Role of Tryptophan-63 of the Kringle 2 Domain of Tissue-Type Plasminogen Activator in Its Thermal Stability, Folding, and Ligand Binding Properties†

Yuan Chang, Jaroslav Zajicek, and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received January 28, 1997; Revised Manuscript Received April 14, 1997⊗

ABSTRACT: Conservative (F and Y) and radical (H and S) mutations have been engineered at a rigidly conserved aromatic residue, W^{63}, of the isolated recombinant kringle 2 domain of tissue-type plasminogen activator (r-$K2_{tPA}$), an amino acid residue predicted from the X-ray crystal structure to be important in the ligand binding properties of this isolated protein domain. The variants were expressed in *Pichia pastoris* cells. The binding constants of ϵ-aminocaproic acid (EACA), 7-aminoheptanoic acid (7-AHpA), and *trans*-(aminomethyl)cyclohexanecarboxylic acid (AMCHA) to each of these mutant polypeptides were determined by titrations of the alterations in intrinsic fluorescence of the variant kringles with the ligands. As compared to wild-type r-$K2_{tPA}$, increases in the K_d (dissociation) values of approximately 15-fold and 20−200-fold were found for the $W^{63}F$ and $W^{63}Y$ mutants, respectively, toward these three ligands. Neither the $W^{63}H$ nor the $W^{63}S$ variant interacted with these same ligands. Differential scanning calorimetric analyses were also performed on each of the peptides to determine whether the alterations affected the conformational stability of wtr-$K2_{tPA}$. The data demonstrated that all of these mutants were thermally destabilized, possessing temperatures of maximum heat capacity (T_m) values that were 12−20 °C lower than that of wtr-$K2_{tPA}$. Addition of EACA resulted in increases (≈12 °C) in the T_m values of r-[$W^{63}F$]-$K2_{tPA}$ and r-[$W^{63}Y$]$K2_{tPA}$, a result showing that EACA stabilized the native conformations adopted by these kringle domains. As expected from its greatly diminished binding to r-[$W^{63}H$]$K2_{tPA}$ and r-[$W^{63}S$]-$K2_{tPA}$, high concentrations of EACA had little effect on the T_m of thermal denaturation of these latter mutants. ^{1}H-NMR analysis of the two aromatic mutant kringles was employed to assess their overall comparative folding properties. The high upfield chemical shifts (−0.98 ppm) of the $CH_3^{\delta'}$ protons of L^{47}, a major signal of proper kringle folding, were slightly lowered to −0.83 to −0.86 ppm in the cases of all of the mutants. This is due to alterations in the W^{25}−L^{47} side-chain spatial orientations, possibly the result of slight conformational alterations that affect the distance relationships of these two amino acid side chains. Assignments of nearly all of the protons of the aromatic residues in the $W^{63}F$ and $W^{63}Y$ mutants were accomplished, and few additional differences from their wild-type counterpart were noted. Reactivities of the mutants against four different monoclonal antibodies directed to wtr-$K2_{tPA}$ revealed the possibility that some small local conformational alterations might have resulted from the residues that have replaced the W^{63}. We conclude that W^{63} possesses an important direct role in the ligand binding properties of r-$K2_{tPA}$. This residue also contributes significantly to the stability of the native conformation of this kringle domain and perhaps to maintenance of local conformations.

Kringles are structural motifs found in a number of proteins related to those involved in blood coagulation and clot dissolution. These modules contain approximately 80 amino acids and are covalently stabilized by three rigidly conserved disulfide bonds in a 1−6, 2−4, 3−5 pattern. These modules occur singly, as in uPA[1] (Gunzler et al., 1982), and with great multiplicity, as in apo-Lp(*a*), where 11−41 copies have been found (McLean et al., 1987; Koschinsky et al., 1991; Kraft et al., 1992; Marcovina et al., 1996). Of the proteins involved in fibrinolysis, Pg contains five kringles (Sottrup-Jensen et al., 1978), one exists in uPA (Gunzler et al., 1982), and tPA possesses two such structural units (Pennica et al., 1983).

The kringle modules in serine proteases of these types do not constitute part of their catalytic domains but principally function as recognition units for other proteins in solution and for components of cell surfaces. As examples, the kringle modules of Pg and Pm interact with platelets (Miles et al., 1988), peripheral blood cells (Miles et al., 1988), endothelial cells (Wu et al., 1992), and bacterial cells

† Supported by Grant HL-13423 from the National Institutes of Health and the Kleiderer/Pezold family endowed professorship (to F.J.C.).

* To whom to address correspondence. Telephone: (219) 631-6456. Telefax: (219) 631-8017.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: tPA, tissue-type plasminogen activator; $K2_{tPA}$, the kringle 2 region (residues C^{180}−C^{261}) of tissue-type plasminogen activator; Pg, human plasminogen; Pm, human plasmin; $K1_{Pg}$, the kringle 1 region (residues C^{84}−C^{162}) of human plasminogen; $K2_{Pg}$, the kringle 2 region (residues C^{166}−C^{243}) of human plasminogen; $K3_{Pg}$, the kringle 3 region (residues C^{256}−C^{333}) of human plasminogen; $K4_{Pg}$, the kringle 4 region (residues C^{358}−C^{435}) of human plasminogen; $K5_{Pg}$, the kringle 5 region (residues C^{462}−C^{541}) of human plasminogen; uPA, urokinase-type plasminogen activator; K_{uPA}, the kringle region (residues C^{50}−C^{131}) of human urokinase; BSA, bovine serum albumin; Lp(*a*), lipoprotein(*a*); MAb, monoclonal antibody; EACA, 6-aminohexanoic acid; 7-AHpA, 7-aminoheptanoic acid; t-AMCHA, *trans*-(aminomethyl)cyclohexanecarboxylic acid; DSC, differential scanning calorimetry; ELISA, enzyme-linked immunoadsorbent assay; C_{50}-MAb, total concentration of monoclonal antibody necessary for binding to 50% of the molecules containing its epitope; TOF-MALDI-DE-MS, time-of-flight matrix-assisted laser desorption ionization with delayed-extraction mass spectrometry; T_m, temperature of maximum change in heat capacity; TOCSY, total correlation spectroscopy; NOESY, nuclear overhauser enhancement spectroscopy.

S0006-2960(97)00197-9 CCC: $14.00

(Ullberg et al., 1990). The kringles of HPm bind to its plasma inhibitor, α_2-antiplasmin (Sugiyama et al., 1988; Hortin et al., 1989), to its substrates, fibrinogen (Lucas et al., 1983) and fibrin (Thorsen et al., 1981; Lucas et al., 1983), to the plasma protein effectors, histidine-rich glycoprotein (Lijnen et al., 1980) and tetranectin (Clemmensen et al., 1986), and to cell-bound actin (Lind & Smith, 1991) and enolase (Miles et al., 1991). Further, the K_{uPA} and $K2_{tPA}$ modules have been shown to mediate binding of tPA and uPA to their fast-acting plasma inhibitor, plasminogen activator inhibitor 1 (Wilhelm et al., 1990; Kaneko et al., 1991; Mimuro et al., 1992). In the case of uPA, a region of the molecule that includes its K_{uPA} domain interacts with components of the cell matrix (Stephens et al., 1992a), and for tPA, its K2 region also interacts with fibrin (Ichinose et al., 1986; van Zonneveld et al., 1986; Wilhelm et al., 1990). Additionally, this module also participates in the binding of tPA to its endothelial cell receptor (Hajjar et al., 1994) and to other cell types (Bizik et al., 1993). In a final example, the Lp(*a*) kringles mediate binding of this protein to low-density lipoprotein (Frank et al., 1996) and to fibrin (Scanu & Edelstein, 1994).

Some of the above interactions are inhibited by ω-amino acids, such as EACA and lysine, thus providing a molecular basis for the hypothesis that proteins containing carboxy-terminal lysine residues are able to interact with proteins containing ω-amino acid binding kringles. The importance of this mode of protein−protein interaction is emphasized by the observation that digestion of virgin fibrin clots by plasmin, thereby exposing carboxyl-terminal lysine residues, enhances binding of Pg and tPA to these clots and, in this manner, stimulates clot lysis (Christensen, 1985; Tran-Thang et al., 1986). Such a mechanism also provides a means for plasma carboxypeptidase B-type enzymes to attenuate clot dissolution potential (Bajzar et al., 1995). Other proteins that contain carboxyl-terminal lysine residues (Hortin et al., 1988; Miles et al., 1991), or even internal lysine ϵ-amino groups with carboxylate groups of D and E side chains, appropriately spatially positioned to mimic free EACA, also interact with plasmin (Sugiyama et al., 1988). The kringle domains that are known to bind these types of ω-amino acid ligands, and thus probably involved in these interactions, are $K1_{Pg}$ (Lerch et al., 1980; Menhart et al., 1991), $K2_{Pg}$ (Marti et al., 1994), $K4_{Pg}$, (Lerch et al., 1980; Rejante et al., 1991; McCance et al., 1994), $K5_{Pg}$ (Novokhatny et al., 1989; McCance et al., 1994), $K2_{tPA}$ (Cleary et al., 1989; De Serrano & Castellino, 1992a,b; De Serrano et al., 1992b), and several of the Lp(*a*) kringles (LoGrasso et al., 1994; Scanu et al., 1994; Klezovitch & Scanu, 1996; Mikol et al., 1996).

These types of observations have stimulated detailed study of the nature of the binding of ω-amino acids to kringle domains. Such work has been facilitated by the successful large-scale expression of these modules in bacterial cells (Menhart et al., 1991; De Serrano & Castellino, 1992b; De Serrano et al., 1992a; Menhart et al., 1993; Marti et al., 1994; McCance et al., 1994; Chenivesse et al., 1996; Klezovitch & Scanu, 1996) and determination of the three-dimensional structures of several of these ligand-bound isolated domain units in the crystal state (de Vos et al., 1992; Mulichak et al., 1991; Arni et al., 1994; Mathews et al., 1996; Mikol et al., 1996) and in solution (Atkinson & Williams, 1990; Byeon et al., 1991; Li et al., 1994). Information forwarded from these structural studies was of predictive value in identification of the amino acid side chains that interacted with the ligand, and site-directed mutagenesis investigations provided a great deal of direct information on this topic (De Serrano & Castellino, 1990, 1992a,b, 1993, 1994a,b; De Serrano et al., 1992a,b; Hoover et al., 1993; McCance et al., 1994).

The amino acid positioned at W^{63} in $K2_{tPA}$ is invariant in all kringles. As revealed through X-ray crystal structures, this amino acid side chain is situated in such a manner as to interact with the hydrophobic regions of ω-amino acids, and NMR analysis shows that this side chain is perturbed by addition of these types of ligands. While these methods suggest that W^{63} functions in ligand binding, its relative importance in this regard has not been assessed by these previous studies. Specifically, only predictions of side-chain involvement in binding can be made from X-ray analysis, and whether perturbations of NMR resonances of the polypeptide by ligand are direct or indirect are not readily ascertained. However, these techniques, in combination with site-directed mutagenesis studies, are invaluable as a cluster of approaches to rigorously resolve these questions. The amino acid residue, W^{63}, is strategically ideal for mutagenesis studies because it exists in a spatial location in the crystal structure that predicts its involvement in ligand binding, its ^{1}H-NMR resonances are perturbed by ligand binding, and NOE connectivities with the ligands are observed. Additionally, the rigid conservation of this amino acid residue in both ligand binding and non ligand binding kringles also suggests that W^{63} might be a determinant of proper kringle folding and/or stability of the native conformation of these modules. In order to evaluate this broad range of possible functions of W^{63} in kringles, we have undertaken a site-directed mutagenesis-based study of the effects of replacements of W^{63} in $K2_{tPA}$ on the properties of this domain. The results of this study are reported herein.

MATERIALS AND METHODS

Materials. Bovine fXa was donated by Enzyme Research Laboratories, Inc. (South Bend, IN). Restriction endonucleases were obtained from Fisher Scientific Co. (Springfield, NJ). Recombinant *Pfu* DNA polymerase was purchased from Invitrogen (San Diego, CA). T4 DNA ligase was a product of New England Biolabs (Beverly, MA).

Hybridoma cell lines expressing MAbs 364, 623, 663, and 700, all directed to wtr-$K2_{tPA}$, were obtained from Genentech, Inc. (South San Francisco, CA).

The *Pichia pastoris* expression kit and the yeast transfer vector, pPIC9K, were purchased from Invitrogen.

Descriptions of the plasmids, pUC119[Pg] and pSVI7/SG-[$K2_{tPA}$P], containing the entire coding sequences of Pg and the $K2_{tPA}$-protease region of tPA, respectively, were provided earlier (Whitefleet-Smith et al., 1989; Nilsen et al., 1997).

Construction of the Fusion Protein Expression Plasmids. The cDNAs of $K2_{tPA}$P and plasminogen were used as templates for PCR-based mutagenesis. The first step consisted of construction of the tandem $K1_{Pg}$-IEGR-$K2_{tPA}$ fusion polypeptide. For this, two pairs of oligonucleotides were synthesized that contained restriction endonuclease sites for *Avr*II site at the 5′-end and *Not*I at the 3′-terminal region of the final cDNA construct.

The first pair of PCR primers was used with pUC118-Pg as the template.

1. 5′ forward primer, 5′-GAT C′CT AGG TCA GAG TGC AAG ACT GGG (*Avr*II site underlined). After cleavage by *Avr*II and insertion of the product into the same restriction site of the *P. pastoris* transfer vector, pPIC9K, this primer encodes the sequence PRSEC[1]KTG. The sequence is preceded by the *Avr*II recognition site. The first two amino acids restore the 3′-end of the polylinker site of the transfer vector, and the next two amino acids flank the first C residue of $K1_{Pg}$. The next three amino acids are residues 2−4 of $K1_{Pg}$.

2. 3′ reverse primer, 5′-TGC ATT GGA tct tcc ttc gat ATC ACA CTC AAG A (the fXa cleavage site, IEGR, is in lower case letters). This primer encodes the sequence LEC^{79}-DIEGRSNA, where LEC^{79} represents the COOH terminus of $K1_{Pg}$, followed by DIEGR′SNA (the fXa-sensitive cleavage site which is excised at R′ by the enzyme).

The second set of PCR primers was employed with plasmid pSVI7/SG[$K2_{tPA}$P].

3. 5′-*GGA AGA TCC AAT GCA* GAC TGC TAC TTT GGG. The first 15 bases (italicized) of this forward PCR primer overlap those of primer 2, above, and encode the sequence GRSNA. The first two amino acids originate from the carboxy terminus of the fXa-sensitive cleavage site (GR). The next three (SNA) begin the construction of $K2_{tPA}$. These are then followed by amino acids at the amino terminus of $K2_{tPA}$, *viz.*, DC[1]YFG.

4. 5′-AT GC′GGCCGC TTA CTA GGA GCA GGA GG. This primer codes for the sequence PSC^{82}S(stop)(stop), where PSC^{82} represents the carboxyl terminus of $K2_{tPA}$, followed by two stop codons and the 3′ *Not*I site (underlined).

The final PCR product was then digested with AvrII/*Not*I and cloned into the polylinker site of pPIC9K through these same restriction sites.

Oligonucleotide-Directed Mutagenesis. The QuickChange protocol (Stratagene) was employed to alter the codon for W^{63} of $K2_{tPA}$ to those that individually code for H, F, S, and Y. The sequences of the pairs of oligonucleotides used in this procedure were as follows (the codons used for the W^{63} mutants are in lower case letters):

W^{63}F: 5'-GAT GCC AAG CCC ttc TGC CAC GTG CTG-3'

3'-CTA CGG TTC GGG aag ACG GTG CAC GAC-5'

W^{63}H: 5'-GAT GCC AAG CCC cat TGC CAC GTG CTG-3'

3'-CTA CGG TTC GGG gta ACG GTG CAC GAC-5'

W^{63}S: 5'-GAT GCC AAG CCC agt TGC CAC GTG CTG-3'

3'-CTA CGG TTC GGG tca ACG GTG CAC GAC-5'

W63Y:5'-GAT GCC AAG CCC tac TGC CAC GTG CTG-3'

3'-CTA CGG TTC GGG atg ACG GTG CAC GAC-5'

The plasmid containing the $K1_{Pg}$-IEGR-$K2_{tPA}$ coding sequence in pPIC9K was amplified from *Escherichia coli* strain DH5α. This plasmid was thermally denatured and reannealed to the above oligonucleotide primer pairs containing the desired mutagenic nucleotides. Then, using the nonstrand-displacing action of *Pfu* DNA polymerase, the primer was extended with incorporation of the mutagenic primers, a process that resulted in nicked circular DNA strands. The nonmutated parental DNA template, which contained methylated G (G^{m6}), was digested with *Dpn*I (recognition sequence 5′-G^{m6}ATC-3′). The circular, nicked double-stranded DNA was then transferred into XL2-Blue competent cells, which repaired the nicks in the mutated plasmid. The final product was amplified in XL2-Blue and subjected to complete nucleotide sequencing to confirm the mutations and the integrity of the entire constructs.

Expression and Purification of the Fusion Polypeptide $K1_{Pg}$-IEGR-$K2_{tPA}$. The plasmid pPIC9K[$K1_{Pg}$-IEGR-$K2_{tPA}$] and the mutant constructions were linearized with the restriction enzymes *Bgl*II, *Sal*I, or *Sac*I and used for transformation of *P. pastoris* strains GS115 or KM71 by means of electroporation. A two-step procedure was employed for selection of transformants that were His^+ and G418 resistant. First, after transformation, the cells were plated on His^- plates and were allowed to grow for 2−5 days. Colonies were selected from these plates and transferred to nutritionally rich plates with variable concentrations (0.25, 2.0, and 4.0 mg/mL) of G418. The colonies that grew on 4 mg/mL G418 were considered as positive transformants with multiple copies of G418-resistant genes and target genes.

The multicopy His^+/GS115 transformants were selected for the Mut^s (methanol utilization slow) and Mut^+ (methanol utilization plus) phenotypes as described in the *P. pastoris* expression kit manual, version C (Invitrogen). The His^+/KM71 transformants are naturally of the Mut^s phenotype (because of interruption of the *AOX1* gene by that of *ARG4*), and further determination of the methanol growth properties was not necessary.

High biomass fermentation of the selected yeast clones (Mut^+ colonies were used for large-scale fermentations whenever possible) was accomplished with a BioFlo 3000 bench-top fermentor (New Brunswick Scientific, Edison, NJ). The inoculum for the fermentation was obtained from shaker flasks containing 200 mL of phosphate-buffered YNB (yeast nitrogen base)/1% (v/v) glycerol and was grown overnight at 30 °C to an OD of 2−6 absorbance units. The details of our fermentation protocols have been published earlier (Nilsen et al., 1997). The only differences in the procedures between wtr-$K2_{tPA}$ and the mutants involved the time of fermentation. While the wild-type polypeptide construct was allowed to ferment for 48−60 h after methanol induction, some of the mutants, *viz.*, $K1_{Pg}$-IEGR-[W^{63}H]$K2_{tPA}$ and $K1_{Pg}$-IEGR-[W^{63}S]$K2_{tPA}$, were found to be degraded in the $K2_{tPA}$ portions under these conditions. When this occurred, shorter fermentation times of 14 h after methanol induction were used. Despite the resulting lower yields of polypeptide, the quality of the material obtained was very high.

Purifications of the fusion polypeptides were achieved by affinity chromatography. Specifically, the supernatant was adjusted to pH 7.4 with NaOH and loaded onto a lysine-Sepharose column, equilibrated and washed with a buffer containing 50 mM Tris-HCl/50 mM NaCl, pH 7.7. The fusion polypeptides were adsorbed to the column by nature of the specific affinity of $K1_{Pg}$ (and in some cases also through the $K2_{tPA}$ portion of the construct) and were then eluted after adjusting the column wash buffer to 0.2 M EACA. The purified polypeptides were then dialyzed against H_2O and lyophilized.

Bovine fXa-Catalyzed Digestion of the Fusion Polypeptides and Isolation of the $K2_{tPA}$ Region. The $K1_{Pg}$-IEGR-$K2_{tPA}$ constructs were mixed with bovine fXa at a peptide:enzyme

ratio of 50:1 (w:w) and allowed to incubate at 37 °C for 3 h. The digest was then applied to a lysine-Sepharose column and washed with a solution containing 50 mM Tris-HCl/50 mM NaCl, pH 7.7. After the baseline absorbance (280 nm) decreased to <0.05, a gradient of EACA (start solution, 50 mM Tris-HCl/50 mM NaCl, pH 7.7; limit solution, 50 mM Tris-HCl/50 mM NaCl/100 mM EACA, pH 7.7) was applied in order to resolve the two kringles by virtue of their differential lysine binding capacities.

Intrinsic Fluorescence Titrations. The binding of ω-amino acid ligands to the kringle domains was measured by titration of the intrinsic fluorescence change in the kringle that accompanied the kringle/ligand interaction. The procedures used have been described in earlier reports from this laboratory (Menhart et al., 1991; De Serrano & Castellino, 1992a,b, 1993). The experiments herein 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 $K2_{tPA}/\omega$-amino acid binding were calculated from the fluorescence titrations by nonlinear least squares iterative curve fitting of the titration data (Menhart et al., 1991).

Differential Scanning Calorimetry. The samples were dissolved in a solution containing 100 mM sodium phosphate, pH 7.4, or 50 mM sodium phosphate/50 mM EACA, pH 7.4. Thermograms were obtained with use of a nano differential scanning calorimeter (Calorimetry Sciences Corp., Salt Lake City, UT). Thermal denaturation scans were conducted between the temperature range of 25 and 100 °C at a scan rate of 1 °C/min. The baseline for each run was obtained in an identical experiment with the buffer in each cell. The temperature of maximum heat capacity (T_m) was obtained from computer software that accompanied the equipment.

^{1}H-NMR. The samples were prepared for NMR analysis by first dissolving the polypeptides in a solution composed of 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.

Homonuclear ^{1}H-NMR spectra were obtained at 37 °C on a Varian (Palo Alto, CA) Unity*Plus* 600 MHz spectrometer. Two-dimensional TOCSY (mixing times, 30 and 85 ms) and NOESY (mixing time, 200 ms) data sets were collected with the usual pulse sequences (Bodenhausen et al., 1988; Griesinger et al., 1988) at a spectral window of 6755.6 Hz using the hypercomplex phase-sensitive method (States et al., 1982). Typically, 484 t_1 increments of 96 scans each were sampled in 2048 complex data points. Spectra were obtained and processed as 4K × 2K complex data sets with baseline corrections and Gaussian weighting functions applied using Varian software that accompanied the spectrometer. Suppression of the residual ^1H_2O signal was achieved by presaturation.

The proton resonances were assigned by combining the procedures of spin-system identification using TOCSY, followed by sequential assignments through NOE connectivities (Wuthrich, 1986). Chemical shift resonances are reported in δ (ppm) with respect to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), which was set to 0 ppm.

ELISA Assays. A solution (0.15 mL of 3 μg/mL) of the $K2_{tPA}$ variant dissolved in 0.15 M sodium borate, pH 8.3, was placed in individual wells of a polyvinyl 96-well assay plate (Costar, Cambridge, MA) and allowed to remain on the plate overnight at 4 °C. The wells were then blocked with 200 μL of 9% milk for 1 h at room temperature and then washed three times with a solution of 0.05 M Tris-HCl/0.1 M NaCl, pH 7.4 (TBS), containing 0.05% Tween 20. Various concentrations of anti-$K2_{tPA}$ MAbs, 364, 623, 663, or 700, dissolved in TBS/1% BSA, pH 7.4, were added (150 μL/well) at varying concentrations and incubated at room temperature for 2 h. The wells were washed three times with TBS/0.05% Tween 20. Alkaline phosphatase, conjugated to goat anti-mouse IgG, diluted 1:1000 with TBS/1% BSA, was added to each well (150 μL/well) and incubated at room temperature for 1 h. The plates were washed with TBS/0.05% Tween 20 three times. Sigma substrate 104 (Sigma Chemical Co.), at 0.4 mg/mL in 10% diethanolamine/$MgCl_2$ (pH 9.8), was added (100 μL/well), and the absorbance at 405 nm was measured in a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA) at different times (absorbancies increased linearly with time up to a least 1 h, and the data collected at the 30 min time point were used herein). Data were fitted by nonlinear least squares analysis to simple saturation binding behavior, and C_{50} values (total concentration of MAb required for 50% saturation) for the $K2_{tPA}$-MAb interaction were obtained from the plots.

Mass Spectra of Polypeptides. Molecular weight analysis of the $K2_{tPA}$ samples was measured by MALDI-TOF-DE-MS on a Voyager-DE spectrometer (PerSeptive Biosystems, Framingham, MA). Samples were prepared by mixing 0.5 μL of a $10^{-4}-10^{-5}$ M aqueous solution of polypeptide and 0.5 μL of 50 mM α-cyano-4-hydroxycinnamic acid in 50% CH_3CN/0.1% TFA on a 100-well sample plate. The 1 μL drops were air-dried and analyzed in the instrument after irradiation with a nitrogen laser (337 nm, 4 ns pulse time). The instrument was operated in the delayed extraction mode (Vestal et al., 1995). The accelerating voltage was +20 or −20 kV. Signal transients were recorded with a digitizer at a time resolution of 5 ns. Spectra were generated from the sum of 50−100 laser pulses using external calibration. Estimated mass accuracy over the observed mass range was ±0.05%. Linear mode positive and negative ionizations were used.

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

RESULTS

Fusion polypeptides containing the $K1_{Pg}$ module linked through a fXa-cleavable peptide (IEGR) to the $K2_{tPA}$ domain were prepared by recombinant DNA technology. This construct allowed separate mutagenic manipulations of the cDNA of $K2_{tPA}$ while still retaining the ability for a one-step purification of the resulting translated product through the specific interaction of $K1_{Pg}$ with a column containing an immobilized amino acid ligand, specifically lysine. After purification of the fusion polypeptide, fXa-catalyzed cleavage resulted in a symmetrical excision between the two kringle domains. Their separation was then accomplished by repassage over the affinity column. With wtr-$K2_{tPA}$, which also adsorbs to this column, gradient elution with EACA resulted in simple purification of the products with baseline separation of $K1_{Pg}$, which elutes first, from $K2_{tPA}$. However, in cases, *viz.*, with r-[$W^{63}H$]$K2_{tPA}$ and r-[$W^{63}S$]$K2_{tPA}$, where

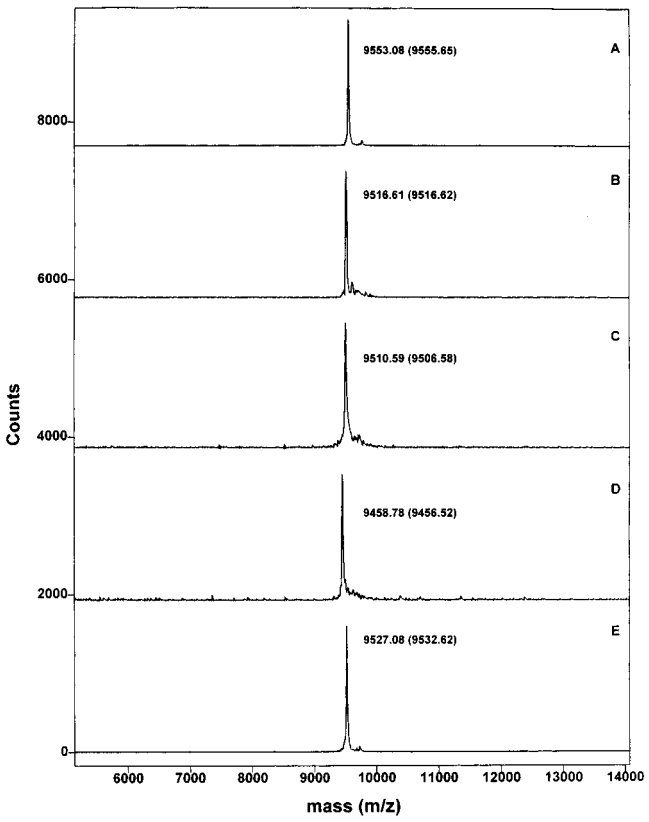

FIGURE 1: Positive ion linear mode TOF-MALDI-DE-MS analysis of the W^{63}-based mutants of $K2_{tPA}$. The samples were irradiated with a nitrogen laser (337 nm, 4 ns pulse time). The instrument was operated in the delayed extraction mode, with a delay time of 100 μs. The accelerating voltage was 20 kV. Signal transients were recorded with a digitizer at a time resolution of 5 ns. Spectra were generated from the sum of 94 laser pulses using external molecular weight calibrations with hen egg white lysozyme. The panels refer to wtr-$K2_{tPA}$ (A), r-[W^{63}F]$K2_{tPA}$ (B), r-[W^{63}H]$K2_{tPA}$ (C), r-[W^{63}S]-$K2_{tPA}$ (D), and r-[W^{63}Y]$K2_{tPA}$ (E).

nearly complete downregulation of ligand binding is a consequence of the mutations, the liberated $K2_{tPA}$ mutants eluted early in the column wash. With the other mutants, *viz.*, r-[W^{63}F]$K2_{tPA}$ and r-[W^{63}Y]$K2_{tPA}$, where partial loss of the binding resulted from the mutations, their elutions occurred late in the column wash step.

Highly purified samples of the $K2_{tPA}$ mutants were obtained by these procedures. Amino-terminal amino acid sequences of each of the mutants, as well as that of wtr-$K2_{tPA}$ that was produced by these same procedures, demonstrated single amino acids at each cycle with the sequences through 10 amino acids of SNADC[1]YFGNG. These results are exactly as expected from the nature of the constructions. Additionally, the mutations were verified by TOF-MALDI-DE-MS. In this case, as shown in Figure 1, single mass peaks were obtained for each of the samples. Their molecular weights were 9553.1 (calculated, 9555.7) for wtr-$K2_{tPA}$ (A), 9516.6 (calculated, 9516.6) for r-[W^{63}F]$K2_{tPA}$ (B), 9510.6 (calculated, 9506.6) for [W^{63}H]r-$K2_{tPA}$ (C), 9458.8 (calculated, 9456.5) for r-[W^{63}Y]$K2_{tPA}$ (D), and 9527.1 (calculated, 9532.6) for r-[W^{63}S]$K2_{tPA}$ (E).

The binding of three ω-amino acid ligands, EACA, 7-AHpA, and t-AMCHA, to the $K2_{tPA}$ variants was examined by titration of the fluorescence alterations that accompany this binding. No fluorescence change was observed for mutants r-[W^{63}H]$K2_{tPA}$ and r-[W^{63}S]$K2_{tPA}$ with any of these

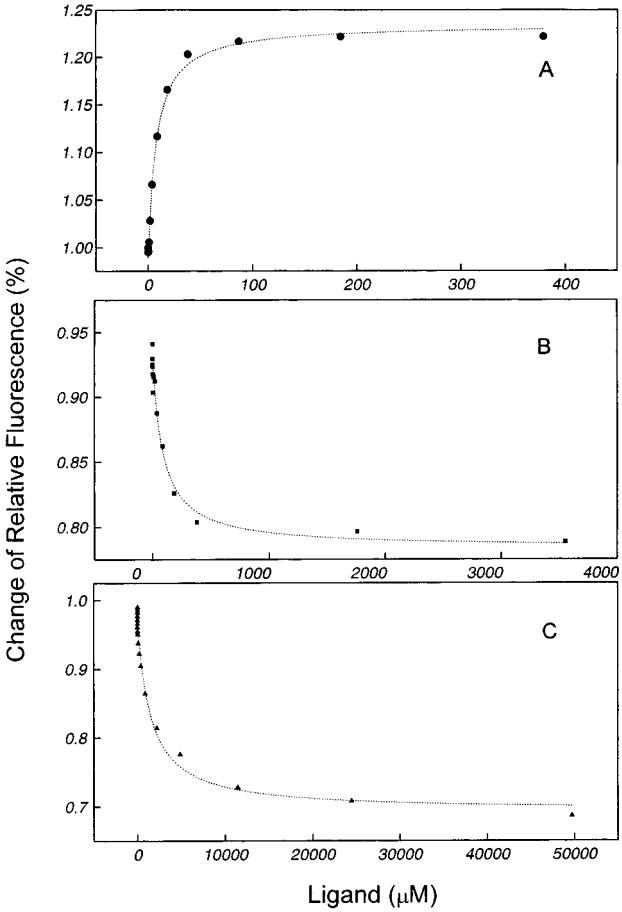

FIGURE 2: Titration of the change in relative intrinsic fluorescence of r-$K2_{tPA}$ (5 μM) mutants against the free concentration of 7-AHpA: (A) wtr-$K2_{tPA}$, (B) r-[W^{63}F]$K2_{tPA}$, and (C) r-[W^{63}Y]-$K2_{tPA}$. The experimental points are best fit to lines generated employing values of K_d = 7.9 μM (A), 92.3 μM (B), and 1500 μM (C). The stoichiometry was set at 1.0 for these iterations, and the calculations provided values of the K_d, saturating concentration of ligand, and free ligand concentrations.

Table 1: Dissociation Constants (K_d) for ω-Amino Acids to W^{63}-Based Variants of $K2_{tPA}$ at 25 °C

	K_d (μM) for		
peptide	EACA	7-AHpA	t-AMCHA
wtr-$K2_{tPA}$	84	8	24
r-[W^{63}H]$K2_{tPA}$			
r-[W^{63}F]$K2_{tPA}$	134	92	307
r-[W^{63}S]$K2_{tPA}$			
r-[W^{63}Y]$K2_{tPA}$	2700	1500	520

ligands, suggesting that binding did not occur. This conclusion is fortified by the observation that these mutants were not significantly retained by the lysine−Sepharose column. However, in the cases of r-[W^{63}F]$K2_{tPA}$ and r-[W^{63}Y]$K2_{tPA}$, titratable decreases in fluorescence took place with all three ligands (Figure 2), and a higher degree of retardation occurred on the lysine−Sepharose column. The K_d values calculated from these titrations are listed in Table 1. In general, large decreases in binding affinity of the ligands to $K2_{tPA}$ accompany the conservative alterations at W^{63}, with the W^{63}Y variant showing considerably higher downregulation of ligand binding than the W^{63}F mutant.

The effects of these mutations on the stability of the native conformation of $K2_{tPA}$ was examined by DSC analysis. The results obtained for all of the mutants are illustrated in Figure

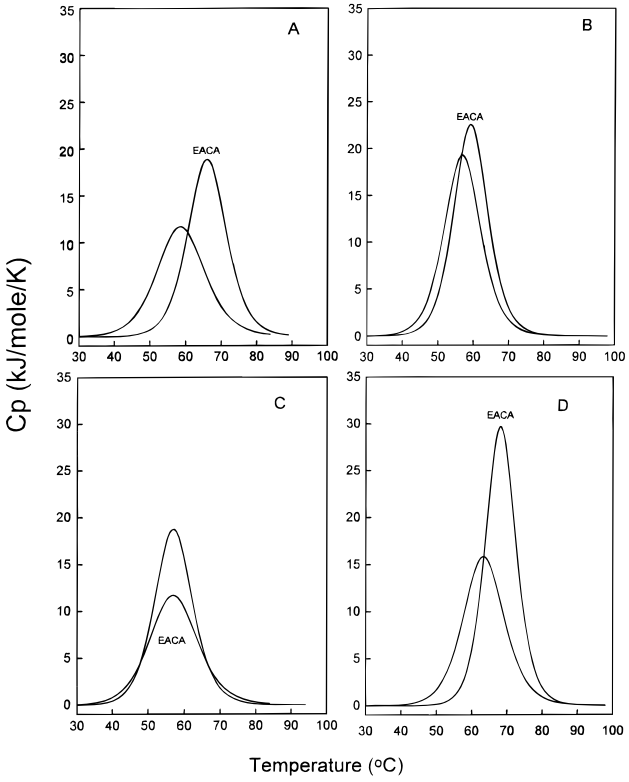

FIGURE 3: DSC thermograms of r-$K2_{tPA}$ mutants and their changes as a result of addition of EACA. The change in heat capacity at constant pressure (ΔC_p) is plotted against the temperature. The buffers employed were 100 mM sodium phosphate, pH 7.4, or 50 mM sodium phosphate/50 mM EACA, pH 7.4. The temperature of maximum heat capacity (T_m) for each sample was determined as the temperature of maximal ΔC_p. The concentrations of the peptides were approximately 0.5 mg/mL. Panels: (A) r-[W^{63}F]-$K2_{tPA}$, (B) r-[W^{63}H]$K2_{tPA}$, (C) r-[W^{63}S]$K2_{tPA}$, and (D) r-[W^{63}Y]-$K2_{tPA}$.

3. The T_m (and calorimetric ΔH) of wtr-$K2_{tPA}$, of 75.6 °C (68 kcal/mol) (De Serrano & Castellino, 1994b), was dramatically lowered to 58.5 °C (40 kcal/mol), 56.6 °C (56 kcal/mol), 56.6 °C (57 kcal/mol), and 63.3 °C (45 kcal/mol) for the mutants r-[W^{63}F]$K2_{tPA}$, r-[W^{63}H]$K2_{tPA}$, r-[W^{63}S]$K2_{tPA}$, and r-[W^{63}Y]$K2_{tPA}$, respectively. This value of the T_m for r-[W^{63}H]$K2_{tPA}$ was only minimally altered (60 °C) by a high concentration of EACA and that for r-[W^{63}S]$K2_{tPA}$ was unchanged (56.4 °C) in the presence of EACA. On the other hand, this same T_m for the W^{63}F and W^{63}Y variants was increased to 66.0 and 68.1 °C as a result of addition of EACA. The T_m value for wtr-$K2_{tPA}$ was altered to 86.1 °C in the presence of saturating levels of EACA (De Serrano & Castellino, 1994b). In all cases, the T_m values were independent of the thermal scan rates of the samples. Thus, stabilization of the native conformation occurs with all mutants that interact with the ligand. The lack of any significant change in the T_m values by EACA for r-[W^{63}H]-$K2_{tPA}$ and r-[W^{63}S]$K2_{tPA}$, at least up to 50 mM EACA, adds further proof that this ligand binding site is nearly completely downregulated by these particular mutagenic changes. In the cases of the ΔH values, all were increased by approximately 25%−50% in the presence of EACA. The lone exception was for [W^{63}S]$K2_{tPA}$, wherein the ΔH was apparently decreased in the presence of EACA by approximately 25%. It is difficult to account for this aberrant behavior of the S^{63} mutant without knowledge of the initial and final conformations of the polypeptide. Also, at this particular pH, these scans are not reversible and the ΔH values are very low, with uncertainties of at least 15%. Further, since EACA does not interact with the S^{63} mutant, it is not easy to rationalize the manner in which it can alter in a specific fashion the thermal denaturation profile of the mutant kringle. For these reasons, we are not normally concerned with calorimetric ΔH values in these cases, and our purpose in these experiments is to obtain the T_m values, which are highly reproducible and informative.

A series of MAbs generated against tPA, which were found to interact with the $K2_{tPA}$ domain,[2] were employed to assess possible changes in the isolated kringle region as a result of the mutations. Binding isotherms of these MAbs to wtr-$K2_{tPA}$ are illustrated in Figure 4, and the C_{50} values characteristic of this interaction are provided in Table 2. Similar experiments performed with the mutants lead to C_{50} values also summarized in Table 2. The data show that three of these MAbs, *viz.*, 623, 663, and 700, react nearly identically to that of wtr-$K2_{tPA}$, whereas another, 364, displays greatly diminished reactivities to three (F, S, and Y) of the mutants.

Lastly, a combination of standard TOCSY and NOESY methods was employed to assign the ^{1}H-NMR resonances of key residues of the two aromatic mutants of $K2_{tPA}$ to determine whether more subtle conformational changes occurred as a result of these amino acid substitutions. Examination of the chemical shift data in Table 3 demonstrates that major changes do not occur in the environments of these protons. Of the alterations that do exist, those involving W^{25} and L^{47} are of interest since the close spatial proximity of the side chains of these two residues is a crucial monitor of proper folding of kringle domains. Regarding these residues, the data indicate that the upfield protons of the $CH_3^{\delta'}$ group of L^{47} are not as influenced as is wtr-$K2_{tPA}$ by the ring current of W^{25} in the mutants. This indicates that small changes do occur in this region of $K2_{tPA}$ as a result of these conservative mutations at W^{63}. When the resonance positions of these L^{47} methyl protons are examined in the W^{63}H an W^{63}S variants, the chemical shifts (−0.84 and −0.83 ppm, respectively) observed for the $CH_3^{\delta'}$ protons are similar as those for the W^{63}F and W^{63}Y mutants.

An example of some of the strategies used for spin assignments is detailed here for wtr-$K2_{tPA}$ and its Y^{63} mutant. Both of these polypeptides contain only one F residue at sequence position 3. The resonances of the aromatic protons of this residue were easily identified in the TOCSY spectrum (mixing time = 85 ms) as shown in Figure 5 for r-[W^{63}/Y]$K2_{tPA}$. Both the H_3 and H_5 protons resonated at the same chemical shift, of 7.38 ppm, and exhibited cross-peaks to the H_4 proton (7.31 ppm) and the H_2 and H_6 protons. These two later protons also resonated at the same chemical shift, of 7.25 ppm. In the NOESY spectrum (mixing time = 200 ms) illustrated in Figure 6, the resonance signal of the H_2 and H_6 protons showed cross-peaks to the CH_2^{β} protons at 2.94 and 3.06 ppm. Finally, using the TOCSY spectrum, the resonance signal of the H^{α} proton (4.90 ppm) was assigned, as both methylene proton signals exhibited cross-peaks with the H^{α} proton signal. The proton resonances of the F^{63} residue in r-[W^{63}F]$K2_{tPA}$ were assigned in the same way.

The resonances corresponding to the aromatic protons H_4, H_5, H_6, and H_7 of the W residues showed mutual cross-peaks

[2] B. Keyt, Genentech, Inc., unpublished data.

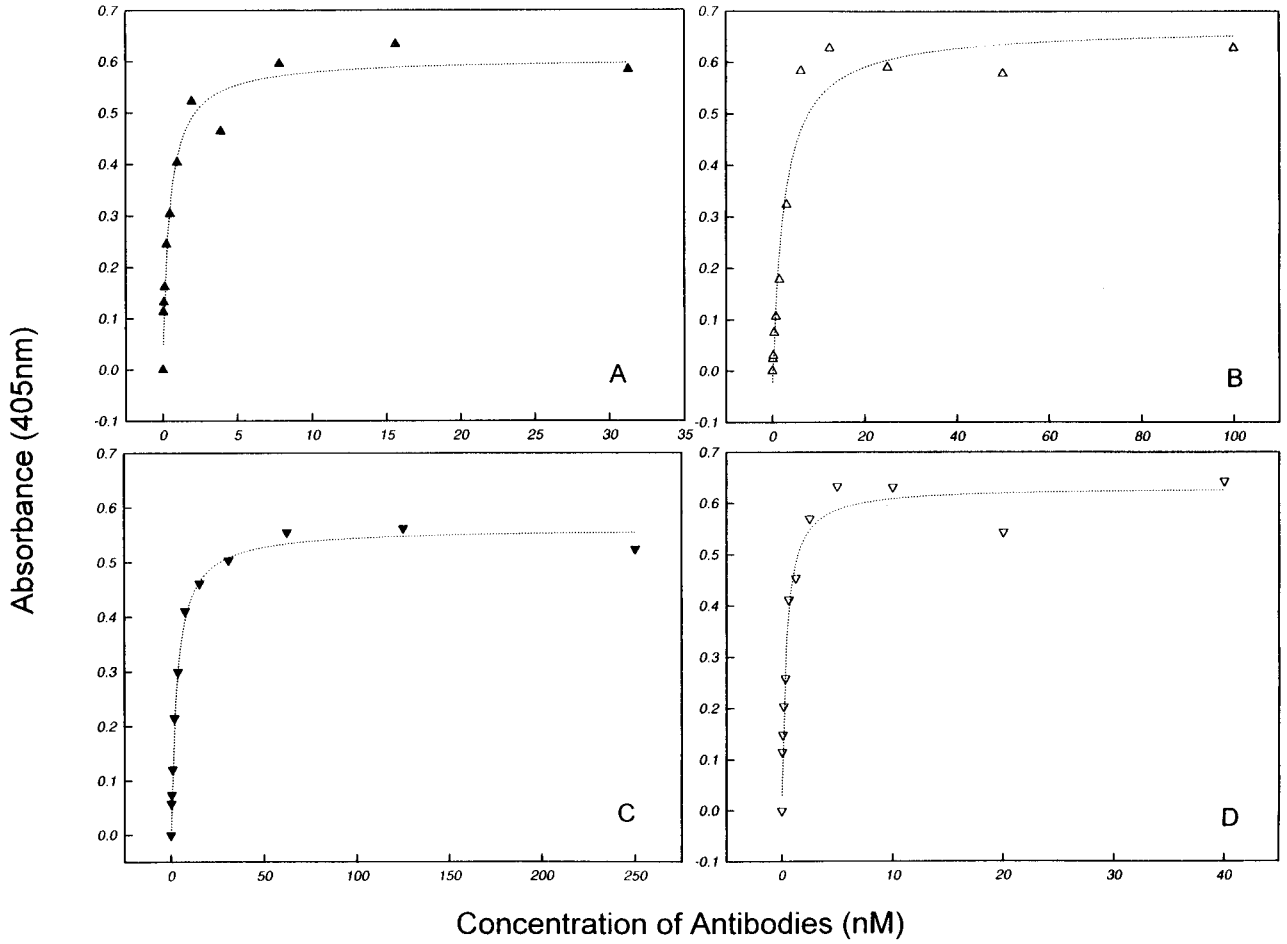

FIGURE 4: The reactivity of wtr-$K2_{tPA}$ with MAbs at 25 °C. ELISA assays were performed at different levels of the various MAbs with a constant amount of the kringle polypeptide adsorbed to the plate. Panels: (A) (▲) MAb-364, (B) (△) MAb-623, (C) (▼) MAb-663, and (D) (▽) MAb-700.

Table 2: Binding of MAbs to r-$K2_{tPA}$ Variants at 25 °C

peptide	C_{50}-MAb[a] (nM) for MAb			
	364	623	663	700
wtr-$K2_{tPA}$	0.7	4.1	3.2	0.3
r-[$W^{63}F$]$K2_{tPA}$	8.7	1.5	3.9	0.5
r-[$W^{63}H$]$K2_{tPA}$	0.6	1.0	10.2	0.4
r-[$W^{63}S$]$K2_{tPA}$	167	0.6	16.9	0.6
r-[$W^{63}Y$]$K2_{tPA}$	5.6	0.7	12.6	0.3

[a] Concentration (total) of MAb required for 50% binding to the kringle domain.

in the TOCSY spectrum, as evident in Figure 5 for the Y^{63} mutant. The H_2 signals of W residues exhibited cross peaks with the CH_2^{β} protons in the NOESY spectrum (Figure 6). Identification of the CH_2^{β} proton signals enabled assignment of the CH^{α} resonances from the TOCSY spectrum in the usual manner.

The H_2 and H_6 protons and the H_3 and H_5 protons of a given Y residue resonated at the same chemical shift, respectively. Therefore, the TOCSY spectrum exhibited one cross-peak between aromatic proton signals for each Y residue. The cross-peaks between the same pairs of aromatic protons also appeared in the NOESY spectrum. The signals of the CH_2^{β} and CH^{α} protons were then assigned in a similar manner as in the case of the F residues.

We were not able to identify all H side-chain proton resonances, as only spectra of the lyophilized samples in ^2H_2O-exchanged buffer were measured. The assignments of proton resonances given in Table 1 are based on the observation of NOEs between side-chain protons as follows: L^{31} and H^{65}; H^{50} and L^{47}, Y^{52}.

The proton signals of L^{47} were unambiguously assigned using the TOCSY spectra. Several NOEs were observed between the CH_3^{δ} protons of L^{47}, W^{25}, and Y^{35} aromatic protons for the wild-type peptide and both mutants. In the NOESY spectrum of the wild-type protein, the protons of both CH_3^{δ} methyl groups exhibited cross-peaks to the H_4 (weaker) and H_7 (stronger) protons of W^{25}. Meanwhile the Y^{63} mutant showed only a NOE connection between the $CH_3^{\delta'}$ protons at −0.83 ppm and H_4 of W^{25}. In this case, the CH_3^{δ} at 0.53 ppm displayed a NOE connectivity with H_7 of W^{25}. For r-[$W^{63}F$]$K2_{tPA}$, no NOE was observed between the $CH_3^{\delta'}$ protons at −0.82 ppm and the W^{25} aromatic protons. Further, the CH_3^{δ} at 0.52 ppm exhibited a NOE only to the H_7 of W^{25}. The NOE between the CH_3^{δ} at 0.52 ppm (0.53 ppm for r-[$W^{63}Y$]$K2_{tPA}$) of the L^{47} and the $H_{2,6}$ protons of Y^{35} was observed for all three polypeptides.

DISCUSSION

We have recently shown that the *P. pastoris* expression system is very suitable for production of large amounts of recombinant kringles that are correctly folded (Nilsen et al., 1997). We have further explored in this paper whether this same expression system would be suitable for production of tandem kringles that are linked *via* a readily exscisible

Table 3: Proton Chemical Shifts of Aromatic Residues of r-[$K2_{tPA}$] Mutants at pH* 7.4, 37 °C

		chemical shifts (ppm)		
residue	proton	wtr-$K2_{tPA}$	r-[$W^{63}F$]$K2_{tPA}$	r-[$W^{63}Y$]$K2_{tPA}$
Tyr^{2}	H^{α}	5.32	5.30	5.33
	H^{β}	3.13, 2.34	3.06, 2.42	3.12, 2.34
	$H_{2,6}$	6.47	6.53	6.48
	$H_{3,5}$	6.96	6.94	6.96
Phe^{3}	H^{α}	4.98	4.90	4.97
	H^{β}	3.07, 2.94	3.06, 2.94	3.06, 2.91
	$H_{2,6}$	7.26	7.25	7.24
	$H_{3,5}$	7.39	7.38	7.38
	H_4	7.41	7.31	7.31
Tyr^{9}	H^{α}	4.42	4.46	4.43
	H^{β}	3.18, 3.08	3.18, 3.08	3.18, 3.11
	$H_{2,6}$	6.78	6.70	6.77
	$H_{3,5}$	7.18	7.19	7.20
Trp^{25}	H^{α}	4.07	4.11	4.09
	H^{β}	3.79, 3.15	3.74, 3.22	3.78, 3.22
	H_2	7.43	7.46	7.53
	H_4	7.83	7.86	7.95
	H_5	7.04	6.51	6.80
	H_6	5.58	7.22	7.30
	H_7	7.65	7.82	7.88
Tyr^{35}	H^{α}	4.48	4.62	4.48
	H^{β}	3.76, 2.95	3.67, 2.92	3.37, 2.82
	$H_{2,6}$	7.12	7.04	7.02
	$H_{3,5}$	6.80	6.80	6.71
Leu^{47}	H^{α}	3.54	3.66	3.63
	H^{β}	1.60, 0.44	1.60, 0.50	1.59, 0.48
	H^{γ}	1.12	1.12	1.13
	H^{δ}	0.52, −0.98	0.52, −0.82	0.53, −0.83
His^{50}	H^{α}	4.50	4.52	4.51
	H^{β}	2.82, 1.63	2.94, 1.66	2.79, 1.65
	H_2	*a*	7.64	7.64
	H_4	*a*	*a*	6.84
Tyr^{52}	H^{α}	5.15	5.16	5.21
	H^{β}	3.30, 2.64	3.30, 2.63	3.32, 2.64
	$H_{2,6}$	6.30	6.30	6.31
	$H_{3,5}$	6.72	6.74	6.73
Trp^{63}	H^{α}	5.60	4.71 (Phe^{63})	4.29 (Tyr^{63})
	H^{β}	3.70, 3.39	3.15, 3.04	3.01, 2.72
	H_2	7.72		
	H_4	7.29		
	H_5	5.66		
	H_6	7.23		
	H_7	6.66		
	$H_{2,6}$		7.24	6.70
	$H_{3,5}$		6.86	7.10
	H_4		6.82	
His^{65}	H^{α}	5.16	5.14	5.02
	H^{β}	2.86, 2.76	2.86	2.80, 2.68
	H_2	7.64	a	7.78
	H_4	6.78	a	6.64
Trp^{74}	H^{α}	5.57	5.70	5.62
	H^{β}	3.27, 3.14	3.40, 3.10	3.34, 3.08
	H_2	7.06	7.09	7.10
	H_4	6.78	6.74	6.66
	H_5	5.44	5.46	5.50
	H_6	6.83	6.86	6.86
	H_7	7.18	7.26	7.22
Tyr^{76}	H^{α}	5.18	4.96	4.86
	H^{β}	3.11, 2.82	3.16, 2.84	3.10, 2.81
	$H_{2,6}$	7.36	7.38	7.36
	$H_{3,5}$	6.89	6.96	6.93

[a] We were not able to assign these residues.

protease-sensitive site and have selected the fXa recognition site (IEGR) for this purpose. Our intention was to link a lysine binding kringle, in this case, $K1_{Pg}$, through this fXa-sensitive site to another kringle which was the subject of the current mutagenesis investigations, *viz.*, $K2_{tPA}$. The construction strategy was based on the fact that the tandem kringle would be readily purified by affinity chromatography by nature of the strong lysine binding affinity of the $K1_{Pg}$ domain, especially in cases where the lysine binding affinity of the target kringle was downregulated by the mutations. These predictions were confirmed in this report. Subsequent cleavage of the tandem kringle construct by fXa, followed by repassage over the affinity chromatography resin (lysine−Sepharose), then provided the target kringle, as was also found in previous studies (De Serrano & Castellino, 1992b; De Serrano et al., 1992a; McCance et al., 1994). In cases where the lysine binding capacity of $K2_{tPA}$ was not compromised by the chosen mutations, or when wtr-$K2_{tPA}$ was desired, gradient elution from the affinity chromatography column, as described herein, was successful.

Four mutations at W^{63} of $K2_{tPA}$ were selected for this study. Two of these, F and Y, were aromatic and conserva-

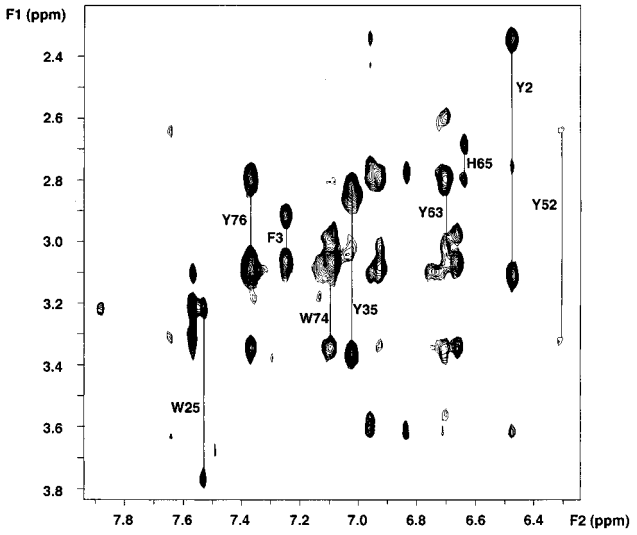

FIGURE 5: Region of the TOCSY spectrum (mix = 85 ms) of r-[$W^{63}Y$]$K2_{tPA}$ showing connectivities between the aromatic protons for selected aromatic residues.

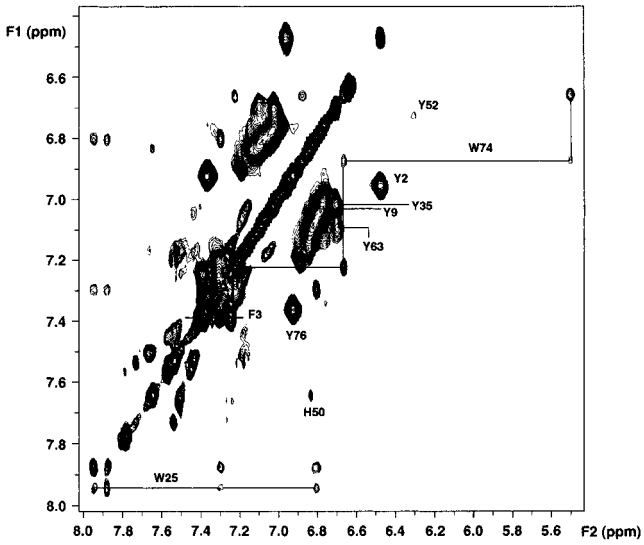

FIGURE 6: Region of the NOESY spectrum (mix = 200 ms) of r-[$W^{63}Y$]$K2_{tPA}$ displaying NOE correlations between the aromatic and H^{β} protons for selected aromatic residues. The intraresidue NOE correlations are labeled.

Four mutations at W^{63} of $K2_{tPA}$ were selected for this study. Two of these, F and Y, were aromatic and conserva-

tive, one, H, was nonaromatic and more radical, and the final, S, was clearly a radical mutation. All tandem kringles were expressed in yeast cells, and a very small amount (<10%) of glycosylated material was present at N^5 of $K2_{tPA}$ (this latter subpopulation could be removed by affinity chromatography on concanavalin A, if desired). Fermentation conditions, initially chosen on the basis of our prior work with isolated $K2_{tPA}$ (Miele et al., 1997; Nilsen et al., 1997), were found to be generally suitable for the expressions conducted in the present study, with some modifications. Specifically, the pH was lowered to 5.0, and the fermentation times decreased to 14 h. These changes were made because we observed that the IEGR bond in the tandem kringle could be cleaved by yeast-derived proteases, and this was minimized by the lowered pH and fermentation times. The yields of material were considerably decreased by these changes but were nonetheless very suitable for the studies conducted herein (*ca.* 100 mg/L for the construct containing wtr-$K2_{tPA}$, *viz.*, $K1_{Pg}$-IEGR-$K2_{tPA}$, and 20−50 mg/L for those containing the mutants). After cleavage and reisolation of the target kringle, quantities of 30−60 mg of $K2_{tPA}$ mutants were readily obtained from a 4 L fermentation.

The final isolated $K2_{tPA}$ mutants were subjected to amino-terminal sequence analysis and molecular weight determination. In all cases, amino-terminal sequences were completely consistent with the expected cleavage of the tandem kringle, *viz.*, between the R of the fXa-sensitive site at the COOH terminus of $K1_{Pg}$ and the S that initiated the stretch of four amino acids that were placed upstream of C^1 of $K2_{tPA}$ in the particular construction employed. In addition, molecular weight analysis by mass spectrometry in each case provided molecular weight values that were within 5 (*ca.* 0.05%) of the calculated molecular masses of the mutants. This also attests to the integrity of the samples.

It has been previously postulated through NMR investigations of ligand/$K2_{tPA}$ complexes that W^{63} is one of the aromatic ligands that should participate in ligand binding in $K2_{tPA}$, along with the Y^{35}, H^{65}, W^{74}, and Y^{76} aromatic rings and the aliphatic side chain of V^{34} (Byeon et al., 1995). The positioning of these amino acid side chains in the X-ray crystal structure of the lysine/$K2_{tPA}$ complex is presented in Figure 7 (de Vos et al., 1992). Site-directed mutagenesis investigations confirm the direct involvement of D^{57}, D^{59} (Weening-Verhoeff et al., 1990; De Serrano & Castellino, 1993), and K^{33} (De Serrano & Castellino, 1992a; De Serrano et al., 1992b) as coordination partners for the substrate amino and carboxylate groups of the ligand and W^{74} as providing a hydrophobic environment energetically favorable for the positioning of the ligand methylene groups (De Serrano & Castellino, 1992b; Menhart & Castellino, 1995). These findings are in agreement with their distance relationships with the ligand as observed in the crystal structure and with information provided by NMR studies. Similar investigations from this laboratory have demonstrated that Y^{76} has less direct importance in ligand binding but appears to influence ligand binding through long-range effects on residues that are directly involved, in particular through an influence on the orientation of W^{63} and/or D^{59}, as argued in an earlier work (De Serrano & Castellino, 1994a). Other site-directed mutagenesis studies demonstrate that H^{65} influences the specificity of ligand binding *via* effects on the shape of the binding pocket but does not likely directly interact with the ligand (Kelley & Cleary, 1989; De Serrano & Castellino, 1992a). Regarding W^{25}, mutagenesis studies show that conservative alterations in this residue did not affect the ligand binding properties of $K2_{tPA}$ (De Serrano & Castellino, 1994b). Thus, of the amino acid residues implicated in ligand binding by NMR studies, only K^{33}, D^{57}, D^{59}, and W^{74} were previously shown to participate directly in interactions with the ligand. These findings are in excellent agreement with predictions that can be made from the crystal structure of the ligand/$K2_{tPA}$ complex and are generally applicable to results obtained from mutagenesis of other kringles (Hoover et al., 1993; McCance et al., 1994; Scanu & Edelstein, 1994).

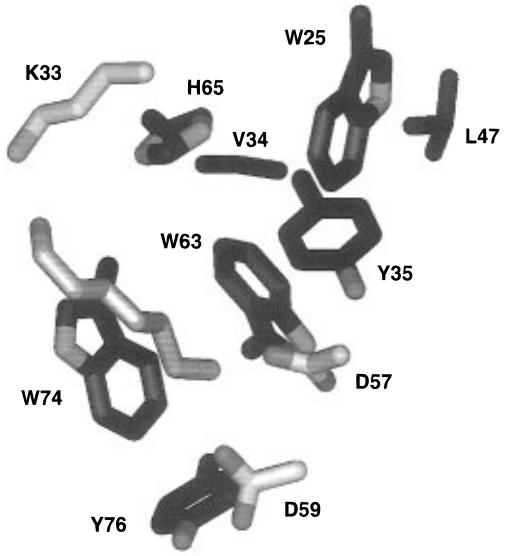

FIGURE 7: Representation of the X-ray structure of wtr-$K2_{tPA}$ emphasizing the steric relationships between important residues that are believed to directly or indirectly influence ligand binding (K^{33}, V^{34}, Y^{35}, D^{57}, D^{59}, W^{63}, H^{65}, W^{74}, and Y^{76}) and residues that are known conformational determinants (W^{25} and L^{47}). Residues are shown from their β-carbons, outward. The sequence position of the amino acids begins at C^1 of the $K2_{tPA}$ sequence and continues consecutively to the last C (C^{82}) of this domain. Hydrogen atoms are excluded to minimize overcrowding. Aromatic and aliphatic hydrophobic amino acid side-chain carbon atoms (plus H^{65}) are in black, nitrogen atoms are in blue, and oxygen atoms are in red. Amino acid side-chain carbon atoms from charged residues (K^{33}, D^{57}, and D^{59}) are in green. The side-chain carbon atoms for the pseudoligand, lysine residue (this is K^{49} from another $K2_{tPA}$ molecule in the unit cell that is inserted into the ω-amino acid binding pocket) are in orange.

A further extension of these conclusions can be made from the current work regarding the participation of W^{63} of $K2_{tPA}$ in ligand binding. In this case, radical mutations (to H and S) at this residue appear to nearly eliminate binding of three different ω-amino acid-derived ligands, and conservative mutations (to F and Y) greatly diminish these ligand binding energies (Table 1). In these latter instances, the Y^{63} mutation resulted in a much greater deleterious effect on the binding process than did the F^{63} substitution. The most reasonable interpretation of this observation is that an alteration to Y might establish new subtle active center conformational relationships with the substrate due to the potential for hydrogen bonding through the phenolate hydroxyl group of Y^{63} with other side-chain residues of the kringle. Consequently this residue can play an active role in affecting ligand binding. In this case then, a larger problem may occur by virtue of the residue that is inserted, rather than by the residue that is eliminated by the mutagenic change. An additional

consideration that requires emphasis is the fact that not all ligands are affected equally by the mutations at W^{63} (Table 1). Detailed analysis of these findings would only be possible with the knowledge of exact structures of the mutant kringles, but it does appear from the binding data that the aromatic group of F can partially compensate for the loss of W^{63} in providing a favorable environment for the hydrophobic region of some ligands. Thus, the integrity of W^{63} is important, but not essential, for ligand binding, and the F^{63} variant to a larger degree than the Y^{63} mutant can support these interactions. Consequently, it appears that some subtle redesign of the binding pocket may accompany the mutation of W^{63} to F, in that there is little difference in the K_d for EACA and 7-AHpA in this mutant, as compared to the case of wtr-$K2_{tPA}$ which shows a strong preference for binding of 7-AHpA over EACA. This situation is similar to that found when H^{65} was mutated (Kelley & Cleary, 1989; De Serrano & Castellino, 1992a).

To assess possible conformational changes in $K2_{tPA}$ that may have been brought about by the mutations at W^{63}, one- and two-dimensional ^{1}H-NMR experiments were conducted on the variants. One strong determinant of proper kringle folding is the large upfield chemical shift of the $CH_3^{\delta'}$ protons of L^{47} due to the close proximity of one of its methyl groups to the aromatic ring currents of W^{25} and Y^{35}. The steric relationships between these residues can be visualized in Figure 7. The side chain of L^{47} is so conformationally restricted that chemical shifts of its two equivalent CH_3^{δ} and $CH_3^{\delta'}$ proton resonances exist at the widely different values, of 0.52 and -0.98 ppm, respectively. In all four of the mutants, the chemical sift of the L^{47} resonance of the CH_3^{δ} protons is maintained at 0.50$-$0.52 ppm, but that from the $CH_3^{\delta'}$ protons exists at -0.82 to -0.84 ppm, a small ($+0.15$ ppm), but distinct, change from their chemical shifts in wtr-$K2_{tPA}$. From the cross-peaks observed in NOESY experiments (Figure 6), the protons in the mutants seem slightly less influenced by W^{25}, with the L^{47} methyl protons in the F^{63} mutant seemingly less influenced by W^{25} than those in wtr-$K2_{tPA}$ and r-[W^{63}Y]$K2_{tPA}$. From these data, we conclude that it is likely that a slight reorientation of the side chain of W^{25} occurs as a result of the loss of W^{63}, such that its ring current effects on the $CH_3^{\delta'}$ protons of L^{47} in the mutants are slightly less intense. Since the environments of the CH_3^{δ} protons, as revealed by their chemical shifts, are not affected by any of the mutations, it is likely that their chemical environments are largely unchanged by alterations in W^{63}. This suggests that the upfield chemical shift changes in L^{47} are not due to direct effects of the mutations on L^{47} but probably on W^{25}.

Additional possible conformational changes in the variants were explored by a fuller assignment of aromatic proton resonances in the F^{63} and Y^{63} mutants using a combination of TOCSY and NOESY pulse sequences. We did not pursue in this same detail the NMR characteristics of the W^{63}H an W^{63}S, since an aromatic group at that position appeared essential for ligand binding to occur, regardless of whether conformational integrity was maintained. As seen from Table 3, nearly all of the aromatic proton resonances were located, and the largest differences in the mutants from wtr-$K2_{tPA}$ are seen in the H_5 and H_6 resonances of W^{25}. None of the other identified chemical shifts were significantly different from those of wtr-$K2_{tPA}$. These findings demonstrate that replacement of W^{63} does not lead to substantial rearrangement of the conformations of aromatic residues in the mutants, except possibly in the case of W^{25}, which is not part of the ligand binding site. Especially noteworthy is the preservation of the local environment of W^{74}, which constitutes the hydrophobic wall opposite to that of W^{63} in the ligand binding pocket.

These findings are generally supported by results from analysis of the reactivity of the mutants with $K2_{tPA}$-directed MAbs. Four different antibodies were examined, and the results of Table 2 demonstrate that one of these, MAb-700, possessed C_{50}-MAb values for the four W^{63} mutants that were very similar to that of wtr-$K2_{tPA}$ and to each other. Another, MAb-364, displayed a C_{50}-MAb value for the S^{63} mutant that was greatly diminished (*ca.* 100-fold), when compared to wtr-$K2_{tPA}$. In this case, the Y^{63} mutant also showed weaker binding, by *ca.* 6-fold, and the F^{63} variant was marginally diminished in its avidity. These results could indicate that r-[W^{63}S]$K2_{tPA}$ and r-[W^{63}Y]$K2_{tPA}$ possess local conformations that are somewhat different than the wild-type counterpart and that these differences are revealed by this MAb. Interestingly, both these mutants possess a side-chain hydroxyl group, which could generate new local conformations by means of hydrogen-bonding interactions with suitably placed side chains of other residues. On the other hand, it is possible that these MAb-364 avidity differences between the mutants are the result of W^{63} being an amino acid directly involved in the interaction with the antibody, and other amino acids at that location can substitute for W^{63} to different extents. In any case, if new conformations are indeed formed with the S^{63} and Y^{63} mutants, they do not directly result from the removal of W^{63}, because the H^{63} variant interacts identically to wtr-$K2_{tPA}$ with this MAb. The reactivities of the polypeptides with MAb-623 are of interest in two different respects. First, all of the mutants possessed nearly equal avidities to this antibody, and the C_{50}-MAb values for the mutants were considerably lower (5$-$7-fold) than that of wtr-$K2_{tPA}$. It is also noteworthy that this particular MAb was not entirely specific for $K2_{tPA}$, since it also interacted strongly with $K1_{Pg}$ (C_{50}-MAb = 3.0 nM). These data show that the epitope recognized by MAb-623 is not optimized in $K2_{tPA}$, that W^{63} is not a necessary amino acid for interaction with this MAb, and that upregulated binding epitopes could be constructed in $K2_{tPA}$ for this MAb. Lastly, C_{50}-MAb data for the mutants (Table 2) with MAb-663 demonstrate that the H^{63} avidity constant is diminished by *ca.* 7-fold, whereas that for the S^{63} and Y^{63} mutants is less affected, and the value for the F^{63} nearly identical to wtr-$K2_{tPA}$. Once again, the weaker binding mutants could result from generation of localized conformational differences between the mutants and the wild-type kringle and/or from the different abilities of different amino acid residues to substitute for W^{63} as a direct ligand for this MAb. Due to the small differences between the Y^{63} and S^{63} mutants, the changes brought about by the mutations in the location of the epitope for MAb-663 are not seemingly major.

In general, the MAb suggests that major changes in conformation of the kringle polypeptide do not result from the amino acid substitutions at W^{63}, at least to the degree that would influence ligand binding to such a large extent. In the single case where such a conclusion might be questioned, *i.e.*, binding of the S^{63} mutant to MAb-364, it is

[3] S. L. Nilsen, and F. J. Castellino, unpublished data.

emphasized that the H^{63} mutant interacted normally with this MAb, but ligand binding did not occur with this latter variant. Thus, from the combination of NMR and MAb data, we believe that W^{63} plays a direct role in stabilizing ligand binding in the binding pocket.

The effects of W^{63} on the stability of the conformation of this kringle module have been examined by DSC analysis. The changes in the value of the T_m for the mutants range from approximately 12 °C ($W^{63}Y$) to over 20 °C ($W^{63}H$, $W^{63}S$, and $W^{63}F$), as compared to wtr-$K2_{tPA}$. These are rather remarkable alterations for replacement of a single amino acid, especially in the case of the Y mutation, which at the least preserves the aromatic character of this sequence position. Thus, we believe that W^{63} is an important determinant for stability of the conformational state of the kringle, as was the case with our previous studies with W^{25} (De Serrano & Castellino, 1994b) and to a lesser degree with Y^{76} (De Serrano & Castellino, 1994a), but not with W^{74}, which did not seem to play as significant role in this regard (De Serrano & Castellino, 1992b). The absolute conservation of W^{63}, even in kringles that do not interact with ω-amino acid ligands, is not based on this residue being a critical conformational determinant but as a residue that contributes greatly to the stability of the native conformation. We observe that the lowest T_m obtainable for kringles with amino acid mutations is approximately 55 °C, as was also the case with the mutants that we were able to construct (F and Y) at W^{25} (De Serrano & Castellino, 1994a) and as we are finding to be the case with Y^9 mutations.[3] Perhaps, because of the three disulfide bonds that stabilize this polypeptide conformation, this is the lowest T_m attainable by single amino acid substitutions of non-C residues.

Despite the substantially lowered T_m values for the $W^{63}F$ and $W^{63}Y$ variants, saturation of their ligand binding sites with EACA leads to an approximate 9−12 °C rise in the T_m value for thermal denaturation, a value similar to that obtained for wtr-$K2_{tPA}$ under these same circumstances. Thus, binding of EACA stabilizes the native conformation by the same amount regardless of the initial value of the T_m. While this method is not of high resolution in identifying small conformational alterations, these data nonetheless add another argument to the consensus position that changes in W^{63} do not lead to conformational adjustments in the resulting mutants that are of a significantly profound nature as to influence ligand binding.

Thus, in conclusion, the complete conservation of this residue in all kringle structures identified to date is not due to its necessity for adoption of the native kringle conformation, but is due to maintenance of the stability of that conformational state. With regard to ligand binding, an aromatic residue at this location, preferably tryptophan, appears to be essential.

REFERENCES

Arni, R. K., Padmanabhan, K., Padmanabhan, K. P., Wu, T. P., & Tulinsky, A. (1994) *Chem. Phys. Lipids 67* (8), 59−66.

Atkinson, R. A., & Williams, R. J. P. (1990) *J. Mol. Biol. 212,* 541−552.

Bajzar, L., Manuel, R., & Nesheim, M. E. (1995) *J. Biol. Chem. 270,* 14477−14484.

Bizik, J., Stephens, R. W., Grofova, M., & Vaheri, A. (1993) *J. Cell. Biochem. 51,* 326−335.

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1988) *J. Magn. Reson. 58,* 370−388.

Byeon, I.-J., Kelley, R. F., & Llinas, M. (1991) *Eur. J. Biochem. 197,* 155−165.

Byeon, I. J. L., Kelley, R. F., Mulkerrin, M. G., An, S. S. A., & Llinas, M. (1995) *Biochemistry 34,* 2739−2750.

Chenivesse, X., Huby, T., Chapman, J., Franco, D., & Thillet, J. (1996) *Protein Expression Purif. 8,* 145−150.

Christensen, U. (1985) *FEBS Lett. 182,* 43−46.

Cleary, S., Mulkerrin, M. G., & Kelley, R. F. (1989) *Biochemistry 28,* 1884−1891.

Clemmensen, I., Petersen, L. C., & Kluft, C. (1986) *Eur. J. Biochem. 156,* 327−333.

De Serrano, V. S., & Castellino, F. J. (1990) *J. Biol. Chem. 265,* 10473−10478.

De Serrano, V. S., & Castellino, F. J. (1992a) *Biochemistry 31,* 11698−11706.

De Serrano, V. S., & Castellino, F. J. (1992b) *Biochemistry 31,* 3326−3335.

De Serrano, V. S., & Castellino, F. J. (1993) *Biochemistry 32,* 3540−3548.

De Serrano, V. S., & Castellino, F. J. (1994a) *Biochemistry 33,* 3509−3514.

De Serrano, V. S., & Castellino, F. J. (1994b) *Biochemistry 33,* 1340−1344.

De Serrano, V. S., Menhart, N., & Castellino, F. J. (1992a) *Arch. Biochem. Biophys. 294,* 282−290.

De Serrano, V. S., Sehl, L. C., & Castellino, F. J. (1992b) *Arch. Biochem. Biophys. 292,* 206−212.

de Vos, A. M., Ultsch, M. H., Kelley, R. F., Padmanbhan, K., Tulinsky, A., Westbrook, M. L., & Kossiakoff, A. A. (1992) *Biochemistry 31,* 270−279.

Frank, S., Durovic, S., & Kostner, G. M. (1996) *Eur. J. Clin. Invest. 26,* 109−114.

Griesinger, C., Otting, G., Wuthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110,* 7870−7872.

Gunzler, W. A., Steffens, G. J., Otting, F., Kim, S. M. A., Frankus, E., & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363,* 1155−1165.

Hajjar, K. A., Jacovina, A. T., & Chacko, J. (1994) *J. Biol. Chem. 269,* 21191−21197.

Hoover, G. J., Menhart, N., Martin, A., Warder, S., & Castellino, F. J. (1993) *Biochemistry 32,* 10936−10943.

Hortin, G. L., Gibson, B. L., & Fok, K. F. (1988) *Biochem. Biophys. Res. Commun. 155,* 591−596.

Hortin, G. L., Trimpe, B. L., & Fok, K. F. (1989) *Thromb. Res. 54,* 621−632.

Ichinose, A., Kisiel, W., & Fujikawa, K. (1986) *J. Clin. Invest. 78,* 163−169.

Kaneko, M., Mimuro, J., Matsuda, M., & Sakata, Y. (1991) *Biochem. Biophys. Res. Commun. 178,* 1160−1166.

Kelley, R. F., & Cleary, S. (1989) *Biochemistry 28,* 4047−4054.

Klezovitch, O., & Scanu, A. M. (1996) *Arterioscler., Thromb., Vasc. Biol. 16,* 392−398.

Koschinsky, M. L., Tomlinson, J. E., Zioncheck, T. M., Schwartz, K., Eaton, D. L., & Lawn, R. M. (1991) *Biochemistry 30,* 5044−5051.

Kraft, H. G., Kochl, S., Menzel, H. J., Sandholzer, C., & Utermann, G. (1992) *Hum. Genet. 90,* 220−230.

Lerch, P. G., Rickli, E. E., Lergier, W., & Gillessen, D. (1980) *Eur. J. Biochem. 107,* 7−13.

Li, X., Bokman, A. M., Llinas, M., Smith, R. A. G., & Dobson, C. M. (1994) *J. Mol. Biol. 235,* 1548−1559.

Lijnen, H. R., Hoylaerts, M., & Collen, D. (1980) *J. Biol. Chem. 255,* 10214−10222.

Lind, S. E., & Smith, C. J. (1991) *J. Biol. Chem. 266,* 5273−5278.

LoGrasso, P. V., Cornell-Kennon, S., & Boettcher, B. R. (1994) *J. Biol. Chem. 269,* 21820−21827.

Lucas, M. A., Fretto, L. J., & McKee, P. A. (1983) *J. Biol. Chem. 258,* 4249−4256.

Marcovina, S. M., Hobbs, H. H., & Albers, J. J. (1996) *Clin. Chem. 42,* 436−439.

Marti, D., Schaller, J., Ochensberger, B., & Rickli, E. E. (1994) *Eur. J. Biochem. 219,* 455−462.

Mathews, I. I., Vanderhoff-Hanaver, P., Castellino, F. J., & Tulinsky, A. (1996) *Biochemistry 35,* 2567−2576.

McCance, S. G., Menhart, N., & Castellino, F. J. (1994) *J. Biol. Chem. 269,* 32405−32410.

McLean, J. W., Tomlinson, J. E., Kuang, W.-j., Eaton, D. L., Chen, E. Y., Gless, G. M., Scanu, A. M., & Lawn, R. M. (1987) *Nature (London) 330,* 132−137.
Menhart, N., & Castellino, F. J. (1995) *Int. J. Pept. Protein Res. 46,* 464−470.
Menhart, N., Sehl, L. C., Kelley, R. F., & Castellino, F. J. (1991) *Biochemistry 30,* 1948−1957.
Menhart, N., McCance, S. G., Sehl, L. C., & Castellino, F. J. (1993) *Biochemistry 32,* 8799−8806.
Miele, R. G., Nilsen, S. L., Brito, T., Bretthauer, R. K., & Castellino, F. J. (1997) *Biotechnol. Appl. Biochem. 25,* 151−157.
Mikol, V., LoGrasso, P. V., & Boettcher, B. R. (1996) *J. Mol. Biol. 256,* 751−761.
Miles, L. A., Dahlberg, C. M., & Plow, E. F. (1988) *J. Biol. Chem. 263,* 11928−11934.
Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., & Plow, E. F. (1991) *Biochemistry 30,* 1682−1691.
Mimuro, J., Kaneko, M., Murakami, T., Matsuda, M., & Sakata, Y. (1992) *Biochim. Biophys. Acta 1160,* 325−334.
Mulichak, A. M., Tulinsky, A., & Ravichandran, K. G. (1991) *Biochemistry 30,* 10576−10588.
Nilsen, S. L., DeFord, M. E., Prorok, M., Chibber, B. A. K., Bretthauer, R. K., & Castellino, F. J. (1997) *Biotechnol. Appl. Biochem. 25,* 63−74.
Novokhatny, V. V., Matsuka, Y. V., & Kudinov, S. A. (1989) *Thromb. Res. 53,* 243−252.
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., & Collen, D. (1983) *Nature (London) 301,* 214−221.
Rejante, M. R., Byeon, I.-J. L., & Llinas, M. (1991) *Biochemistry 30,* 11081−11092.
Scanu, A. M., & Edelstein, C. (1994) *Clin. Genet. 46,* 42−45.
Scanu, A. M., Pfaffinger, D., Lee, J. C., & Hinman, J. (1994) *Biochim. Biophys. Acta 1227,* 41−45.
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis 3,* 191−209.
States, D. I., Haberkorn, R. A., & Rubeu, D. J. (1982) *J. Magn. Reson. 48,* 286−293.
Stephens, R. W., Bokman, A. M., Myohanen, H. T., Reisberg, T., Tapiovaara, H., Pedersen, N., Grondahl-Hansen, J., Llinas, M., & Vaheri, A. (1992) *Biochemistry 31,* 7572−7579.
Sugiyama, N., Sasaki, T., Iwamoto, M., & Abiko, Y. (1988) *Biochim. Biophys. Acta 952,* 1−7.
Thorsen, S., Clemmensen, J., Sottrup-Jensen, L., & Magnusson, S. (1981) *Biochim. Biophys. Acta 668,* 377−387.
Tran-Thang, C., Kruithof, E. K. O., Atkinson, J., & Bachmann, F. (1986) *Eur. J. Biochem. 160,* 599−604.
Ullberg, M., Kronvall, G., Karlsson, I., & Wiman, B. (1990) *Infect. Immun. 58,* 21−25.
van Zonneveld, A.-J., Veerman, H., & Pannekoek, H. (1986) *J. Biol. Chem. 261,* 14214−14218.
Vestal, M. L., Juhasz, P., & Martin, S. A. (1995) *Rapid Commun. Mass Spectrom. 9,* 1044−1050.
Weening-Verhoeff, E. J. D., Quax, P. H. A., van Leeuwen, R. T. J., Rehberg, E. F., Mariotti, K. R., & Verheijen, J. H. (1990) *Protein Eng. 4,* 191−198.
Whitefleet-Smith, J., Rosen, E., McLinden, J., Ploplis, V. A., Fraser, M. J., Tomlinson, J. E., McLean, J. W., & Castellino, F. J. (1989) *Arch. Biochem. Biophys. 271,* 390−399.
Wilhelm, O. G., Jaskunas, S. R., Vlahos, C. J., & Bang, N. U. (1990) *J. Biol. Chem. 265,* 14606−14611.
Wu, H. L., Wu, I. S., Fang, R. Y., Hau, J. S., Wu, D. H., Chang, B. I., Lin, T. M., & Shi, G. Y. (1992) *Biochem. Biophys. Res. Commun. 188,* 701−711.
Wuthrich, K. (1986) *NMR of Proteins and Nucleic Acids,* John Wiley & Sons, Inc., New York.

BI970197G