The Cationic Locus on the Recombinant Kringle 2 Domain of Tissue-Type Plasminogen Activator That Stabilizes Its Interaction with ω-Amino Acids†

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ABSTRACT: The properties of the cationic locus within the recombinant (r) kringle 2 domain (residues 180–261) of tissue-type plasminogen activator ([$K2_{tPA}$]) that are responsible for stabilization of its interaction with the carboxylate moiety of ω-amino acid ligands have been assessed by determination of the binding constants of several such ligands to a variety of r-[$K2_{tPA}$] mutants obtained by oligonucleotide-directed mutagenesis. We have generated, expressed in *Escherichia coli*, and purified alanyl mutants of individual histidyl, lysyl, and arginyl residues of r-[$K2_{tPA}$] and determined the dissociation constants of several ω-amino acids, viz., 6-aminohexanoic acid (6-AHxA), 7-aminoheptanoic acid (7-AHpA), L-lysine (L-Lys), and *trans*-(aminomethyl)cyclohexane-1-carboxylic acid (AMCHA), to each of the r-[$K2_{tPA}$] variants. We find that K33 plays the most significant role as a cationic partner of the complementary carboxylate group of these ligands. When K33 is altered to a variety of other amino acids, the K33R mutant best stabilizes binding of all of these ligands. However, the r-K33L and r-K33F variants selectively interact with 7-AHpA almost as strongly (ca. 2-fold reduction in binding strength) as wild-type r-[$K2_{tPA}$]. Increased polarity (K33Q) or a negative charge (K33E) at this sequence position significantly destabilizes binding of ω-amino acids to the muteins. We also found that the r-K33E mutant and, to a lesser extent, the r-K33Q variant selectively interact with a new ligand, 1,6-diaminohexane. These observations show that the ω-amino acid binding site of wtr-[$K2_{tPA}$] could be redesigned to provide a new binding specificity. The results reported herein demonstrate that the cationic donor of the kringle 2 domain of tissue-type plasminogen activator that is responsible for partial stabilization of the binding of ω-amino acids to this region of the protein consists solely of K33. However, the presence of a cation at this site is not an absolute requirement for ω-amino acid binding stability, since hydrophobic amino acids at position 33 allow such binding to occur in a selective manner for certain ligands of this class.

Kringles are protein modules containing approximately 80 amino acids that are partially stabilized by three disulfide bonds (Magnusson et al., 1975). These domains have been found in a variety of proteins, many of which contain multiple copies. For example, a single kringle domain is found in coagulation factor XII (McMullen & Fujikawa, 1985) and in urokinase (Steffens et al., 1982), two kringles have been identified in tPA[1] (Pennica et al., 1983) and prothrombin (Magnusson et al., 1975), four kringles have been discovered in a human hepatocyte growth factor (Nakamura et al., 1989), five kringles are present in HPg (Sottrup-Jensen et al., 1978), and 38 kringles have been located in apolipoprotein(*a*) (McLean et al., 1987).

The most important property of many of these kringles resides in their abilities to bind ω-amino acids, ligands that compete with many physiologically important interactions of proteins that contain these domains. Examples of such relevant interactions are the binding of kringle domains of HPg (Thorsen, 1975; Wiman & Wallen, 1977; Thorsen et al., 1981) and tPA (van Zonneveld et al., 1986; de Munk et al., 1989) to fibrin; the initial binding of HPm to its fast-acting inhibitor, α_2-antiplasmin (Wiman et al., 1978); the binding of HPg and tPA to certain types of cells (Miles et al., 1988); and the binding of the activation regulator, Cl^-, to HPg (Urano et al., 1987).

While the kringle regions of these proteins are highly homologous, they do not all interact similarly with ω-amino acids. To this time, the only kringles found to bind to ω-amino acids are [$K1_{HPg}$] (Lerch & Rickli, 1980; Lerch et al., 1980; Menhart et al., 1991), [$K4_{HPg}$] (Lerch et al., 1980; DeMarco et al., 1987, 1989; Sehl & Castellino, 1990), [$K5_{HPg}$] (Novokhatny et al., 1989; Thewes et al., 1990), [$K2_{tPA}$] (Cleary et al., 1989; De Serrano et al., 1991), and possibly some of the apolipoprotein(*a*) kringles. The strength of binding of these ligands, as well as their specificities for different ω-amino acid analogues, differs among the various kringles.

A number of past and more recent studies have appeared that contribute to our understanding of the manner of binding of ω-amino acids to isolated kringle domains. From chemical modification studies, it was found that D56 and R69 of [$K4_{HPg}$] formed ion-pair interactions with the complementary amino and carboxyl groups of 6-AHxA (Trexler et al., 1982). On the other hand, the cationic ligand binding center of [$K1_{HPg}$] has also been proposed to include R34 (Vali & Patthy, 1984). Regarding [$K2_{tPA}$], site-directed mutagenesis studies in our laboratory have directly implicated K33 as the principal cationic center for interaction of this kringle domain with ω-amino acids (De Serrano et al., 1991). In addition to these ion-pair interactions, ^1H NMR investigations have shown that

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[1] Abbreviations: tPA, tissue-type plasminogen activator; HPg, human plasminogen; HPm, human plasmin; [$K1_{HPg}$], the kringle 1 region (residues C84–C162) of human plasminogen; [$K4_{HPg}$], the kringle 4 region (residues C358–C435) of human plasminogen; [$K5_{HPg}$], the kringle 5 region (residues C462–C541) of human plasminogen; [$K2_{tPA}$], the kringle 2 region (residues C180–C261) of human tissue-type plasminogen activator; 7-AHpA, 7-aminoheptanoic acid; 6-AHxA, 6-aminohexanoic acid; AMCHA, *trans*-(aminomethyl)cyclohexane-1-carboxylic acid; r, recombinant; wt, wild type; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; ES/MS, electrospray/mass spectrometry.

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aromatic residues in HPg kringles (Motta et al., 1986; Ramesh et al., 1987; Tulinsky et al., 1988a; Thewes et al., 1990), and in [$K2_{tPA}$], are influenced by addition of ω-amino acids to these domains. In [$K2_{tPA}$], the relevant aromatic residues are W25, W63, H65, W74, and Y76 (Byeon et al., 1990). In this regard, we have employed recombinant DNA technology to substitute different classes of amino acids at W74 and have demonstrated that an aromatic residue at this sequence position best stabilizes binding to ω-amino acids, with a W residue being much more effective than an F or a Y residue for this purpose (De Serrano & Castellino, 1992).

The X-ray crystal structures of both r-[$K2_{tPA}$] and [$K4_{HPg}$] have been determined. In the case of r-[$K2_{tPA}$],[2] the unit cell contained three r-[$K2_{tPA}$] molecules, with the side chain of K49 from two r-[$K2_{tPA}$] of these inserted into the putative ω-amino acid binding pockets of two other r-[$K2_{tPA}$] molecules, thus providing a working model of the kringle/ligand complex (de Vos et al., 1991). Additionally, the structure of the [$K4_{HPg}$]/6-AHxA complex[3] has been solved (Wu et al., 1991). These significant advances allow some generalizations to be made regarding the nature of ω-amino acid binding to kringle domains. In order to test these models, we have employed site-directed mutagenesis to generate strategically altered molecules. In the current paper, we mutated each of the positively charged residues in this polypeptide and examined the binding constants of a variety of ω-amino acid ligands to these variants, in order to thoroughly examine the possibility that the cationic donor for ω-amino acid binding stability in r-[$K2_{tPA}$] was exclusively accounted for by K33, as has been proposed earlier (De Serrano et al., 1991). The results of this investigation are reported herein.

MATERIALS AND METHODS

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

DNA Analytical Methods. 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, and references therein).

Construction of Expression Plasmids. The starting plasmid for the mutations was pSTII/[$K2_{tPA}$], an *Escherichia coli* expression vector that contains the alkaline phosphatase promoter, the heat-stable enterotoxin signal sequence present upstream of, and in frame with, the cDNA encoding wtr-SD[$K2_{tPA}$]S. The polypeptide expressed after cleavage of the signal sequence consists of residues C180–C261 of tPA (kringle numbering, C1–C81), flanked on the amino terminus by the dipeptide SD and on the carboxyl terminus by the amino acid S. All steps in the construction of this expression vector have been described in detail (De Serrano et al., 1991).

This plasmid was the starting point for most of the r mutants of this kringle domain, which were obtained by oligonucleotide-directed mutagenesis. Screenings of positive transformants were accomplished by the gain or loss of specific restriction endonuclease cleavages. In some cases, it was best served for either screening purposes and/or for subsequent molecular weight analysis of the r polypeptides to employ one of the kringle mutants as a starting point for construction of other mutants. These cases will be elaborated upon where applicable.

Expression and Purification of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] Variants. E. coli DH5α cells were employed for all expressions, which were conducted as previously described (De Serrano et al., 1991; Menhart et al., 1991).

For purification of the recombinant materials, the *E. coli* cells were separated into the periplasmic and oxidatively refolded fractions (Menhart et al., 1991) and passed over a column of Sepharose–lysine equilibrated with low salt buffer (25 mM Tris-HCl, pH 8.0). Use of this buffer resulted in adherence to the column of even weakly bound r-[$K2_{tPA}$] mutants. Elution of the variant kringles was accomplished with 6-AHxA as previously described (Menhart et al., 1991). The eluant was then dialyzed against a solution of 10 mM NaOAc, pH 5.0, and placed on a column of MonoS HR 5/5 (0.5 cm × 5 cm) equilibrated with the same buffer at room temperature. The adsorbed r-[$K2_{tPA}$] variants were then eluted at 1 mL/min with a salt gradient. The elution conditions were a linear gradient of start solution A (10 mM NaOAc, pH 5.0) to a limit solution of 25% (v/v) solution B (10 mM NaOAc/450 mM NaCl, pH 5.0)/solution A, over 2 min, during which time a minor impurity was eluted. This was followed by a linear gradient of 25% (v/v) solution B/solution A (start solution) to 60% (v/v) solution B/solution A (limit solution), over a time of 30 min. The r-[$K2_{tPA}$] mutants were eluted as the major components at various positions in this gradient, depending in large part on the charge alterations resulting from the mutations. The final linear gradient employed was 60% (v/v) solvent B/buffer A to 100% solvent B over 2 min, which in some cases resulted in elution of another impurity. In all cases, highly homogeneous r-[$K2_{tPA}$] mutants resulted from this purification method.

Intrinsic Fluorescence Titrations. Titrations of the effect of ω-amino acids on the intrinsic fluorescence of r-[$K2_{tPA}$] and r-[$K2_{tPA}$] mutants were accomplished at 25 °C in a buffer containing 50 mM TrisOAc/150 mM NaOAc, pH 8.0, as previously described (Menhart et al., 1991). These titrations were conducted by employing a SLM Aminco SPF-500 spectrofluorometer. The excitation and emission wavelengths were 285 and 340 nm, respectively.

The binding (dissociation) constants (K_d) were calculated from the fluorescence titrations by nonlinear least squares curve fitting to an equation developed for a two-state model of binding with a 1:1 stoichiometry, as described in detail in an earlier publication (Menhart et al., 1991).

Molecular Modeling. Molecular models of the complex of 1,6-diaminohexane with r-[$K2_{tPA}$/K33E] and r-[$K2_{tPA}$/K33Q] were constructed on the basis of the X-ray crystal structure of r-[$K2_{tPA}$]. This latter structure was displayed on a Silicon Graphics workstation in the Insight II program (Biosym Technologies, San Diego, CA). Residue K33 was then replaced with either E or Q, and this new side chain was allowed to minimize, constraining all other atoms in the molecule. A second minimization was then performed, allowing structural perturbations to occur within 5 Å of the modified residue, and allowed to proceed until a rms derivative of 0.01 kcal mol^{-1} $Å^{-1}$ was reached. Next, 1,6-diaminohexane was constructed as an extended chain and docked into the lysine binding pocket of [$K2_{tPA}$/K33E] or r-[$K2_{tPA}$/K33Q] by employing the docking module of the Insight II program. Subsequently, 1,6-diaminohexane was minimized in the

[2] The crystal coordinates have been provided to this laboratory by Dr. A. B. de Vos, South San Francisco, CA. The MSU PROLSQ refined coordinates, provided by Dr. Alexander Tulinsky, East Lansing, MI, were used for modeling.

[3] A. Tulinsky, unpublished data. The crystal coordinates (Wu et al., 1991) have been provided to this laboratory by Dr. Alexander Tulinsky, East Lansing, MI.

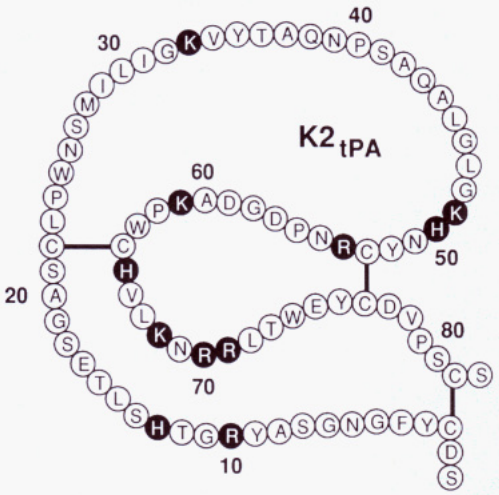

FIGURE 1: Amino acid sequence of the recombinant wild-type [$K2_{tPA}$] construct expressed in *E. coli* cells. Single-letter codes for amino acids are used. The sequence locations of the positively charged (at pH 8.0) amino acids (including H residues) are indicated by black-white color reversals.

binding pocket using the Discover program (Biosym Technologies), holding protein atoms constant until a rms derivative of 0.01 kcal mol^{-1} $Å^{-1}$ was attained.

Analytical Methods. ES/MS analysis for determination of the molecular weight of the samples was conducted using a JOEL (Peabody, MA) AX505 HA mass spectrometer equipped with the JOEL electrospray ionization source. Samples were dissolved in a solution containing equal volumes of methanol and 2% (v/v) acetic acid. A solution of approximate concentration of 10 μM was injected at a flow rate of 2 μL/min. Usually, two species were observed, representing +7 and +6 charge states of the polypeptide. Molecular masses were calculated from each of these peaks and averaged.

$NaDodSO_4$/PAGE was performed as described (Laemmli, 1970). FPLC was conducted employing a Pharmacia (Piscataway, NJ) system.

RESULTS

On the basis of a molecular model that we forwarded for r-[$K2_{tPA}$], accompanied by some mutagenesis and ω-amino acid binding experiments, we suggested that K33 of this kringle domain is the most likely candidate for the cationic center presumably needed for stabilization of binding of the carboxylate moiety of the ligand to this kringle (De Serrano et al., 1991). With the availability of the X-ray crystal structure of r-[$K2_{tPA}$] (de Vos et al., 1991) to assist interpretation of structure-binding relationships, we undertook a complete investigation of this topic and generated r mutants of each of the charged residues of r-[$K2_{tPA}$]. The K_d values of a series of ω-amino acids to the variant kringles were then determined by fluorescence titrations. The locations of these residues in the primary structure of the r-[$K2_{tPA}$] construct that we have employed are provided in Figure 1, and their orientations in the crystal structure (de Vos et al., 1991) of r-[$K2_{tPA}$] are illustrated in Figure 2.

Table I provides a listing of the oligonucleotide primers employed to generate the desired mutants, as well as the restriction endonucleases used for screening the positive *E. coli* transformants. In the case of the H50A mutant, two separate steps were needed, the first to provide a cDNA with a convenient restriction endonuclease site and the second to

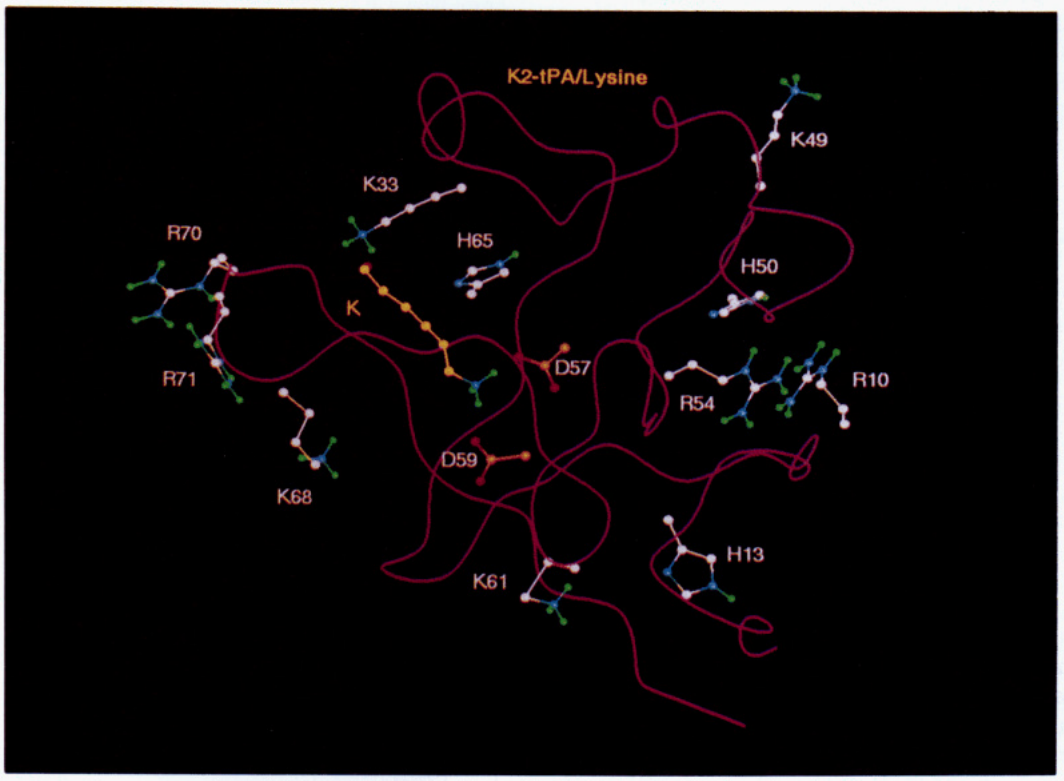

FIGURE 2: X-ray crystal structure of wtr-[$K2_{tPA}$]. Selected amino acid side chains are displayed on the backbone structure, as indicated. The numbering of amino acids beings at C1 of the r-[$K2_{tPA}$] sequence and continues consecutively to C81. Each amino acid is displayed from its β-carbon, and nonexchangeable hydrogen atoms are excluded to minimize overcrowding. The backbone conformation is in magenta. Positively charged (at pH 8.0) amino acid side-chain carbon atoms (including histidyl residues) are in off-white, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms are in green. The lysine (K) side chain (K49) from another molecule of r-[$K2_{tPA}$] that is inserted into the binding pocket in the unit cell is shown with its side-chain carbon atoms in yellow. The two D residues, D57 and D59, implicated as anionic centers for the binding of the amino group of the ligand, are shown for reference, with their carbon atoms in orange.

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

mutation[a]	primer[b]	screen
R10A[c]	5'-GGG TCA GCC TAC gcc GGg ACG CAC AGC CTC	- *Scr*F I
H13A[c]	5'-TAC CGT GGC ACG gct AGC CTC ACC GAG	+ *Nhe* I
K33A[c]	5'-ATC CTG ATA GGC gcc GTT TAC ACA GCA CAG	+ *Nar* I
K33E[d]	5'-ATC CTG ATA GGC Gaa GTT TAC ACA GCA	- *Bsa* I
K33F[e]	5'-ATC CTG ATA GGC ttC GTT TAC ACA GCA	- *Nar* I
K33H[d]	5'-ATC CTG ATA GGC caC GTT TAC ACA GCA	- *Bsa* I
K33L[c]	5'-TG ATC CTG ATA GGt ctc GTT TAC ACA GCA	+ *Bsa* I
K33Q[e]	5'-ATC CTG ATA GGC cag GTT TAC ACA GCA CAG	- *Nar* I
K33R[c]	5'-C ATG ATC CTG ATc GGC cgG GTT TAC ACA GCA	+ *Eag* I
K33T[c]	5'-TG ATC CTG ATA GGt Acc GTT TAC ACA GCA CAG	+ *Kpn* I
K49A[c]	5'-TG GGC CTG GGt gca CAT AAT TAC T	+ *HgiA* I
H50A[c]	5'-GC CTG GGC AAA gcT tAT TAC TGC CGG	+ *Hind* III
	5'-G GGC AAA GCa aAT TAC TGC C	- *Hind* III
R54A[c]	5'-CAT AAT TAC TGC gca AAT CCT GAT GGG	+ *Fsp* I
H65Y[c]	5'-GCC AAG CCC TGG TGC taC GTa CTG AAG AAC CGC AGG	+ *SnaB* I
K68A[c]	5'-TGC CAC GTG CTG gcc AAC CGC AGG CTG	+ *Eae* I
R70A[c]	5'-AC GTG CTG AAG AAC gcg cGc CTG ACG TGG GAG TA	+ *BssH* II
R71A[c]	5'-TG AAG AAC CGC gcG CTG ACG TGG G	+ *BssH* II

[a] The column below indicates the wild-type amino acid in r-[$K2_{tPA}$] and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position. [b] The mutated bases are represented by lower-case lettering. [c] The starting DNA was the wild-type kringle 2, and mutated bases are referenced to the wild-type cDNA. [d] The starting DNA was K33L, and the mutated bases are referenced to this cDNA. [e] The starting DNA was K33A, and the mutated bases are referenced to this cDNA.

generate the desired mutation using the loss of the first restriction site as a screening procedure. All cDNAs were expressed in *E. coli* cells, and the expressed polypeptides were purified by Sepharose–lysine affinity chromatography and FPLC, as described above and in previous papers from this laboratory (De Serrano et al., 1991; Menhart et al., 1991; De Serrano & Castellino, 1992). Regarding the Sepharose–lysine step, even those mutants that exhibited the weakest binding to ω-amino acids interacted with this column, as long as the column was equilibrated and washed with a low salt buffer, i.e., 25 mM Tris-HCl, pH 8.0. However, washing with this buffer resulted in a more impure kringle preparation after elution with 6-AHxA than would result from a high salt buffer wash (Menhart et al., 1991), and a simple FPLC step was needed to resolve the components.

These procedures resulted in purification of the r-[$K2_{tPA}$] positively charged r variants listed in Table II. The yields were dependent upon the particular mutant, but most were in the range of 20–30 mg/100 g of *E. coli* cells, with approximately 80% of the material obtained from the refolded fraction. Notable exceptions were the r-K61 mutants, which we were not able to express despite attempts with r-K61A, r-K61G, and r-K61N constructions. The r-H50A variant was obtained at a very low expression level (ca. 0.2 mg/150 g of cells), and the r-K33A variant only resulted in a final yield of approximately 2–4 mg/100 g of *E. coli* cells. In these latter two cases, the amounts, while low, were nonetheless sufficient for all studies to be described.

ES/MS was employed to determine accurate mass numbers for all of the variants. A listing of the values obtained is provided in Table II. The data clearly show that the intended mutants were indeed obtained, that the signal sequence was cleaved in all cases, and that modifications other than those intended were highly unlikely.

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

variant[a]	molecular weight calcd	molecular weight exptl
r-SD[$K2_{tPA}$]S[b]	9365.69	9363.4
r-SD[$K2_{tPA}$/R10A]S[c]	9280.58	9279.6
r-SD[$K2_{tPA}$/H13A]S[c]	9299.62	9300.3
r-SD[$K2_{tPA}$/K33A]S[c]	9308.59	9306.3
r-SD[$K2_{tPA}$/K49A]S[c]	9308.59	9307.0
r-SD[$K2_{tPA}$/H50A]S[c]	9299.62	9297.5
r-SD[$K2_{tPA}$/R54A]S[c]	9280.58	9277.4
r-SD[$K2_{tPA}$/H65Y]S[c]	9391.72	9393.0
r-SD[$K2_{tPA}$/K68A]S[c]	9308.59	9306.3
r-SD[$K2_{tPA}$/R70A]S[c]	9280.58	9277.7
r-SD[$K2_{tPA}$/R71A]S[c]	9280.58	9278.7

[a] Each of the H, K, and R residues have been mutated, except for K61 mutants, which we were not able to express. [b] Refers to the wild-type recombinant molecule. The bracketed portion is the [amino acid sequence of $K2_{tPA}$ (from C1 to C81)]. Flanking the amino terminus of C1 is the dipeptide SD, and flanking the carboxyl terminus of C81 is the single amino acid S. [c] The bracketed portion is the [amino acid sequence of $K2_{tPA}$/the wild-type amino acid, and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position]. Flanking the amino terminus of C1 of the kringle is the dipeptide SD, and flanking the carboxyl terminus of C81 of the kringle is the single amino acid S.

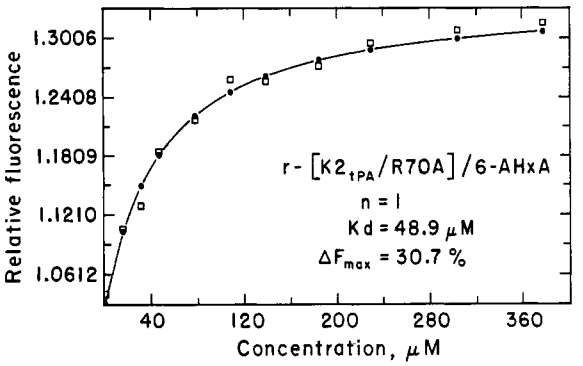

FIGURE 3: Titration of the relative change in intrinsic fluorescence of r-[$K2_{tPA}$/R70A] (6.95 μM) with 6-AHxA. The experimental points (*q*) are best fit to a line (|) generated by employing values of $n = 1.0$, $K_d = 48.9$ μM, and a maximal relative fluorescence change (ΔF_{max}) of 30.7%. The buffer was 50 mM TrisOAc/150 mM NaOAc, pH 8.0, at 25 °C. The excitation and emission wavelengths were 285 and 340 nm, respectively.

The K_d values for ω-amino acids to each of the r-[$K2_{tPA}$] variants listed in Table II were determined by titration of the fluorescence change accompanying such binding (Menhart et al., 1991; De Serrano & Castellino, 1992). With this set of mutants, maximum intrinsic fluorescence changes accompanying binding ranged from approximately 7% for the interaction of AMCHA with r-[$K2_{tPA}$/H50A] to 47% for the binding of 7-AHpA to wtr-[$K2_{tPA}$]. Most of the fluorescence changes were in the range of 20%–40%, alterations quite sufficient for titration and analysis. Since all of the r-variants were purified by affinity chromatography employing the ligands of interest, it could also confidently be assumed that the stoichiometry of the ligand/kringle interaction was 1.0. This fact also eases interpretation of the titrations. An example of the type of data obtained for interaction of 6-AHxA with r-[$K2_{tPA}$/R70A] is shown in Figure 3. The K_d values provided in Table III are averages of triplicate titrations at different ranges of ligand concentrations, with variances in K_d values of replicate experiments being <15%. Examination of the data of Table III shows that only K33 is a firm candidate for the cationic site that stabilizes interaction of the kringle with the carboxylate moiety of the ligand, a conclusion reached earlier on the basis of molecular modeling of [$K2_{tPA}$] and on

Table III: Dissociation Constants for ω-Amino Acids to r-[$K2_{tPA}$] Variants

variant[a]	dissociation constant (μM)			
	6-AHxA	7-AHpA	L-Lys	AMCHA
wt[b]	43	6	85	27
R10A[c]	42	8	64	16
H13A[c]	33	8	71	32
K33A[c]	258	32	146	209
K49A[c]	38	3	85	21
H50A[c]	37	6	71	55
R54A[c]	33	6	71	19
H65Y[c]	20	67	188	nd
K68A[c]	71	16	89	25
R70A[c]	49	9	66	22
R71A[c]	47	9	52	32

[a] Each of the H, K, and R residues have been mutated, except for K61 mutants, which we were not able to express. [b] Refers to the wild-type recombinant molecule. [c] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position.

results of mutational analysis with a much smaller number of variants on fewer ligands (De Serrano et al., 1991).

The results in Table III regarding the K_d values for the r-H65Y mutant are of great interest. In the case of all other mutants, the strength of ligand binding follows the order 7-AHpA > AMCHA > 6-AHxA > lysine. However, with r-[$K2_{tPA}$/H65Y], a selective weakening of the interaction of 7-AHpA and, to a smaller extent, of lysine with the variant molecule is obtained. This results in a change of specificity of binding strength to 6-AHxA > 7-AHpA > lysine (the fluorescence change accompanying binding of AMCHA to this mutant was too small for accurate titrations). This latter observation confirms a conclusion reached earlier on this point (Kelley et al., 1991). In all of the above cases, K_d values were nearly identical for the materials isolated from the cell periplasm and from the refolded cellular fraction. Since the former fraction did not require an oxidative refolding step, the results indicate that the refolding procedures employed did not affect the nature of the ω-amino acid binding site.

With the obvious importance of K33 as a functional residue in ω-amino acid binding, we generated a series of recombinant mutants at this locus to probe further into its importance in stabilizing overall binding. The mutants constructed, the oligonucleotides employed, and the screening procedures of the positive transformants are listed in Table I. Expression in *E. coli* and purification of the mutant kringles were conducted as described above. In all of these cases, the yields were considerably lower than those for wtr-[$K2_{tPA}$], ranging in these cases from 1 to 4 mg/100 g of cells, again with the bulk of the material isolated from the refolded fraction of the cells.

ES/MS was employed to accurately determine the molecular mass of the purified materials, and the results are listed in Table IV. In all cases, very close agreement with the calculated values is obtained, clearly showing that the expected mutants were expressed and isolated. Since, for two mutants, viz., those with r-K33E and r-K33Q substitutions, the molecular mass values would be virtually indistinguishable from that of wtr-[$K2_{tPA}$], the starting material for the mutagenesis experiments in these cases was the cDNA encoding r-[$K2_{tPA}$/K33A]. Thus, the products r-[$K2_{tPA}$/K(A)33E] and r-[$K2_{tPA}$/K(A)33Q] possess molecular mass values that are sufficiently different from the starting material, and this technique could be employed to confirm that the intended mutation occurred.

Table IV: Molecular Weights for K33-Derived Variants of r-[$K2_{tPA}$]

variant	molecular weight	
	calcd	exptl
r-SD[$K2_{tPA}$]S[a]	9365.69	9363.4
r-SD[$K2_{tPA}$/K33A]S[b]	9309.59	9306.3
r-SD[$K2_{tPA}$/K33E]S[b] (K33A)[c]	9366.63 (9308.59)	9365.3
r-SD[$K2_{tPA}$/K33F]S[b] (K33A)[d]	9384.69 (9308.59)	9384.4
r-SD[$K2_{tPA}$/K33H]S[b] (K33A)[d]	9374.66 (9308.59)	9375.1
r-SD[$K2_{tPA}$/K33L]S[b]	9350.67	9348.5
r-SD[$K3_{tPA}$/K33Q]S[b] (K33A)[c]	9365.65 (9308.59)	9362.9
r-SD[$K2_{tPA}$/K33R]S[b]	9393.70	9391.3

[a] Refers to the wild-type recombinant molecule. The bracketed portion is the [amino acid sequence of $K2_{tPA}$ (from C1 to C81)]. Flanking the amino terminus of C1 is the dipeptide SD, and flanking the carboxyl terminus of C81 is the single amino acid S. [b] The bracketed portion is the [amino acid sequence of $K2_{tPA}$/the wild-type amino acid and its sequence position beginning from C1 to the kringle, followed by the amino acid replacement at that position]. Flanking the amino terminus of C1 of the kringle is the dipeptide SD, and flanking the carboxyl terminus of C81 of the kringle is the single amino acid S. [c] The starting cDNA was that for the r-K33A mutant (its molecular weight is in parentheses), since the molecular weights of the r-[$K2_{tPA}$/K33E] and r-[$K2_{tPA}$/K33Q] mutants would be experimentally indistinguishable from the wt material. [d] The starting cDNA was that for the K33A mutant (its molecular weight is in parentheses), as a matter of convenience of screening mutant colonies.

Table V: Dissociation Constants for ω-Amino Acids to K33-Derived Variants of r-[$K2_{tPA}$]

variant	dissociation constant (μM)			
	6-AHxA	7-AHpA	L-Lys	AMCHA
wt[a]	43	6	85	27
K33A[b]	258	32	146	209
K33E[b]	1146	921	106	925
K33F[b]	184	11	155	301
K33H[b]	220	83	224	79
K33L[b]	151	13	148	178
K33Q[b]	1195	126	203	534
K33R[b]	89	14	105	22

[a] Refers to the wild-type recombinant molecule. [b] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position.

Table V provides a listing of the K_d values for each of the r-K33 mutants as obtained from intrinsic fluorescence titrations of the variant kringles with the appropriate ligands. In these cases, maximal fluorescence changes ranged from 9% for the binding of AMCHA to r-[$K2_{tPA}$/K33F] to nearly 50% for the 6-AHxA/r-[$K2_{tPA}$/K33Q] interaction. An example of binding data for one member of this set of mutants is shown in Figure 4, for the titration of r-[$K2_{tPA}$/K33L] with 7-AHpA. Analysis of the K_d values listed in of Table V show that the r-K33R variant was nearly as effective in ligand binding as wtr-[$K2_{tPA}$]. This was as expected, since a positive charge at this site is retained at pH 8. Of great surprise, the r-K33L variant showed only slightly diminished ability to interact with 7-AHpA and lysine and was much less effective in binding 6-AHxA and AMCHA, as compared to wtr-[$K2_{tPA}$]. The K_d for binding of 6-AHxA to the K33L variant reported in Table V is slightly lower than that reported previously (De Serrano et al., 1992). Decreasing the size (and hydrophobicity) of the side chain by employing the r-K33A mutant led to a general weakening of the binding interactions with these ligands, and placement of a hydrophobic aromatic side chain (F) as residue 33 resulted in some weakening of the binding constant for AMCHA but retained the K_d values for the other ligands as compared to the r-K33L variant. Regarding other variants in this set, large increases in the K_d values were found when

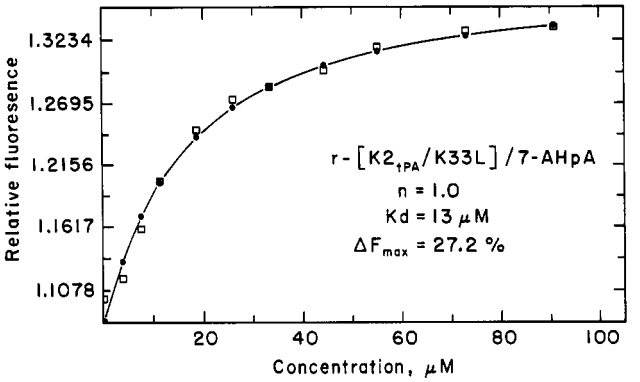

FIGURE 4: Titration of the relative change in intrinsic fluorescence of r-[$K2_{tPA}$/K33L] (5.90 μM) with 7-AHpA. The experimental points (q) are best fit to a line (|) generated by employing values of n = 1.0, K_d = 13 μM, and a maximal relative fluorescence change (ΔF_{max}) of 27.2%. The buffer was 50 mM TrisOAc/150 mM NaOAc, pH 8.0, at 25 °C. The excitation and emission wavelengths were 285 and 340 nm, respectively.

Table VI: Binding of 1,6-Diaminohexane to K33-Derived Variants of r-[$K2_{tPA}$]

variant	dissociation constant (μM)	
	1,6-DHA	6-AHxA
wt[a]	1706	43
K33A[b]	768	258
K33E[b]	13	1146
K33F[b]	283	184
K33Q[b]	279	1195

[a] Refers to the wild-type recombinant molecule. [b] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position.

neutral or negatively charged polar residues were inserted at residue 33, as is seen from examination of the K_d values for the r-K33Q and r-K33E variants. Additionally, with this mutant set 7-AHpA was the most tightly bound ligand with one exception, viz., the case of the interaction of L-lysine with r-[$K2_{tPA}$/K33E], which was the most tightly bound ligand to this variant. This may represent a special case in that the presence of the α-amino group on this ligand may be in position to interact with the newly present carboxylate from the inserted E33 residue.

In order to evaluate whether E at sequence position 33 could serve as a potential functional group for binding to an appropriate ligand, and whether it was properly situated in the bindig pocket, we investigated binding of 1,6-diaminohexane to a series of these mutants. In these cases, maximal fluorescence changes ranged from approximately 14% for the binding to r-[$K2_{tPA}$/K33E] to approximately 47% for binding to r-[$K2_{tPA}$/K33L]. The results listed in Table VI show that this substrate was poorly bound to wtr-[$K2_{tPA}$], and somewhat better to molecules with K33 replaced with hydrophobic amino acids (A and F) and with a variant in which K33 is replaced with Q. It is very clear from the data that 1,6-diaminohexane interacted much tighter with the r-K33E variant, showing that the binding pocket has been successfully redesigned to accommodate another type of ligand.

The final set of variants that were constructed contained deletion of positively charged (at pH 8.0) amino acids from the r-K33L variant in order to determine whether another cationic group could substitute for K33 in mutants that lacked K at this residue. We centered our attention on the positively charged cluster of K68, R70, and R71, since similar amino acid residues in this location of [$K1_{HPg}$] and [$K4_{HPg}$] constitute

Table VII: Molecular Weights for r-[$K2_{tPA}$/K33L]-Derived Variants

variant	molecular weight	
	calcd	exptl
wtr-SD[$K2_{tPA}$]S[a]	9365.69	9363.4
r-SD[$K2_{tPA}$/K33L]S[b]	9350.67	9348.5
r-SD[$K2_{tPA}$/K33L,K49A]S[b]	9293.57	9289.0
r-SD[$K2_{tPA}$/K33L,H65Y]S[b]	9376.70	9375.2
r-SD[$K2_{tPA}$/K33L,K68A]S[b]	9293.57	9292.0
r-SD[$K2_{tPA}$/K33L,R71A]S[b]	9265.56	9264.0
r-SD[$K2_{tPA}$/K33L,R70A,R71A]S[b]	9180.45	9179.5

[a] Refers to the wild-type recombinant molecule. The bracketed portion is the [amino acid sequence of $K2_{tPA}$ (from C1 to C81)]. Flanking the amino terminus of C1 is the dipeptide SD, and flanking the carboxyl terminus of C81 is the single amino acid S. [b] The bracketed portion is the [amino acid sequence of $K2_{tPA}$/the wild-type amino acid and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position; this continues for other amino acid replacements made in the $K2_{tPA}$ sequence]. Flanking the amino terminus of C1 of the kringle is the dipeptide SD, and flanking the carboxyl terminus of C81 of the kringle is the single amino acid S.

part of the ω-amino acid binding pocket in these kringles. We also focused on amino acid H65, since this residue is important in the specificity of binding (vide supra). The variant r-[$K2_{tPA}$/K33L,K49A] was used as a control for general effects of removal of a second charged residue, since K49 is clearly not a member of the ω-amino acid binding site (Figure 2 and Table III). The oligonucleotide primers employed to generate these variants, as well as the screening procedures for positive bacterial transformants, are as in Table I, except that the cDNA encoding r-[$K2_{tPA}$/K33L] was the starting material for the mutations. In the only case not referred to in Table I, i.e., the cDNA that codes for r-[$K2_{tPA}$/K33L,R70A,R71A], the following oligonucleotide primer was employed, beginning with the cDNA encoding r-[$K2_{tPA}$/K33L,R71A]:

5′-GTG CTG AAG AAC gca GCG CTG ACG TGG

This primer directs placement of an A residue at position 70 and also eliminates the *Bss*HII site previously incorporated into the cDNA as a result of the mutagenesis strategy employed for construction of the mutation of R71A (see Table I). This latter feature was used for screening purposes.

Similar to the above, the purified mutants were characterized by molecular mass analysis using ES/MS methodology. As seen from the results of Table VII, the expected values were found, assuring that the proper mutants have been obtained.

The K_d values for binding of various ω-amino acids to this set of mutants have been determined by intrinsic fluorescence titrations and are listed in Table VII. Once again, maximum fluorescence changes accompanying binding were in the range of 9%–40%, with the lower values found for AMCHA/kringle interactions. An example of titration data for one member of this r-mutant set, i.e., the titration of 7-AHpA with r-[$K2_{tPA}$/K33L,H65Y], is illustrated in Figure 5. Examination of the binding data of Table VIII shows that removal of a second charge in addition to K33, viz., at K49, K68, or R71, or two additional charged groups, in r-[$K2_{tPA}$/K33L,R70A,R71A], did not result in significant diminishment of the binding interactions, as compared to the r-K33L variant. In fact, in most cases binding was either essentially unchanged or slightly stronger. Additionally, the specificity of binding was maintained, in that 7-AHpA was the most tightly bound member of the ω-amino acids studied. Of interest, altered specificity in binding between 6-AHxA and 7-AHpA is seen with r-[$K2_{tPA}$/K33L,H65Y], as was the case with the single r-H65Y

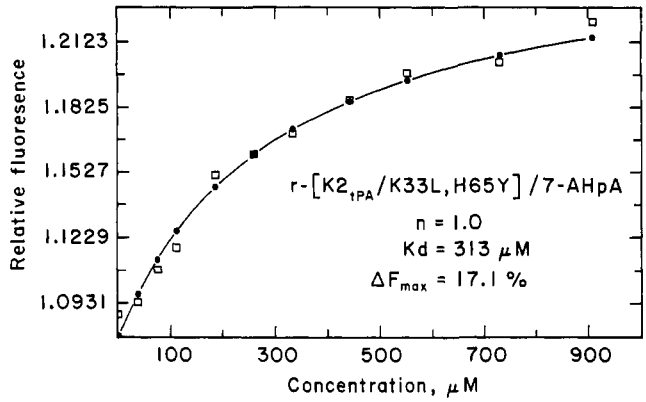

FIGURE 5: Titration of the relative change in intrinsic fluorescence of r-[$K2_{tPA}$/K33L,H65Y] (6.22 μM) with 7-AHpA. The experimental points (*q*) are best fit to a line (|) generated by employing values of $n = 1.0$, $K_d = 313$ μM, and a maximal relative fluorescence change (ΔF_{max}) of 17.1%. The buffer was 50 mM TrisOAc/150 mM NaOAc, pH 8.0, at 25 °C. The excitation and emission wavelengths were 285 and 340 nm, respectively.

Table VIII: Dissociation Constants for ω-Amino Acids to r-[$K2_{tPA}$/K33L]-Derived Variants

variant	dissociation constant (μM)			
	6-AHxA	7-AHpA	L-Lys	AMCHA
wt[a]	43	6	85	27
K33L[b]	151	13	148	178
K33L,K49A[b]	87	8	149	192
K33L,H65Y[b]	130	293	127	nd[c]
K33L,K68A[b]	137	12	107	222
K33L,R71A[b]	104	12	85	148
K33L,R70A,R71A[b]	107	22	90	200

[a] Refers to the wild-type recombinant molecule. [b] The mutation made in wtr-SD[$K2_{tPA}$]S: the amino acid in the wt molecule and its sequence position beginning from C1 of the kringle, followed by the amino acid replacement at that position; this continues for other amino acid replacements made in the $K2_{tPA}$ sequence. [c] Not determined (nd) due to inadequate fluorescence changes.

mutation (Table III). Thus, replacement of the cationic binding center in r-[$K2_{tPA}$] with the noncharged and hydrophobic L residue accommodates binding of ω-amino acids, particularly that of 7-AHpA, in a more selective fashion.

DISCUSSION

The goal of this study was to thoroughly define the nature of the cationic center of the isolated r-[$K2_{tPA}$] domain that complements the amino group of the important class of ω-amino acid ligands. On the basis of molecular modeling of [$K2_{tPA}$] from reference crystal structures of the kringle 1 domain of prothrombin (Tulinsky et al., 1988b) and the [$K4_{HPg}$]/6-AHxA complex (Wu et al., 1991), and upon analysis of a small set of r-[$K2_{tPA}$] variants, we proposed that K33 was the primary candidate for the binding locus of the carboxylate group of the ligand. In the current investigation, we have mutated to alanyl residues all basic amino acid residues of r-[$K2_{tPA}$] and showed that the strength of binding of 6-AHxA is decreased only in the case of the K33A mutation. While we were not successful in expressing a r-K61 mutant, the X-ray crystal structure shown in Figure 2 does not implicate this residue in directly stabilizing binding of ω-amino acids. However, since substitutions at this position did not appear to be tolerated, this residue may be important in the stability of the r-[$K2_{tPA}$] structure.

In other ω-amino acid binding kringles, positively charged residues are present in locations analogous to K33 in [$K2_{tPA}$], e.g., R34 in [$K1_{HPg}$], R32 and K35 in [$K4_{HPg}$], and R32 in [$K5_{HPg}$]. Chemical modification studies (Vali & Patthy, 1984) have implicated R34 of [$K1_{HPg}$] as an important contributor to the binding site of ω-amino acids (Vali & Patthy, 1984). In [$K4_{HPg}$], chemical modification investigations have suggested that R69 plays a role in ω-amino acid binding (Trexler et al., 1982), and examination of the crystal structure of this domain implicates R69, as well as K35, as potentially important residues in interaction with the carboxylate group of the ligand. Regarding [$K2_{tPA}$], it is clear from the data of Table III, and the structure of Figure 2, that while the equivalent R71 is present in [$K2_{tPA}$], it does not serve as a binding locus for the ligand. This function is exclusively reserved for K33. The conclusions from these mutagenesis experiments are consistent with the X-ray crystal structure of the domain (de Vos et al., 1991).

The binding data of Tables III and V show that while K33 is the only positively charged locus for direct binding energy stabilization to all ligands tested, neutral hydrophobic amino acids at this site can function with certain of these ligands. The most important example of this is the interaction of r-[$K2_{tPA}$/K33L] with 7-AHpA. While this kringle mutant shows an approximate 4-fold decrease in its ability to interact with 6-AHxA, it binds to 7-AHpA with only a 2-fold increase in K_d as compared to wtr-[$K2_{tPA}$]. The ability of the r-K33L mutant to interact effectively with 7-AHpA probably resides in the increased stabilization of the complex due to hydrophobic interactions of the leucyl side chain with the more hydrophobic ligand 7-AHpA. When the hydrophobicity of the residue at position 33 is reduced, as is the case with r-[$K2_{tPA}$/K33A], the binding to 7-AHpA is reduced by approximately 3-fold, as compared to the r-K33L mutant, and when a residue of F is placed at this position, the K_d for interaction of this latter mutant with 7-AHpA is approximately the same as for the r-K33L variant. Placement of a noncharged polar residue, i.e., Q, at position 33, reduces binding strength of 7-AHpA by >10-fold, and placing a negatively charged residue, i.e., E, at position 33 results in a 90-fold reduction in binding strength for this ligand.

The data of Table V suggested that residue 33 might be a location useful for attempts at redesign of the substrate binding pocket of [$K2_{tPA}$] by substitution of other amino acids at this sequence position. We reasoned that this hypothesis could be more dramatically tested since the substitution of E for K at sequence position 33 raised the possibility that a ligand with two positively charged substituents, separated by appropriate distances by methylene groups, might effectively bind to this mutant if the E33 residue was properly situated in an otherwise intact binding pocket. Alternatively, substitution of a Q for K at sequence position 33 might also stabilize binding of appropriate diamino-derived ligands as a result of hydrogen bonding between one of the amino groups of the ligand and the carbonyl moiety of the side-chain amide of the Q residue. Thus, we examined the binding of 1,6-diaminohexane to a series of r-K33 variants. As seen from Table VI, this potential ligand interacted very poorly with wtr-[$K2_{tPA}$], presumably due to the charge repulsion of K33 with one of the amino groups of the ligand. Replacing K33 with nonpolar groups, i.e., A and F, improved the binding by approximately 2-fold and 6-fold, respectively, most likely due to stabilization of the methylene backbone of the ligand with the hydrophobic residues of the binding pocket and the lack of destabilizing charge repulsions. Binding of this ligand was also enhanced 6-fold over wtr-[$K2_{tPA}$] by placing a neutral polar amino acid (Q) at sequence position 33, most likely due to stabilization by a combination of hydrophobic effects and head-group

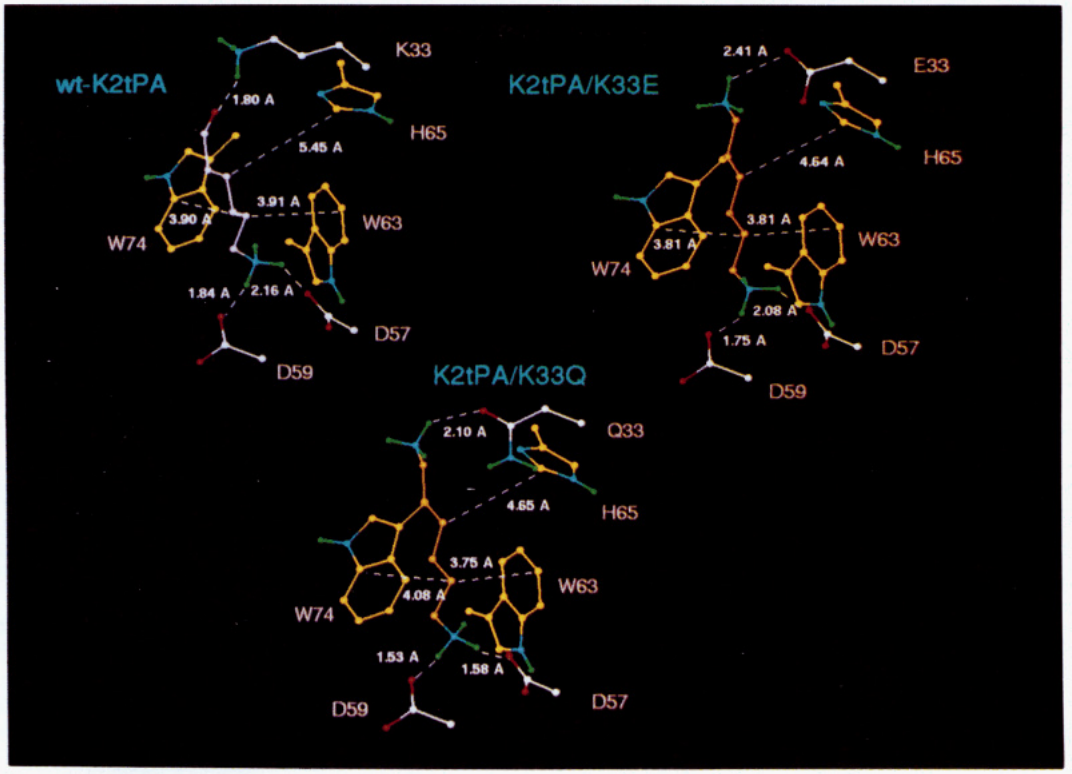

FIGURE 6: Steric relationships of binding site residues and ω-amino acid ligands in r-[$K2_{tPA}$] and K33-derived mutants. A portion of the X-ray crystal structure for the wtr-[$K2_{tPA}$]/lysine complex, consisting of K33 (white carbon backbone), D57 (white carbon backbone), D59 (white carbon backbone), H65 (yellow carbon backbone), W63 (yellow carbon backbone), and W74 (yellow carbon backbone), is shown along with the lysine side chain (mauve carbon backbone) from K49 of another r-[$K2_{tPA}$] molecule in the unit cell bound within the putative binding site (see Figure 2). Also illustrated are molecular models of r-[$K2_{tPA}$/K33E]/1,6-diaminohexane (orange carbon backbone) and r-[$K2_{tPA}$]/K33Q]/1,6-diaminohexane. All molecules are oriented in the same manner by superimposition of all atoms of W74. Oxygen atoms are in red, nitrogen atoms in blue, and exchangeable hydrogen atoms in green. All residues are displayed beginning with their β-carbon atoms.

hydrogen bonding. Although the absolute value of the K_d value for binding of 1,6-diaminohexane to r-[$K2_{tPA}$/K33Q] is not much different than the K_d value for this same ligand to the r-K33F variant, it is emphasized that binding of 1,6-diaminohexane to r-[$K2_{tPA}$]/K33Q] shows nearly a 5-fold stronger interaction than that of EACA to this variant, whereas the K_d for 1,6-diaminohexane to r-[$K2_{tPA}$/K33F] is not reduced relative to the K_d for EACA toward r-[$K2_{tPA}$/K33F]. This strongly suggests that the r-K33Q variant has indeed shown selectively enhanced binding to 1,6-diaminohexane, demonstrating that an additional design feature could be incorporated into the binding site. Finally, and more dramatically, the binding of 1,6-diaminohexane was enhanced over 2 orders of magnitude as compared to its interaction with wtr-[$K2_{tPA}$] as a result of placement of E at sequence position 33, an interaction even stronger than the binding of EACA to wtr-[$K2_{tPA}$].

In an extension of this finding, we employed molecular modeling technology to examine the hypothesis that the newly inserted E and Q residues at amino acid sequence position 33 of [$K2_{tPA}$] were in a favorable position to interact with one of the amino groups of the ligand, 1,6-diaminohexane. A comparison of important binding site residues and their steric relationships to the relevant ligands is provided in Figure 6. Here, the X-ray crystal structure for the r-[$K2_{tPA}$]/lysine complex, and for molecular models of r-[$K2_{tPA}$/K33Q]/1,6-diaminohexane and r-[$K2_{tPA}$/K33E]/1,6-diaminohexane complexes, is illustrated. Examination of the models reveals that one NH_2 group from 1,6-diaminohexane is easily within a distance favorable for an ion-pair interaction with the side-chain carboxyl group of the 33E residue in r-[$K2_{tPA}$/K33E] and for H-bond formation with the side-chain carbonyl group of the 33Q residue in r-[$K2_{tPA}$/K33Q]. Thus, the modeling experiments strongly support the results from mutagenesis.

Another amino acid residue that influences ligand binding specificity is H65 (Tulinsky et al., 1988a), which, as seen in Figure 2, is situated in the putative binding pocket. A previous investigation (Kelley & Cleary, 1989) of the function of this residue has shown that the H-residue at this location serves to define a specificity for binding of 7-AHpA with greater strength than that of 6-AHxA. We confirm this finding by demonstration that alteration of this residue to Y leads to a greater binding energy of 6-AHxA as compared to 7-AHpA (Table III). Of interest, a Y residue is present in that same location in wt-[$K1_{HPg}$], which also binds 6-AHxA with greater affinity than 7-AHpA (Menhart et al., 1991), and an F residue is present in wt-[$K4_{HPg}$], which also shows this latter binding specificity (Sehl & Castellino, 1990). Thus, while the X-ray crystal structure shown in Figure 2 does not suggest that H65–substrate distances are important to direct interaction of H65 with the ligand, clearly this residue influences the specificity of binding, perhaps by directing local conformations, and/or the geometry of the binding pocket.

From these examples we have demonstrated that the substrate binding pocket could be redesigned to accept different ligands or classes of ligands. The results allow a rational means for further design of this binding pocket to interact with other ligands which may prove important for revelation of the role of the K2 domain in intact tPA in structure–function relationships of this protein.

In conclusion, we have shown that K33 is the sole cationic center of r-[$K2_{tPA}$] that stabilizes interaction with ω-amino acids but that such stabilization is minimally required for certain more hydrophobic members of this group of ligands.

In these cases, hydrophobic amino acid residues at this location provide nearly equivalent binding stabilization. While H65 does not likely interact with ligands of this type, a histidyl residue at this location serves to contribute to specificity of ligand binding. Further, different amino acids placed at sequence position 33 mediate binding of other ligand classes to this kringle domain. In addition to assisting our understanding of the structural features of [$K2_{tPA}$] that direct ligand binding, the knowledge gained from this study, when applied to the intact tPA molecule, will be very useful in evaluating the importance of this particular macroscopic binding site toward tPA functional properties that rely on sites common to those that bind ω-amino acids.

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