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Purification and Characterization of Tissue Plasminogen Activator Kringle-2 Domain Expressed in *Escherichia coli*

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ABSTRACT: We have expressed the 174–263 fragment (kringle-2 domain) of human tissue-type plasminogen activator (t-PA) in *Escherichia coli* by secretion into the periplasmic space using the alkaline phosphatase promoter and stII enterotoxin signal sequence. A large portion of the secreted protein is associated with an insoluble cellular fraction. This material can be solubilized by extraction with denaturant and reducing agent and then recovered in active form by refolding in the presence of reduced and oxidized glutathione. Kringle-2 is then easily purified by affinity chromatography on lysine-Sepharose followed by cation-exchange chromatography. The isolated protein has an amino acid composition and N-terminal sequence as expected for the 174–263 fragment of t-PA, indicating that the signal peptide has been properly removed. Circular dichroic spectra suggest that the protein is folded similar to the kringle-4 domain of plasminogen [Castellino et al. (1986) *Arch. Biochem. Biophys. 247*, 312–320]. Equilibrium dialysis experiments indicate a single binding site on kringle-2 for L-lysine having a K_D of 100 μM. Using a method based on elution of kringle from lysine-Separose with ω-aminocarboxylic acids [Winn et al. (1980) *Eur. J. Biochem. 104*, 579–586], we have shown the lysine binding site of t-PA kringle-2 to have a preference for a ligand with 8.8-Å separation between amine and carboxylate functions. Charge interactions with the ϵ-amino group of L-lysine are important in binding since the affinities for N^{ϵ}-acetyl-L-lysine, L-arginine, and γ-guanidinobutyric acid are decreased >2000-fold, 200-fold, and 12-fold, respectively, relative to the affinity for L-lysine. Modification of the ligand α-carboxylate by methylation or peptide bond formation produces only 3- and 7-fold decreases in affinity, respectively, suggesting a less significant role in ligand binding of electrostatic interaction with the ligand α-carboxylate.

Kringles are small (ca. 80 residues) protein domains which have a characteristic three disulfide bonded structure that was first observed in prothrombin (Magnusson et al., 1975). On the basis of sequence homology to prothrombin, kringles were proposed to occur singly in urokinase (Steffens et al., 1982) and factor XII (McMullen & Fujikawa, 1985), twice in tissue-type plasminogen activator (t-PA)[1] (Pennica et al., 1983), and 5 times in plasminogen (Sottrup-Jensen et al., 1978). These domains appear to be independent folding units (Castellino et al., 1981; Trexler & Patthy, 1983; Novokhatny et al., 1984), but a general functional role for kringles is not known. The plasminogen kringles have been extensively studied and serve as prototypes of this domain. One or more of the kringle domains of plasminogen (Thorsen, 1975; Thorsen et al., 1981) are involved in the binding of this protein to fibrin. Plasminogen kringles-1 and -4 have a binding site for L-lysine with kringle-1 having the greater affinity (Vâli & Patthy, 1982; Lerch et al., 1980). Affinity chromatography on immobilized lysine is often employed in the purification of plasminogen (Deutsch & Mertz, 1970). Kringle domains are presumed to interact with fibrin by binding to an exposed lysine side chain. The role of the lysine binding site in fibrin binding is supported by the observation that analogues of lysine such as 6-aminohexanoic acid are potent antifibrinolytic agents (Okamoto et al., 1959).

Human t-PA is thought to be a multidomain protein having a domain homologous to fibronectin type I "finger", an epidermal growth factor-like domain, two kringle domains, and a C-terminal proteolytic domain homologous to the trypsin family of serine proteases (Pennica et al., 1983). Studies of t-PA domain deletion mutants suggest that the kringle-2 domain has a lysine binding site (van Zonneveld et al., 1986a). Both the kringle-2 and the fibronectin type I domain appear to be required for high-affinity binding of t-PA to fibrin (van Zonneveld et al., 1986b; Verheijen et al., 1986). In order to characterize the lysine/fibrin binding site of t-PA kringle-2, we have used recombinant DNA technology to express the

[1] Abbreviations: t-PA, tissue-type plasminogen activator; Gdn-HCl, guanidine hydrochloride; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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isolated domain in *Escherichia coli*. We report here the purification and characterization of the kringle-2 domain expressed by secretion in *E. coli*. Ligand binding experiments suggest a specificity for t-PA kringle-2 that is different from that of plasminogen kringles-1 and -4 (Markus et al., 1978; Lerch et al., 1980; Winn et al., 1980).

Materials and Methods

Materials. Lysine-Sepharose 4B, an FPLC Mono S cation-exchange column, Klenow fragment of DNA polymerase, T4 DNA ligase, polynucleotide kinase, and deoxy- and dideoxynucleotides were purchased from Pharmacia. All restriction enzymes were obtained from New England Biolabs and were used according to manufacturer's instructions. Low melting temperature agarose was purchased from BRL. Bio-Gel P-10 gel filtration media were from Bio-Rad. L-[U-^{14}C]Lysine monohydrochloride (342 mCi/mmol) was purchased from Amersham. Polypropylene equilibrium dialysis cells having a volume of 0.2 mL for each compartment were obtained from Bolabs (Lake Havasau, AZ). SpectraPor/3 dialysis tubing (MWCO 3500) was purchased from Spectrum. All lysine analogues except *trans*-4-(aminomethyl)cyclohexanecarboxylic acid, which was purchased from Aldrich Chemical Co., were obtained from Sigma Chemical Co. Stock solutions were prepared based on dry weight and used without further purification. The dipeptides L-lysylglycine and glycyl-L-lysine, reduced and oxidized glutathione, dithiothreitol, and guanidine hydrochloride were also purchased from Sigma.

Bacterial Strains and Plasmids. E. coli strain 294 (*endA1 thi-1 hsdR* F$^-$ *supE44*; ATCC 31446) was used for routine transformations and plasmid preparations. *E. coli* strain JM101 (Messing, 1979) was used for propagation of M13-derived vectors. *E. coli* strain 16C9 was used for expression of kringle-2 under control of the *phoA* promoter. The plasmid phGH4R and a cDNA of human t-PA were from Genentech. Plasmid pUC119, a pUC19 derivative having an f1 origin of replication, was supplied by J. Messing (University of Minnesota).

DNA Manipulations. Replicative form DNA and plasmid DNA were isolated according to the procedure of Birnboim and Doly (1979). DNA fragments produced by restriction enzyme cleavage were prepared for cloning by electrophoresis in 1.0% low melting agarose gels, visualized by ethidium bromide staining, and recovered from the gel as described by Wieslander (1979). Oligonucleotide-directed mutagenesis was performed essentially as described by Smith and Gillam (1981). DNA sequences were determined by using the dideoxynucleotide chain termination method (Sanger et al., 1977).

Purification of Kringle-2. The expression plasmid pSB-5 was constructed as outlined in Figure 1 and used to transform *E. coli* strain 16C9 to ampicillin resistance. The transformed strain was grown at 37 °C in 10 L of low-phosphate media (Chang et al., 1987) in a fermentor. Cells were harvested at 36 h after inoculation and stored at −20 °C as a cell paste. A typical purification started from 200 g wet weight cell paste and is outlined in Figure 2. The cell paste was thawed and suspended in 1 L of ice-cold homogenization buffer (50 mM Tris-HCl, pH 8, containing 0.5 g/L lysozyme and 0.01 g/L each deoxyribonuclease and ribonuclease A) with the aid of a Tekmar Ultra-turrax. The cells were broken by sonication, the suspension was stirred on ice for an hour, and the solution was centrifuged at 12000 rpm for 1 h in a Sorvall GSA rotor. Both the pellet and supernatant fractions were saved for further processing. The pellet was suspended in 100 mL of 6 M guanidine hydrochloride containing 50 mM Tris-HCl, pH 8, and 5 mM dithiothreitol and stirred overnight at room temperature. This solution was then diluted with 4 volumes of 50 mM Tris-HCl, pH 8, containing 1.25 mM each reduced and oxidized glutathione, incubated at ambient temperature with stirring for 2 h, and then centrifuged at 12000 rpm in the GSA rotor for 20 min. This second soluble fraction was combined with the first supernatant and concentrated by ammonium sulfate precipitation. The pellet obtained between 20 and 70% saturation with ammonium sulfate was resuspended in a minimal volume of 50 mM Tris-HCl, pH 8, dialyzed versus 40 volumes of this solution, and loaded on a 2.5 × 20 cm column of lysine-Sepharose equilibrated with this buffer. After being loaded, the column was washed with buffer containing 0.5 M NaCl until the effluent had an absorbance at 280 nm of less than 0.2 (about 3 column volumes). Kringle-2 was then eluted with buffer containing 0.2 M 6-aminohexanoic acid. Kringle-2-containing fractions, determined by SDS–PAGE, were pooled, dialyzed versus 50 mM sodium acetate, pH 5, and chromatographed on a Pharmacia FPLC Mono S cation-exchange column. Protein was eluted with a linear gradient formed from 0 and 0.25 M NaCl. Kringle-2 eluted at about 0.15 M NaCl and was dialyzed versus 50 mM ammonium bicarbonate and lyophilized.

Composition and Sequence Analysis of Purified Kringle-2. Protein samples were prepared for amino acid analysis by extensive dialysis versus distilled water. The absorbance at 280 nm was recorded, and a portion was hydrolyzed at 110 °C for 20 h in 6 N HCl containing 5% (v/v) phenol under vacuum after the sample was purged with argon. The N-terminal sequence was determined by automated Edman degradation of a protein sample that had been reduced and the cysteines alkylated. Kringle-2 was reduced by incubation with 10 mM dithiothreitol at 45 °C for 2 h followed by alkylation with 50 mM iodoacetamide at 25 °C overnight in the dark. The free thiol content of the native protein was measured by adding 0.2 mL of a 5 mg/mL solution of kringle-2 to 0.8 mL of 6 M guanidine hydrochloride containing 50 mM Tris-HCl, pH 8, and 2 mM DTNB. This mixture was incubated at 37 °C for 10 min and then the reduction of DTNB quantitated by absorbance measurements at 412 nm.

Circular Dichroic Spectroscopy. Circular dichroic spectra were recorded on a Aviv Cary 60 spectropolarimeter using a time constant of 1 s. Jacketed, cylindrical cells were used, and the temperature was maintained at 25 °C using a circulating water bath.

Equilibrium Dialysis. For lysine binding determination, stock solutions of L-lysine of varied concentration were prepared in 50 mM Tris–acetate, pH 8, and 50 mM NaOAc. Each stock solution contained L-[^{14}C]lysine at a final radiochemical concentration of 0.2 μCi/mL. Disks of 1-cm diameter were cut from wet Spectrapor/3 (MWCO 3500) dialysis membrane and mounted in the dialysis cell. The lysine solution was added to one of the dialysis cell compartments, and a solution of 70 μM kringle-2 was added to the other compartment. After overnight equilibration at ambient temperature (ca. 23 °C), the lysine concentration on each side of the dialysis cell was determined by using scintillation counting. Final protein concentration in each compartment was determined by UV absorbance measurements using an ϵ_{280} of 28 mM^{-1} cm^{-1}. In all cases, less than 5% of the total protein passed through the membrane. This passage was ignored in the calculation of the molar binding ratio. All measurements of inhibition of lysine binding by analogues were performed as described above except for the following modifications. A stock solution of 100 μM L-lysine having a specific activity

of 2.0 mCi/mmol was prepared and added to one side of the dialysis cell in each trial. To the other half of the dialysis cell was added a solution containing 70 μM kringle-2 and a varied concentration of an inhibitor.

Lysine-Sepharose Elution Experiments. The method of Winn et al. (1980) was used with the following modifications. A batch of lysine-Sepharose having 1 mg of bound kringle-2 per milliliter of lysine-Sepharose was prepared by mixing equal volumes of lysine-Sepharose and a 2 mg/mL solution of kringle-2 and incubating overnight at 4 °C. For each ligand tested, a complete elution profile was determined by using a single 0.4-mL aliquot of kringle-2/lysine-Sepharose. The final volume of this aliquot was adjusted to 1.4 mL with 50 mM Tris-HCl, pH 8, an aliquot of a concentrated solution of ligand was added, the mixture was incubated at 4 °C for 1 h and then briefly centrifuged, and the amount of eluted protein was determined by absorbance measurements on the supernatant. This cycle of ligand addition and A_{280} measurements was repeated until all of the bound protein was eluted or the solubility limit of the ligand was reached.

Results

Expression of Kringle-2 in E. coli. Since *E. coli* produces very few proteins containing disulfide bonds, and the high intracellular concentrations of reduced glutathione favor disulfide reduction, we decided to express kringle-2 by secretion into the periplasmic space where the redox potential for disulfide bond formation should be more favorable. This approach has been successful for expression of bovine pancreatic trypsin inhibitor in *E. coli* (Marks et al., 1986). The plasmid pSB-5 was constructed as shown in Figure 1 in order to secrete the kringle-2 domain using the alkaline phosphatase promoter and the stII heat-stable enterotoxin signal sequence. In this expression plasmid, the codon for cysteine-264 of t-PA, which is presumed to form a disulfide bond with a cysteine from the protease domain, was changed to a stop signal. The stII signal sequence was joined to the t-PA coding segment so that serine-174 of t-PA occupies the P_1' position of the stII signal sequence cleavage site. Thus, the t-PA fragment targeted for secretion includes the entire kringle-2 domain, six residues preceding the first cysteine of kringle-2, and two residues past the last cysteine of kringle-2. For expression of kringle-2, pSB-5 was transformed into strain 16C9. Western analysis using a polyclonal antibody raised against reduced, alkylated t-PA suggested that kringle-2 was expressed when the transformed strain was grown in low-phosphate media.

For purification of milligram quantities of kringle-2, 16C9.pSB-5 was grown in 10 L of low-phosphate media in a fermentor. A small portion of cell paste from the fermentation was used in experiments to identify the subcellular localization of kringle-2. The cell paste was subjected to freeze–thawing in low ionic strength buffer to obtain an osmotic shock extract. A second soluble fraction was obtained by sonication of the shocked paste. Finally, a third soluble extract was prepared from the cell debris fraction by using the denaturant extraction and refolding procedure described under Materials and Methods. An ELISA based on polyclonal antibody against native t-PA was used to detect kringle-2 in the three fractions. Although we found this assay to be unsatisfactory for quantitation of kringle-2 in crude extracts, it was useful for qualitative assay. Significant amounts of kringle-2 were detected in all three fractions, with the third extract containing about 60% of the total kringle-2 detected by ELISA. Both denaturant and reducing agent were required for solubilization of the cell debris associated kringle-2. Kringle-2 in each of the three extracts was retained on a column of lysine-Sepharose

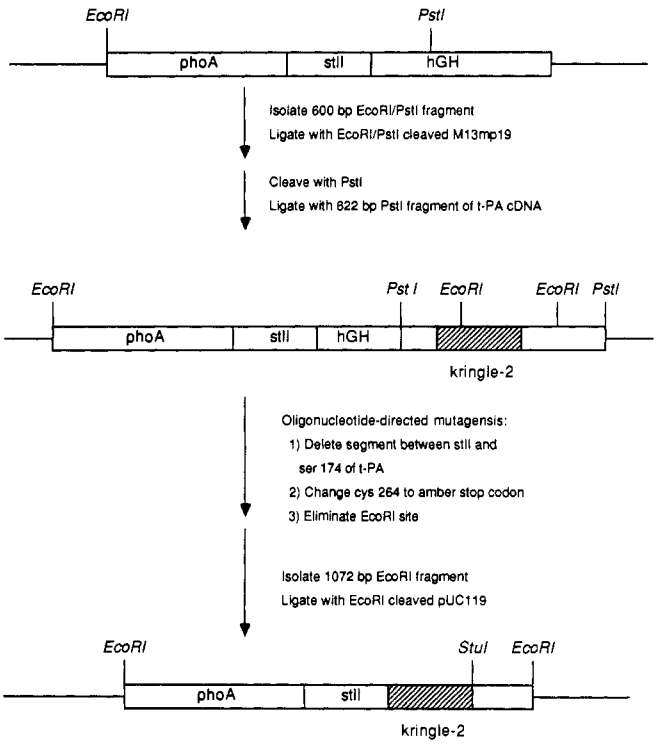

FIGURE 1: Outline of construction of kringle-2 expression plasmid pSB-5. First, a 600 bp *Eco*RI–*Pst*I fragment from the plasmid phGH4R (Chang et al., 1987) carrying the *E. coli* alkaline phosphatase promoter (phoA), the *E. coli* heat-stable enterotoxin II signal sequence (stII), and a portion of the human growth hormone gene (hGH) was cloned into *Eco*RI–*Pst*I-cut M13mp19. Then a 622 bp *Pst*I fragment from a digest of the t-PA cDNA was ligated into the *Pst*I site of this vector. The codons for amino acids 174–264 of t-PA are denoted by the hatched segment. A series of three oligonucleotide-directed mutagenesis steps was performed. In the first step, a deletion was made so that the stII signal sequence was joined to the t-PA segment at serine-174. The codon for cysteine-264 of t-PA was then changed to an amber stop codon which creates the *Stu*I site. Finally, a silent point mutation was introduced in the kringle-2 coding segment in order to remove an *Eco*RI site. The 1072 bp *Eco*RI fragment was ligated into the *Eco*RI site of pUC119 to yield the plasmid pSB-5. The orientation of the *Eco*RI fragment in pSB-5 is such that transcription of the *lacZ* gene is opposite to the direction of transcription from phoA.

and required millimolar concentrations of 6-aminohexanoic acid for elution.

Purification of Kringle-2. Using the protocol outlined in Figure 2, about 50–80 mg of kringle-2 could be purified from a homogenate of 200 g of cell paste. The primary tool in this purification is affinity chromatography on lysine-Sepharose. SDS–PAGE indicated that the affinity step affects a large purification of kringle-2 with the eluted material having a purity of >90% (data not shown). The t-PA ELISA suggests that between 60 and 80% of the kringle-2 applied to the lysine-Sepharose column is recovered upon elution with 6-aminohexanoic acid. However, two peaks are observed upon chromatography of this fraction on a Mono S cation-exchange column run at pH 5 as shown in Figure 3. Both components react with the antibody against native t-PA. The elution positions of the two components are maintained upon rechromatography and are not affected by addition of 6-aminohexanoic acid, indicating that the two forms are not in equilibrium. Both species have an apparent molecular weight of 10 000 by SDS–PAGE and also have similar amino acid compositions and N-terminal sequences. These data demonstrate that the two forms are not related by a difference in proteolytic processing but we have not as yet identified the difference between the two species. Given the difference in

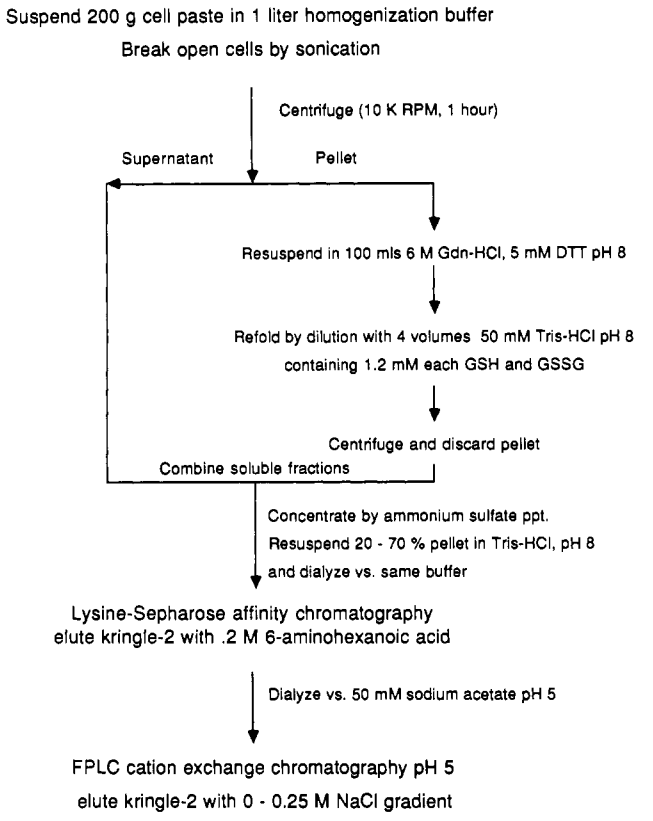

FIGURE 2: Flow chart for purification of kringle-2.

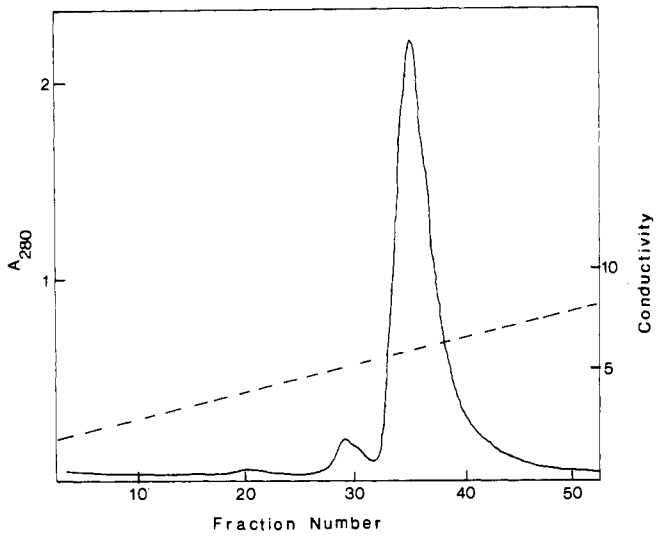

FIGURE 3: Fractionation of the lysine-Sepharose pool by FPLC Mono S cation-exchange chromatography. Protein eluted from the lysine-Sepharose column with 6-aminohexanoic acid was dialyzed against 50 mM sodium acetate, pH 5, and a portion of this solution was loaded onto a FPLC Mono S cation-exchange column equilibrated with the same buffer. A linear gradient formed from 0 and 0.25 M NaCl was used to elute the bound protein, and fractions of 2 mL were collected. The absorbance at 280 nm is shown as the solid line, and conductivity is shown as the dashed line. Fractions 32–40 were pooled and used in further experiments.

ion-exchange properties, the minor peak could represent a form of kringle-2 containing a mixed disulfide or a deamidated residue. Since the later-eluting component is the most abundant form, we chose to perform further experiments using this fraction. This peak usually accounts for about 80% of the A_{280} units loaded onto the column. Thus, the overall recovery of kringle-2 from the two column chromatography steps is about 50–70%. Since the typical yield of a purification is 50–80 mg, these data suggest that kringle-2 is expressed at a level of at least 0.4–0.8 mg/g of cell paste.

Table I: Amino Acid Composition of Purified Kringle-2 Domain[a]

amino acid	residues expected	residues observed
Asx	11	11.73
Thr	5	4.75
Ser	10	8.82
Glx	5	5.17
Pro	5	5.34
Gly	9	9.27
Ala	6	6.23
Cys	6	4.54
Val	3	3.00
Met	1	0.96
Ile	2	1.93
Leu	7	7.27
Tyr	5	4.99
Phe	1	1.00
His	3	2.97
Lys	4	4.03
Arg	4	4.12

[a] A sample of purified kringle-2 was prepared for amino acid analysis by hydrolysis in 6 N HCl containing 5% (v/v) phenol. The sample was purged with argon and hydrolyzed at 110 °C for 20 h under vacuum.

Characterization of Purified Kringle-2. As shown by the data of Table I, the amino acid composition observed for kringle-2 purified from *E. coli* is identical with that expected for the 174–263 fragment of human t-PA. (Serine and cystine are somewhat unstable to the acid hydrolysis procedure used and thus are not quantitatively recovered.) The ultraviolet absorption spectrum of kringle-2 (data not shown) has a peak centered at 280 nm with a shoulder at 290 nm as expected for a protein having both tryptophan and tyrosine. An extinction coefficient at 280 nm of 2.8×10^4 M^{-1} cm^{-1} was calculated for kringle-2 based on quantitative analysis of the composition data and was used throughout these studes to determine protein concentration. The magnitude of the extinction coefficient compares favorably with an ϵ_{280} of 3.1×10^4 M^{-1} cm^{-1} determined for the kringle-4 domain of human plasminogen (Vâli & Patthy, 1982), which has a similar number of aromatic chromophores. Kringle-2 was isolated with all cysteine residues in disulfide bonds as judged by the lack of reaction with DTNB in the presence of concentrated denaturant. A single peak was observed for chromatography of kringle-2 on a 1.5 × 50 cm Bio-Gel P-10 size exclusion column eluted with 50 mM Tris-HCl, pH 8. The peak had an elution volume intermediate between that observed for ribonuclease A (13 700 daltons) and bovine pancreatic trypsin inhibitor (6500 daltons), suggesting that kringle-2 isolated from *E. coli* is monomeric. Automated Edman degradation of a sample of this material after reduction and alkylation indicated the N-terminal sequence S-E-G-N-S-D-C-Y-F-G, as expected if the signal peptide had been properly removed.

Circular Dichroic Spectra of t-PA Kringle-2. Circular dichroic spectra of kringle-2 are shown in Figure 4. The CD spectra of t-PA kringle-2 and plasminogen kringle-4 (Castellino et al., 1986) are nearly identical except the near-UV CD spectrum reported for plasminogen kringle-4 lacks the shoulder at 295 nm observed for t-PA kringle-2. (Kringle-4 was prepared by elastase digestion of human plasminogen.) Kringle far-UV CD spectra are quite different from the spectra commonly observed for a variety of globular proteins (Hennessey & Johnson, 1981) in having positive ellipticity at 220 nm and a negative ellipticity peak around 200 nm. However, these spectra seem to be consistent with the structure reported for the prothrombin fragment 1 kringle domain determined

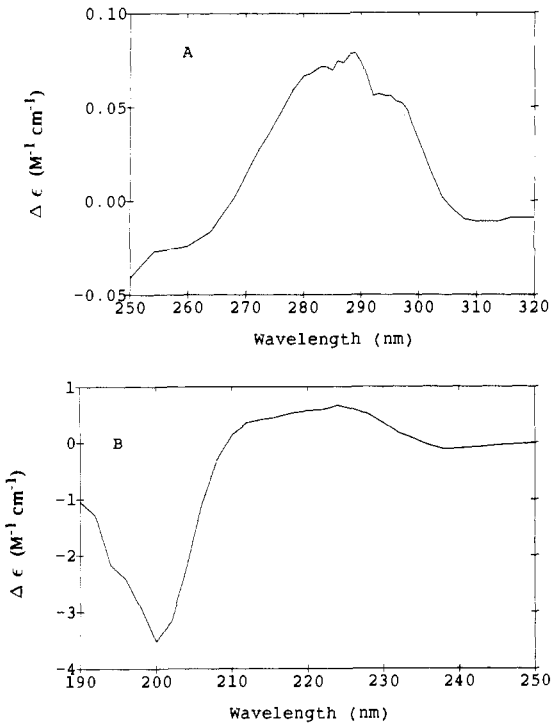

FIGURE 4: Circular dichroic spectra t-PA kringle-2. Spectra were obtained on solutions of kringle-2 containing 50 mM sodium phosphate, pH 7.5, at a temperature of 25 °C. The data shown are the average of 10 spectra collected using a 1.0-cm pathlength cell, a spectral bandwidth of 1.0 nm, and a wavelength interval of 0.5 nm. A mean residue molecular weight of 110 was used in the calculation of $\Delta\epsilon$. (A) Near-ultraviolet CD spectrum of 36 μM kringle-2. (B) Far-ultraviolet CD spectrum observed for a 3.6 μM solution of kringle-2.

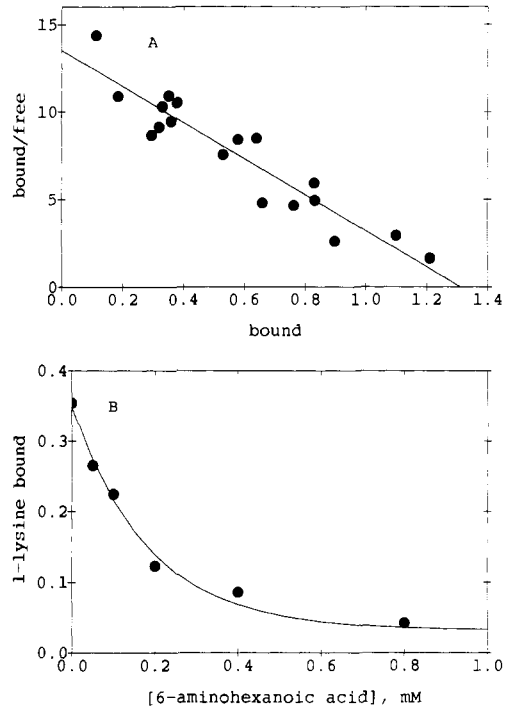

FIGURE 5: Kringle-2–lysine binding. (A) Scatchard plot of data for lysine binding to kringle-2 obtained from equilibrium dialysis experiments. Filled circles are data points for lysine binding to 70 μM kringle-2 in solutions containing 50 mM Tris–acetate, pH 8, and 50 mM sodium acetate at ambient temperature (ca. 23 °C). The solid line is the result of a least-squares analysis of the data. (B) Inhibition of lysine binding to kringle-2 by the lysine analogue 6-aminohexanoic acid determined by using equilibrium dialysis. Filled circles represent the molar binding ratios for L-lysine in the presence of the indicated concentration of 6-aminohexanoic acid. The solid line is the result of a nonlinear least-squares analysis of the data.

by Park and Tulinsky (1986) from X-ray crystallographic measurements. The prothrombin kringle has no α-helix, a small amount of β-sheet, and a high turn content. In addition, a CD spectrum recorded for reduced, alkylated t-PA kringle-2 is missing all of the ellipticity peaks observed for the native protein except for the negative peak at 200 nm (data not shown). This result suggests that the disulfide bonds are necessary for stable folding of t-PA kringle-2. Similar folding of plasminogen kringle-4 and t-PA kringle-2 is also indicated by proton NMR spectroscopy (R. Kelley, I.-J. Byeon, and M. Llinâs, unpublished results). The proton NMR spectrum of t-PA kringle-2 is very similar to the spectra observed for both bovine and human plasminogen kringle-4 in the connectivity pattern of the aromatic resonances and also the upfield shift observed for the Leu-46 methyl resonance, a fingerprint for the kringle fold.

Specificity of Lysine Binding Pocket of Kringle-2. The affinity of kringle-2 for L-lysine was quantitated by using equilibrium dialysis (Myer & Schellman, 1962). Binding of L-lysine at ambient temperature determined by using solutions of 70 μM kringle-2 containing 50 mM Tris–acetate, pH 8, and 50 mM sodium acetate is shown in a Scatchard plot in Figure 5A. As shown by least-squares analysis, these data are adequately described by a single lysine binding site (n = 1.3) on kringle-2 having a K_D of 100 μM. Preliminary equilibrium dialysis experiments with human t-PA suggest that this protein has a single binding site for L-lysine. The dissociation constant of the lysine binding site on t-PA is of similar magnitude to that measured for the isolated kringle-2 domain (R. Kelley, unpublished results).

The affinity of kringle-2 for ω-aminocarboxylic acids was measured by using a protocol adapted from the method described by Winn et al. (1980). In this method, protein is bound to lysine-Sepharose and the affinity of kringle for a lysine analogue tested by determining the concentration of analogue that elutes 50% of the bound protein (C_{50}). A typical elution profile obtained with 7-aminoheptanoic acid is shown in Figure 6A. This method is useful for determining relative ligand affinities and was used to estimate the size of the lysine binding pocket by measuring C_{50} values for a series of ω-aminocarboxylic acids differing in chain length. As displayed in Figure 6B, 7-aminoheptanoic acid, having an 8.8-Å separation between amine and carboxylate groups, is the most effective eluant for kringle-2. Ligands of shorter or longer chain length are poorer eluants. In contrast, the most effective eluant for plasminogen kringles-1 and -4 is *trans*-4-(aminomethyl)-cyclohexanecarboxylic acid (Winn et al., 1980), having a chain length of 6.8 Å. *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid gave an elution potency for t-PA kringle-2 that seemed inconsistent with the trend observed for increasing chain length. We believe this discrepancy results from comparing a cyclic ligand with a series of linear compounds. The lysine-Sepharose elution protocol was also used to determine the relative affinity of human t-PA for the ligands 6-aminohexanoic acid, 7-aminoheptanoic acid, and 8-aminooctanoic acid. These ligands gave C_{50} values of 1, 0.1, and 3 mM, respectively, for elution of t-PA from lysine-Sepharose. The chain length specificity of the kringle-2 domain appears to be the same in intact t-PA as it is in the isolated domain.

Ligand affinities were also determined by using a competition experiment in which lysine binding measured using equilibrium dialysis was inhibited by titration with an analogue. A typical inhibition profile, obtained by titration with 6-aminohexanoic acid, is shown in Figure 5B. The dissociation

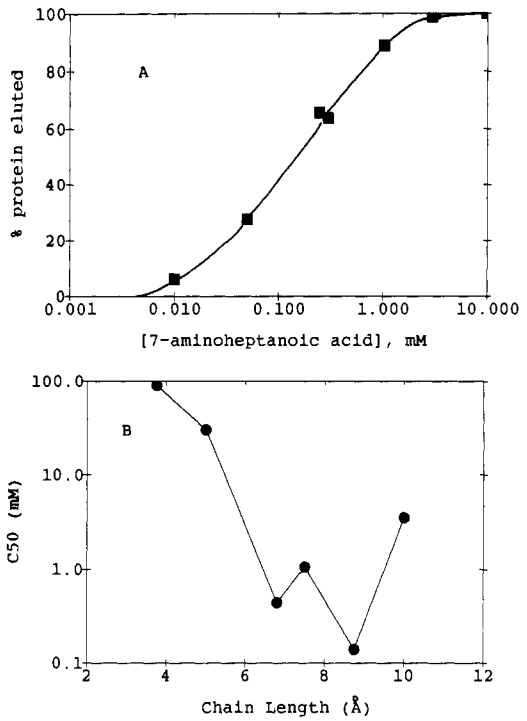

FIGURE 6: Elution of kringle-2 from lysine-Sepharose with ω-aminocarboxylic acids. (A) Profile observed for elution of kringle-2 from lysine-Sepharose with 7-aminoheptanoic acid. (B) Dependence of C_{50}, concentration of ligand required to elute 50% of the bound protein, on the chain length of the ω-aminocarboxylic acid used as eluant. The following ω-aminocarboxylic acids, in order of increasing chain length, were used to determine this dependence: 3-aminopropionic acid, 4-aminobutanoic acid, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid, 6-aminohexanoic acid, 7-aminoheptanoic acid, and 8-aminooctanoic acid. The chain lengths used for these ligands are those calculated by Winn et al. (1980).

constant for binding of the inhibitor, K_I, can be calculated from this profile by using the equation:

$$K_I = [I]_{50}/(1 + [L]/K_D)$$

where $[I]_{50}$ is the free analogue concentration that gives 50% inhibition, [L] is the free L-lysine concentration, and K_D is the dissociation constant for L-lysine binding. K_I values determined for several lysine analogues are given in Table II and are compared with C_{50} values where possible. The two methods for measuring ligand affinity give the same relative ranking of the various ligands for interaction with the lysine binding site of kringle-2; however, the lysine-Sepharose elution method tends to exaggerate differences between ligands.

The charge interactions required for lysine binding were examined by determining the affinity of kringle-2 for lysine derivatives in which the α-amino, α-carboxylate, or ε-amino group was modified. These affinities were determined by competition assay with L-lysine using equilibrium dialysis. As shown in Table II, interaction with the positively charged ε-amino group of lysine is essential for binding since acetylation of this group results in a greater than 2000-fold increase in K_I. Replacement of the ε-amino group with a guanidinium moiety also causes a decrease in affinity. For example, the K_I values for 6-aminohexanoic acid and γ-guanidinobutyric acid differ 12-fold although these ligands have a similar dipole length. This effect is more pronounced for a longer ligand as indicated by the 700-fold difference in K_I values for 7-aminoheptanoic acid and L-arginine. Although part of this difference in K_I might be assigned to a steric or electrostatic repulsion due to the α-amino group on L-arginine, this effect should be small since the dissociation constants for L-lysine and 6-aminohexanoic acid are approximately the same.

Table II: Ligand Binding to t-PA Kringle-2 Domain[a]

ligand	C_{50} (mM)	K_I (mM)
6-aminohexanoic acid	1.05	0.096
7-aminoheptanoic acid	0.14	0.029
L-lysine	1.7	0.100
N^{α}-acetyl-L-lysine	0.33	0.054
L-lysine methyl ester	10.7	0.30
N^{α}-acetyl-L-lysine methyl ester	6.8	
L-arginine	130	21.0
γ-guanidinobutyric acid		1.2
N^{ϵ}-acetyl-L-lysine		>200.0
L-lysylglycine		0.7
glycyl-L-lysine		0.06

[a] "C_{50}" refers to the ligand concentration required to elute 50% of bound kringle-2 from lysine-Sepharose. K_I values were determined from the ligand concentration that inhibited 50% of lysine binding measured by equilibrium dialysis as shown in Figure 5B.

In contrast to effects of modification of the ε-amino group on ligand binding, modification of the α-carboxylate has a smaller effect on ligand affinity. The affinity of kringle-2 for L-lysine methyl ester and the dipeptide L-lysylglycine is reduced 3-fold and 7-fold, respectively, relative to the affinity for L-lysine. These results indicate that ion pair interactions with the α-carboxylate of the lysine ligand are much less important in binding than charge interactions with the ε-amino group of the ligand. Interactions between the protein and the positively charged α-amino group of the lysine ligand also have a minor role in binding. The dissociation constants for binding of 6-aminohexanoic acid and L-lysine to t-PA kringle-2 are equivalent while the K_I for N^{α}-acetyl-L-lysine is decreased 2-fold. A similar increase in affinity is observed for the dipeptide glycyl-L-lysine.

Discussion

We have shown that functional t-PA kringle-2 domain can be produced in *E. coli* using an expression system based on secretion into the periplasmic space. Large amounts of homogeneous kringle-2 can be purified from *E. coli* cell paste by using a relatively simple purification protocol. Kringle-2 purified in this manner is a monomer, has the expected amino acid composition and N-terminal sequence, and has reasonable affinity for L-lysine. A number of investigators have reported methods for production of t-PA fragments using expression of truncated gene segments in mammalian cell culture [cf. van Zonneveld et al. (1986a), Verheijen et al. (1986), and Gething et al. (1988)]. These approaches have an advantage over our method in that any fragment expressed in *E. coli* would not be glycosylated. However, expression in *E. coli* allows one to rapidly generate quantities of homogeneous protein required for biophysical studies such as X-ray crystallography and NMR spectroscopy. In addition, analysis of the effect of a site-specific mutation on protein function is facilitated if the mutant protein can be expressed in a bacterial system.

A potential criticism of these studies is that our refolding procedure may not have generated the conformation of kringle-2 found in native t-PA. In developing the refolding protocol, we expected to recover the native conformation of kringle-2 based on results of others on oxidative refolding of disulfide-containing proteins. For example, both BPTI (Creighton, 1975) and RNase A (White, 1961) are unfolded upon reduction of all disulfide bonds but can be recovered in fully active form by oxidative refolding. Trexler and Patthy (1983) have shown that the lysine binding function of isolated plasminogen kringle-4 can be regenerated from the reduced form of the protein by using a procedure analogous to that

described here. Thermal denaturation experiments have also shown that plasminogen kringle-4 is an independent folding unit (Castellino et al., 1981; Novokhatny et al., 1984). The experimental results we have presented on the conformation of kringle-2 purified from *E. coli* confirm our expectation for recovery of native protein by oxidative refolding. Refolded kringle-2 is recognized by polyclonal antibody raised against native t-PA and has a circular dichroic spectrum similar to that observed for the isolated kringle-4 domain of plasminogen (Castellino et al., 1986). A proton NMR spectrum of kringle-2 displays many of the features, including a fingerprint methyl doublet at −1 ppm for Leu-46, that are characteristic of kringles (R. Kelley, I.-J. Byeon, and M. Llinás, unpublished results). Equilibrium dialysis experiments indicate that t-PA has a single binding site for L-lysine with affinity similar to that measured for the isolated kringle-2 domain (R. Kelley, unpublished results). In addition, both intact t-PA and the isolated kringle-2 domain have optimal affinity for a ligand of 8.8-Å chain length. These data clearly indicate that the conformation of the isolated domain must closely resemble the native conformation found in intact t-PA.

Tulinsky et al. (1988) have modeled the sequences of plasminogen kringles-1 and -4 and t-PA kringle-2 onto the structure determined for prothrombin fragment 1 kringle (Park & Tulinsky, 1986). Using data from chemical modification (Trexler et al., 1982; Vâli & Patthy, 1984) and proton NMR experiments (Motta et al., 1987; Ramesh et al., 1987) on the plasminogen kringles, a putative lysine binding site was constructed. The lysine analogue 6-aminohexanoic acid can be docked in the binding site with the amino group forming an ion pair with Asp-57,[2] the methylene backbone nestled against a cluster of aromatic residues, and the carboxylate interacting with Arg-71 in plasminogen kringle-4 and both Arg-34 and Arg-71 in plasminogen kringle-1. Asp-57 is conserved in t-PA kringle-2, but the arginine residues at 34 and 71 are replaced by Val and Thr, respectively. Tulinsky and colleagues proposed that His-64 of t-PA kringle-2 provides the cationic site for interaction with the ligand carboxylate. However, this hypothesis is inconsistent with the observation that t-PA kringle-2 binds lysine at pH 8—the His side chain should be deprotonated at this pH—with affinity only 2-fold lower than measured for plasminogen kringle-4 and 5-fold lower than measured for plasminogen kringle-1 (Markus et al., 1978; Lerch et al., 1980; Ramesh et al., 1987). Since the ligand specificity experiments indicate a larger lysine binding pocket for t-PA kringle-2 than determined for the plasminogen kringles, the cationic site in t-PA kringle-2 may be supplied by a residue which is further away from Asp-57 and was not included in the set of residues modeled by Tulinsky et al. (1988). Alternatively, the ligand carboxylate could be tethered in the binding pocket by hydrogen bonding, perhaps with the side chain hydroxyl of Thr-71. This hypothesis is consistent with both the chain length specificity of t-PA kringle-2 and the observation that neutralization of the charge on the ligand carboxylate has a relatively small effect on ligand binding.

Our results on the effect of modification of the α-amino group of L-lysine on binding differ from those reported for plasminogen (Winn et al., 1980). Lys-plasminogen has approximately equal affinity for the ligands 6-aminohexanoic acid and N^{α}-acetyl-L-lysine; the affinity for L-lysine is 8-fold lower. Winn et al. (1980) interpreted these results as reflecting reduced binding of L-lysine due to repulsion of the positively charged α-amino group. By comparison, t-PA kringle-2 has equivalent affinity for 6-aminohexanoic acid and L-lysine, indicating that the positive charge of the α-amino group does not interfere with binding of L-lysine to this kringle. Furthermore, as indicated by comparing the dissociation constants measured for binding of L-lysine and N^{α}-acetyl-L-lysine, interactions with the peptide unit on the N-terminal side of a lysine residue may slightly enhance binding of t-PA kringle-2 to fibrin. Although t-PA kringle-2 has optimal affinity for a ligand of 8.8-Å chain length, L-arginine binds very weakly. As suggested by Winn et al. (1980) for the plasminogen kringles, the guanidinium moiety may be excluded from close approach to Asp-57. This conclusion is supported by the observation of a 10-fold difference in affinity for the ligands 6-aminohexanoic acid and γ-guanidinobutyric acid.

Binding of t-PA to clots made from intact fibrinogen is described by a single binding site having a K_D of 380 nM (Higgins & Vehar, 1987). Our results and those of studies of domain deletion mutants of t-PA (van Zonneveld et al., 1986a,b; Verheijen et al., 1986) clearly show that this affinity for fibrin cannot be accounted for solely by interaction of the kringle-2 domain with a lysine residue in fibrin. Clots made from fibrinogen that has been partially degraded by plasmin, thus generating carboxy-terminal lysine residues, have an additional binding site(s) for t-PA. The binding of t-PA to this (these) site(s) is 2–4 orders of magnitude greater than binding of t-PA to the site on clots made from intact fibrinogen (Higgins & Vehar, 1987). Since the difference in affinity of kringle-2 for L-lysine as opposed to L-lysylglycine is about 7-fold, the difference in affinity of kringle-2 for internal versus carboxy-terminal lysine residues in fibrin should be small. Thus, the increased binding of t-PA to plasmin-degraded fibrin is not explained by a simple mechanism requiring only a change in the affinity of the lysine binding site on the kringle-2 domain.

[2] We use a numbering system for kringle sequences proposed by Tulinsky et al. (1988) based on alignment of five kringle sequences to the sequence of plasminogen kringle-5. The first cysteine of the kringle domain is numbered 1.

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Registry No. t-PA, 105913-11-9; lysine, 56-87-1; 3-aminopropionic acid, 107-95-9; 4-aminobutanoic acid, 56-12-2; *trans*-4-(aminomethyl)cyclohexanecarboxylic acid, 1197-18-8; 6-aminohexanoic acid, 60-32-2; 7-aminoheptanoic acid, 929-17-9; 8-aminooctanoic acid, 1002-57-9; N^{α}-acetyllysine, 1946-82-3; lysine methyl ester, 687-64-9; N^{α}-acetylysine methyl ester, 6072-02-2; arginine, 74-79-3; γ-guanidinobutyric acid, 463-00-3; N^{ϵ}-acetyllysine, 692-04-6; lysylglycine, 7563-03-3; glycyllysine, 997-62-6.

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