray structure of wt-r-[$K2_{tPA}$]. The spatial relationships between aromatic amino acids, L^{47}, and the active-site residues are emphasized. Selected amino acid side chains are displayed on the backbone structure, as indicated. The numbering of amino acids begins at C^1 of the r-[$K2_{tPA}$] sequence and continues consecutively to the last C (C^{82}) of this domain. Each amino acid is displayed from its β-carbon (most hydrogen atoms are excluded to minimize overcrowding). The ribbon corresponding to the backbone conformation is in purple. Amino acid side-chain carbon atoms are white, nitrogen atoms are blue, oxygen atoms are red, and hydrogen atoms are green. The side-chain carbon of L^{47} is shown in yellow for emphasis, as are the side-chain carbon atoms for a lysine residue (K^{49}) from another [$K2_{tPA}$] molecule in the unit cell that is inserted into the ω-amino acid binding pocket. The charged amino acids displayed (K^{33}, D^{57}, and D^{59}) have been shown in earlier studies to be involved in ligand binding, as has the influence of H^{65} on the ligand binding specificity of [$K2_{tPA}$].

High-field ^1H NMR spectra for each of the kringle polypeptides are illustrated in Figure 3. The unusual and characteristic chemical shift of the $CH_3^{\delta'}$ protons of L^{47}, observed at approximately −1.0 ppm for wt-r-[$K2_{tPA}$], is absent from both of the W^{25} mutant kringles, suggesting that W^{25} plays a role in defining the spectral shifts of these protons.

DISCUSSION

The amino acid residue W^{25} is strictly conserved in all kringle domains identified to date, and this observation prompted us to evaluate its importance in the binding of ligands to an isolated kringle domain (r-[$K2_{tPA}$]), as well as its possible role in maintaining the structural integrity of the kringle. Despite the fact that several nucleotide substitutions were made in the cDNA of this kringle that would be translated into a variety of amino acid substitutions, only the most conservative of these mutants, *viz.*, $W^{25}F$ and $W^{25}Y$, could be expressed as identifiable kringles. Expressions of this kringle containing other amino acid substitutions were unsuccessful even when such r-[$K2_{tPA}$] mutants were placed in tandem vectors containing another kringle, a strategy that has been employed successfully to express other mutants of r-[$K2_{tPA}$] (De Serrano & Castellino, 1992b), as well as the non-lysine binding kringle, r-[$K1_{tPA}$] (De Serrano et al., 1992a).

Both variants of r-[$K2_{tPA}$] interacted with lysine–Sepharose, providing the first indication that the ω-amino acid binding site was present to some degree. Quantitative binding studies by intrinsic fluorescence titrations with three ligands were then performed. Despite the replacement of W^{25}, intrinsic fluorescence perturbations were still observed, although the direction of the change was opposite to that of wt-r-[$K2_{tPA}$]. However, in the cases of both mutants, these alterations were saturable with the ligands, allowing effective use of this binding method. The results of these titrations showed that conservative substitutions at W^{25} did not substantially disrupt the ligand binding site of [$K2_{tPA}$]. This result is consistent with the X-ray crystal structure of r-[$K2_{tPA}$] (Figure 4), wherein it could be seen that W^{25} is situated at too far of a distance from the ligand to have a direct effect on its binding (*e.g.*, the distances from the CZ3, CE3, and CH2 atoms of W^{25} to the CG atom of the ligand in Figure 4 are approximately 9–10 Å). However, it has also been shown that the ^1H NMR chemical shifts of this residue are affected by ligands of these types (Byeon et al., 1989). Thus, the ligand effects on the environment of W^{25} must be indirect and transmitted through other amino acid residues in the polypeptide.

The very high upfield chemical shifts of the $CH_3^{\delta'}$ protons of L^{47} are believed to be due to the strong ring current effects of W^{25} on the methyl protons of this leucine residue (De Marco et al., 1982; Byeon et al., 1989; Atkinson & Williams, 1990; Thewes et al., 1990; Mulichak et al., 1991; Menhart et al., 1991; De Serrano & Castellino, 1992b, 1993; De Serrano et

al., 1992a). Support for this concept is provided by the X-ray crystal structure of this kringle domain (Figure 4), which clearly shows the very close proximity of the W^{25} aromatic ring to the $CH_3^{\delta'}$ of L^{47} (*e.g.*, a distance of only 2.7 Å is found from the CE3 atom of W^{25} to the HD21 atom of L^{47}).

Confirmation of the importance of W^{25} to the chemical shifts of the $CH_3^{\delta'}$ protons is provided by examination of the methyl proton regions of the ^1H NMR spectra of the two W^{25} mutants (Figure 3). It is seen that the $CH_3^{\delta'}$ protons are shifted from their positions in wt-r-[$K2_{tPA}$]. This is an expected result if W^{25} is indeed so involved, since ring current induced chemical shifts that originate from F^{25} and Y^{25} would be less than that from a W at this position. Of course, it cannot be ruled out that a localized conformational perturbation in these mutants could have affected the spatial relationships between these aromatic rings and the methyl groups of L^{47}, thereby also influencing the chemical shifts of these methyl proton resonances. However, a large conformational alteration resulting from these mutations appears highly improbable, since the binding constants for the ligands to the two mutants are very similar to those of wt-r-[$K2_{tPA}$]. It is most likely that reduced ring current effects, as well as small localized conformational perturbations due to substitutions of F or Y for W, are responsible for the changes seen in the chemical shifts of the protons from the $CH_3^{\delta'}$ group of L^{47}.

DSC analysis of the W^{25} mutants clearly reveals the importance of this amino acid residue in defining the structural stability of wt-r-[$K2_{tPA}$]. Approximate 17–25 °C reductions in the T_m values of thermal denaturations were found as a consequence of these conservative mutations at W^{25}. The T_m values of each mutant were increased by approximately 11 °C consequent to the addition of saturating levels of EACA, as was the case with wt-r-[$K2_{tPA}$] (Table 2). This is consistent with the conclusion reached above, that no major conformational alterations resulted from these conservative mutations, otherwise it would not be expected that similar increases in thermal stabilities to wt-r-[$K2_{tPA}$] would result from addition of EACA to the mutants. Our interpretation of these results is that the conformations of both wt-r-[$K2_{tPA}$] and the wt-r-[$K2_{tPA}$]/EACA complex are substantially stabilized by W^{25}. Conservative mutation of this residue to either F or Y does not markedly alter the overall conformation of this kringle or its complex with ligand, but does substantially alter their structural stabilities.

In conclusion, we have forwarded an explanation for the strict conservation of W^{25} in all known kringle structures. This residue is of great importance in contributing to the conformational stability of [$K2_{tPA}$] and likely to all other kringles as well. The equally strict conservation of an L residue at a position homologous to L^{47} of [$K2_{tPA}$] in all kringles, and the close proximity of W^{25} and L^{47} in [$K2_{tPA}$], suggests that the W^{25}–L^{47} hydrophobic interaction plays a major role in stabilizing the conformations of kringles.

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