Tetranectin-Binding Site on Plasminogen Kringle 4 Involves the Lysine-Binding Pocket and at Least One Additional Amino Acid Residue†

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ABSTRACT: Kringle domains are found in a number of proteins where they govern protein−protein interactions. These interactions are often sensitive to lysine and lysine analogues, and the kringle−lysine interaction has been used as a model system for investigating kringle−protein interactions. In this study, we analyze the interaction of wild-type and six single-residue mutants of recombinant plasminogen kringle 4 expressed in *Escherichia coli* with the recombinant C-type lectin domain of tetranectin and trans-aminomethyl-cyclohexanoic acid (*t*-AMCHA) using isothermal titration calorimetry. We find that all amino acid residues of plasminogen kringle 4 found to be involved in *t*-AMCHA binding are also involved in binding tetranectin. Notably, one amino acid residue of plasminogen kringle 4, Arg 32, not involved in binding *t*-AMCHA, is critical for binding tetranectin. We also find that Asp 57 and Asp 55 of plasminogen kringle 4, which both were found to interact with the low molecular weight ligand with an almost identical geometry in the crystal of the complex, are not of equal functional importance in *t*-AMCHA binding. Mutating Asp 57 to an Asn totally eliminates binding, whereas the Asp 55 to Asn, like the Arg 71 to Gln mutation, was found only to decrease affinity.

Plasminogen (Plg)[1] is the zymogen for the serine protease plasmin, which is active in fibrinolysis and other physiological and pathological processes where highly controlled proteolysis is required, such as inflammation, tissue remodeling, ovulation, and invasive growth of metastases (*1*). Structurally, Plg is organized in seven protein domains, i.e., an N-terminal peptide, five kringle domains, and one serine protease domain (*2*).

Kringle domains consist of approximately 80 amino acid residues with three intradomain disulfide bridges defining three loops. Several of the kringle domains are known to be involved in protein−protein interactions, which often regulate the functional properties of the entire kringle containing mosaic protein. Such interactions are often sensitive to ω-amino acids (e.g., lysine and lysine analogues), and the so-called lysine-binding site is highly conserved among several kringles.

The amino acid residues in Plg kringle 4 involved in binding to the lysine analogue 6-AHA have been identified both through mutagenesis and structural studies. From the crystal structure, it appears as if the main contributors to the interaction with the charged part of 6-AHA are K35, D55, D57, and R71 (*3*). However, mutagenesis analysis showed that K35 is apparently of little importance in solution, since binding strengths of 6-AHA and *t*-AMCHA are not greatly affected when Lys 35 is replaced by Ile, Met (*4*), or Ser (*5*). The finding that only one positively charged amino acid is required for binding of ω-amino acids to kringles has also been reported for Plg kringle 1 (*6*−*8*), for Plg kringle 5 (*5*, *9*), and for tissue type-plasminogen activator kringle 2 (*10*). Furthermore, the mutation D54N in Plg kringle 1, corresponding to D55N in Plg K4, has been found to have a larger effect on binding ω-amino acids relative to the mutation D56N, corresponding to D57N in Plg K4 (*7*). Mutation in the kringle 2 domain of tissue type plasminogen activator of the residue D59, corresponding to D57 in Plg K4, has a larger effect compared to mutation of residue D57, corresponding to D55 in Plg K4 (*11*).

Angiostatin, a fragment of Plg, was found to exert an inhibitory effect on neo-vascularisation and thereby the growth of metastatic tumors, both in vivo and in vitro (*12*). The functional role of individual kringle domains in Angiostatin has been examined both in vitro (*13*, *14*) and in vivo (*15*). Apparently, inhibition of neo-vascularisation resides in kringles 1−3, whereas kringle 4 is involved in inhibition of endothelial cell migration. Also, Plg kringle 5 of plasminogen has been shown to exert an inhibition on neo-vascularisation (*16*). However, the detailed mechanism by which Angiostatin exerts its inhibitory effect remains to be elucidated.

Tetranectin (TN) binds specifically to the Plg kringle 4 domain and binding is sensitive to both lysine and calcium (*17*). TN is a trimeric protein (*18*) exhibiting sequence and, in particular, structural similarity to the C-type lectin family (*19*, *20*). The binding site on TN for Plg kringle 4 has been localized to the so-called carbohydrate recognition domain

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[1] Abbreviations: 5-APA, 5-aminopentanoic acid; 6-AHA, 6-aminohexanoic acid; CRD, carbohydrate recognition domain; ITC, isothermal titration calorimetry; PAGE, polyacrylamide gel electrophoresis; Plg, plasminogen; *t*-AMCHA, *trans*-aminomethyl-cyclohexanoic acid; TN, tetranectin; TN3, TN amino acid residues 45−181.

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(CRD) and three residues (i.e., Lys 148, Glu 150, and Asp 165) in or near calcium-binding site 2 have been identified as important for binding (*17*). TN was originally isolated from plasma (*21*), but the protein is expressed by a variety of cells and in various tissues. TN has been shown to be a useful prognostic marker in cancer treatment (*22*), and TN has been shown co-localized with plasmin/plasminogen at the invasive front of cutaneous melanoma lesions, indicating a coordinated role of these proteins in the invasive process (*23*). In addition, the protein interacts with complex sulfated polysaccharides (*24*, *25*) and with apolipoprotein (a) (*26*), as well as with fibrin in a calcium-dependent manner (*27*). Recent studies indicate that TN also plays a role in osteogenesis and myogenesis (*28*−*30*).

In this study, the Plg kringle 4 and the TN CRD domains are used as a model system for analyzing kringle−protein interactions. The strength of binding of rPlg kringle 4 (rPlg K4) and six single-residue kringle mutants to TN and *t*-AMCHA was determined using isothermal titration calorimetry (ITC). Three of the mutants were described earlier in the analysis of the interaction between Plg K4 and *t*-AMCHA, i.e., K35I, D57N, and R71Q (*4*, *5*). The two additional mutants, R32A and K58A, were constructed to test whether other positively charged residues near the lysine-binding pocket of Plg K4 are involved in binding of TN. The mutant D55N was constructed to assess the importance of this second negative charge present in the lysine-binding pocket for TN and *t*-AMCHA binding.

MATERIALS AND METHODS

Site-Directed Mutagenesis, Expression, Refolding, and Processing of Recombinant Proteins. After excision from the plasmid prPKr4 (*4*) using *Bam*HI and *Hin*dIII, the cDNA encoding rPlg K4 was subcloned into the corresponding sites in pT7H6UB (*31*), yielding the expression plasmid pT7H6UB-K4.

The single-residue mutations in Plg K4 (i.e., R32A, K35I, D55N, D57N, K58A, and R71Q) were introduced using the Quickchange kit (Stratagene, La Jolla, CA) as previously described for rTN single-residue mutations (*17*).

The rPlg K4 derivatives were expressed in *Escherichia coli* DH1 cells, in vitro refolded and purified, partly on lysine-Sepharose, as described (*32*). However, the mutant D57N, which failed to bind lysine-sepharose, was purified by ion-exchange chromatography on SP-sepharose in a 25 mM sodium acetate, pH 5, buffer gradient from 10 to 500 mM NaCl over 20 column volumes.

The TN CRD derivative (rTN3) was produced essentially as described previously (*18*).

Isothermal Titration Calorimetry Binding Analysis. All titrations were performed at 25 °C on an MCS ITC instrument from MicroCal Inc. (Northampton, MA) (*33*). Titrations were performed in a cell with a volume of 1.3187 mL with 21 injections of 13.011 μL of titrator under stirring at 400 rpm. Protein samples and *t*-AMCHA were dissolved in a buffer containing 100 mM NaCl and 50 mM Tris-HCl, pH 8.2, or, for two titrations of Plg K4 wild-type with *t*-AMCHA, 100 mM NaCl and 50 mM Tris-HCl, pH 8.0 and degassed by stirring under vacuum. For each titration, a blank titration of the titrator, rPlg K4 or t-AMCHA, into the buffer was included for reference. The blank titration data was always subtracted before fitting the binding isotherms using the ORIGIN, ver. 4.10, program package from MicroCal Software Inc. (Northampton, MA). The one-site model was used, i.e., a model that assumes identical and independent binding sites.

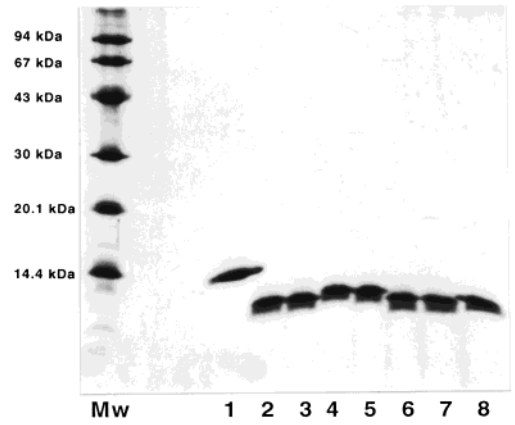

FIGURE 1: SDS−PAGE gel, 16%. Lanes: Mw, molecular mass marker; 1, rTN3; 2, rPlg K4 wild-type; 3, K58A; 4, D57N; 5, D55N; 6, K35I; 7, R32A; 8, R71Q.

RESULTS

All recombinant Plg kringle 4 derivatives exhibited the same expression level, and they all refolded, generating the authentic disulfide bridging pattern, as judged from their virtually identical mobilities on nonreducing SDS−PAGE gels (Figure 1). However, rPlg K4 D55N and D57N exhibited a slightly slower migration rate than the rPlg K4 derivatives, presumably due to the removal of a negative charge. On the basis of comparison of absorbance values at 280 nm and Coomassie brilliant blue staining intensities of bands on SDS−PAGE gels, it was found justified to use the same $A(1\%)_{280}$ value of 30 g L^{-1} cm^{-1} for all the rPlg K4 derivatives. The kringle derivatives that bound *t*-AMCHA and which could be purified on lysine-Sepharose were assumed to be properly refolded. To validate the refolding of rPlg K4 D57N, which, as the only mutant, did not bind lysine-sepharose or *t*-AMCHA, a 1-D ^{1}H NMR spectrum was recorded, and compared to the spectrum of rPlg K4. The spectrum showed the diagnostic dublet peak, arising from the conserved Leu 46, with the high-field position at approximately −1 ppm, indicative of correct folding of Plg kringle domains (*34*). Further, the aromatic region of the spectrum showed a fine-structure pattern also diagnostic of a folded state (S. Nielbo and J. H. G., unpublished material). In summary, the rPlg K4 D57N mutant appears to be correctly folded, as are all the remaining mutants, a conclusion based on ability to bind lysine-Sepharose, indicating that none of the observed affinity changes are in fact due to incorrect folding.

Before analyzing the interactions between rTN3 and *t*-AMCHA with rPlg K4 and the single-residue mutant derivatives, blank titrations of the titrators (i.e., the rPlg K4 derivatives and *t*-AMCHA, respectively) into buffer were performed (Figure 2). Surprisingly, a large enthalpy signal was observed in the blank titration of rPlg K4, which was not observed to the same extent for any of the single-residue mutants (Figure 2A). The enthalpy signal dropped as the rPlg K4 concentration increased in the cell, thus indicating a

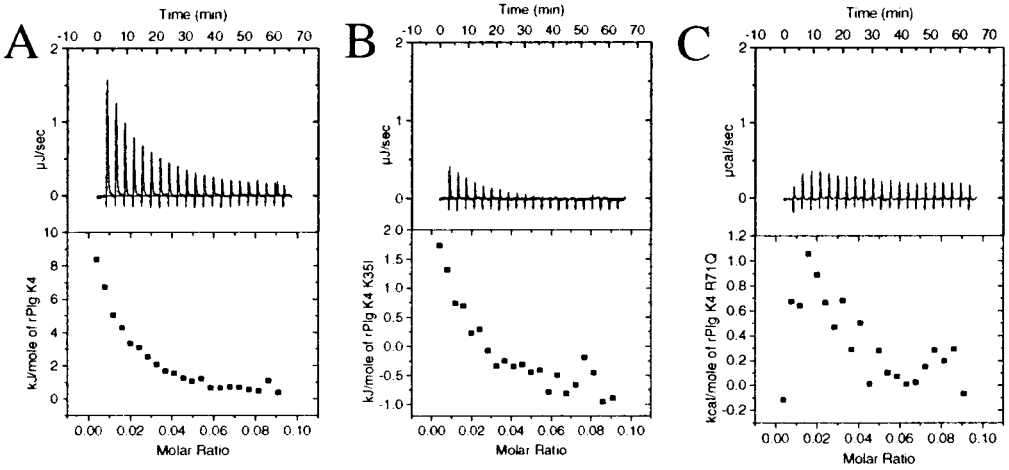

FIGURE 2: Representative thermograms and binding isotherms for blank titrations of kringles at pH 8.2. Kringle concentrations used = 0.398 mM. (a) rPlg K4. (b) rPlg K4 K35I. (c) rPlg K4 R71Q.

possible dissociation reaction of oligomeric assemblies of rPlg K4 present in the syringe. The thermogram resembles that found by Lovatt et al. in studying dissociation of insulin dimers (*35*). We attempted to fit the isotherms obtained for the blank titration of Plg K4 to a equation similar to that used by Lovatt et al., accounting for a possible higher degree of oligomerization, but we could not obtain a consistent set of fitting parameters due to strong statistical correlations. We decided not to pursue this problem further, since it had no major effect on the main conclusions of the present study with regard to a possible involvement of individual amino acid residues of Plg K4 in binding to *t*-AMCHA or rTN3.

The single-residue mutants rPlg K4 K35I and K58A produced significantly smaller responses in the blank titrations than did rPlg K4. The blank titration for rPlg K4 K35I is shown in Figure 2B. No responses were obtained in the blank titrations for any of the other single-residue mutant derivatives. The blank titration for rPlg K4 R71Q is shown in Figure 2C. These findings indicate that a putative oligomerization may involve the region involved in binding *t*-AMCHA and TN and accordingly take place in competition with them. Blank titration of *t*-AMCHA into buffer produced no significant response (not shown).

Because of the putative multimerisation of rPlg K4, which would take place in competition with ligand binding, all association constants determined in the ITC analysis should be referred to as apparent association constants, K'_A.

Representative thermograms and binding isotherms of the titrations of rTN3 with rPlg K4 and the single-residue mutant derivatives are shown in Figure 3, and the calculated thermodynamic parameters are listed in Table 1. The ITC-data from the appropriate blank titration was subtracted from the titration data in each binding analysis prior to fitting to a one-site model. Binding of rPlg K4 and the single-residue mutant derivatives rPlg K4 K35I and K58A could clearly be demonstrated. However, the strength of interaction was found to depend both on pH and, in the case of rPlg K4, on the concentration of the titrator. For the mutants rPlg K4 K35I and K58A, a somewhat smaller K'_A was obtained compared to the K'_A obtained for the wild-type derivative. The interaction between rTN3 and the mutants rPlg K4 K35I and K58A was found to be strongly enthalpy driven under the conditions used with large entropy penalties, like the

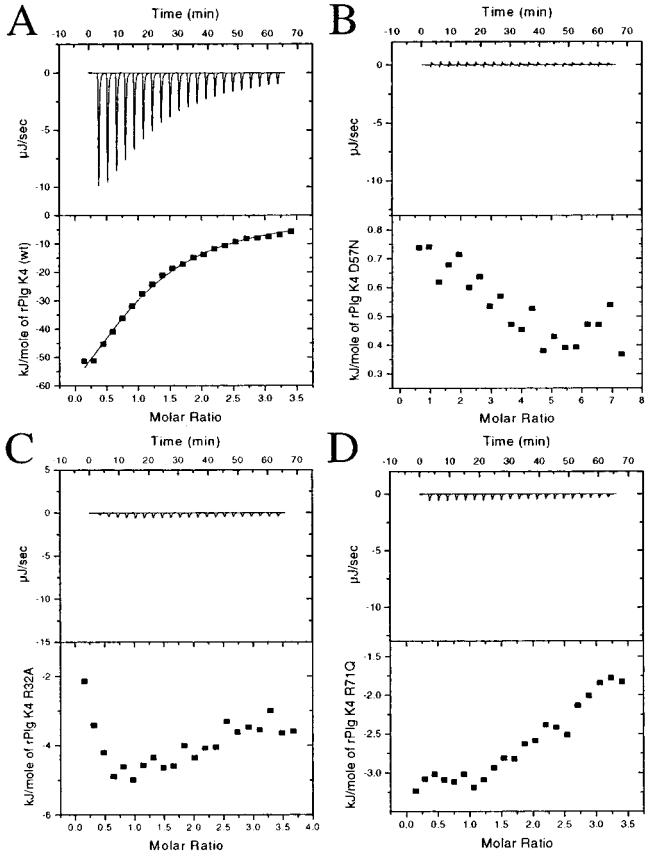

FIGURE 3: Representative thermograms and binding isotherms for the titration of rTN3 with kringles at pH 8.2. (a) $[\text{rPlg K4}]_{\text{Syringe}}$ = 0.398 mM, $[\text{rTN3}]_{\text{chamber}}$ = 0.0248 mM. (b) $[\text{rPlg K4 D57N}]_{\text{Syringe}}$ = 0.398 mM, $[\text{rTN3}]_{\text{chamber}}$ = 0.0248 mM. (c) $[\text{rPlg K4 R32A}]_{\text{Syringe}}$ = 0.398 mM, $[\text{rTN3}]_{\text{chamber}}$ = 0.0248 mM. (d) $[\text{rPlg K4 R71Q}]_{\text{Syringe}}$ = 0.398 mM, $[\text{rTN3}]_{\text{chamber}}$ = 0.0248 mM.

interaction between rTN3 and Plg K4. The small change in affinity obtained for rPlg K4 K35I is due to a difference in entropy for the interaction, since the enthalpy is in fact marginally more favorable. For the mutant rPlg K4 K58A, the enthalpy is less favorable, and the entropy is less unfavorable than for the wild-type, resulting in a minor decrease in affinity. No binding of the single-residue mutant derivatives rPlg K4 R32A, D55N, D57N, and R71Q was observed.

Table 1: Binding Parameters for Binding of rPlg K4 Wild-Type and Mutants to rTN3

	wild-type			K35I	K58A	R32A, D55N, D57N, R71Q
pH	8.2	8.2	8.0[a]	8.2	8.2	8.2
$[rTN3]_{Chamber}$ (mM)	0.025	0.053	0.053	0.025	0.025	0.025
$[K4]_{Syringe}$ (mM)	0.40	0.79	0.79	0.40	0.40	0.40
stoichiometry	1.04 ± 0.03	1.16 ± 0.04	0.95 ± 0.08	1.11 ± 0.07	1.30 ± 0.13	no binding
K'_A (10^4 M^{-1})	5.41 ± 0.29	3.71 ± 0.07	1.93 ± 0.11	2.05 ± 0.10	2.29 ± 0.30	detected
$\Delta G°$ (kJ/mol)	−27.0 ± 0.1	−26.1 ± 0.1	−24.5 ± 0.3	−24.6 ± 0.1	−24.9 ± 0.3	
$-T\Delta S°$ (kJ/mol)	63.0 ± 0.4	68.6 ± 0.9	113 ± 14	77.5 ± 7.9	53.5 ± 11.2	
ΔH (kJ/mol)	−90.0 ± 0.4	−94.7 ± 0.9	−137 ± 14	−102.1 ± 7.9	−78.4 ± 11.2	

[a] Data from ref *17*.

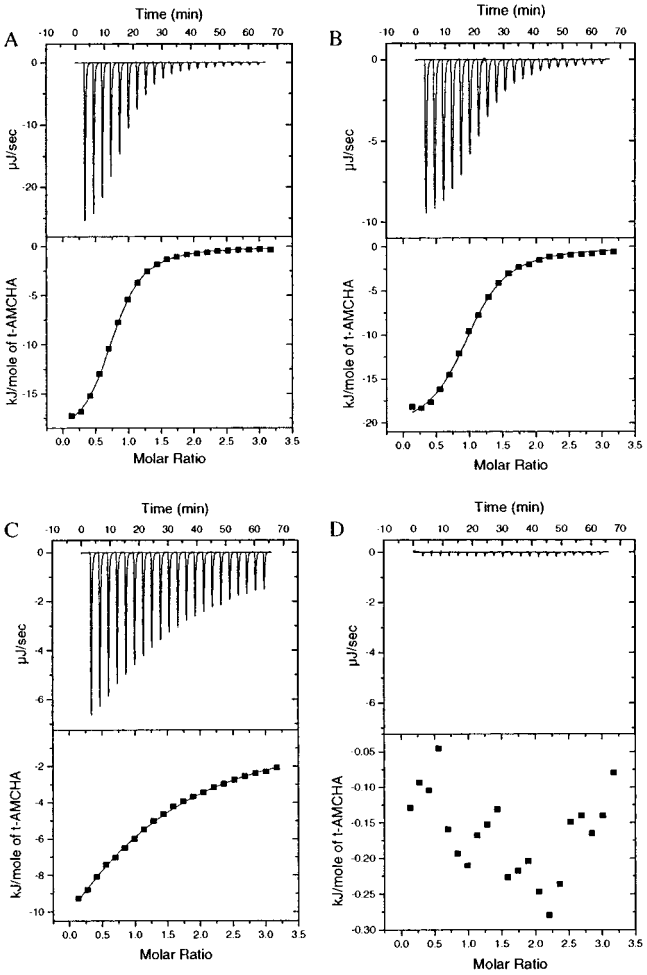

FIGURE 4: Representative thermograms and binding isotherms for titration of kringles with *t*-AMCHA. (a) [rPlg K4] = 0.216 mM, [*t*-AMCHA] = 3 mM. (b) [r Plg K4 R32A] = 0.108 mM, [*t*-AMCHA] = 1.5 mM. (c) [rPlg K4 D55N] = 0.108 mM, [*t*-AMCHA] = 1 0.5 mM. (d) [rPlg K4 D57N] = 0.108 mM, [*t*-AMCHA] = 2.5 mM.

Representative thermograms and binding isotherms of the titrations of rPlg K4 and the single-residue mutants with *t*-AMCHA are shown in Figure 4, and the thermodynamic parameters were determined by fitting to a one-site model (Tables 2 and 3, respectively). Both a favorable enthalpy and entropy drove the reaction between rPlg K4 and *t*-AMCHA with the largest contribution from the enthalpy. However, the binding parameters determined were found to depend on the concentrations of rPlg K4 in the chamber. Particularly, the apparent stoichiometry determined for the reaction increased from 0.751 to 0.922 upon decreasing the concentration of rPlg K4 from 0.216 mM to 0.054 mM at pH 8.2. At pH 8.0, a similar variation with kringle concentration is observed; however, the stoichiometry is 1 or above 1. The reason for this observed stoichiometry above 1 remains unknown. Also, the affinity and the exothermic enthalpy signal increased at lower concentrations of rPlg K4 at both pH 8.0 and 8.2. This variation in binding parameters may well be due to the putative oligomerization of the recombinant derivative, since at lower concentrations fewer rPlg K4 oligomers dissociate (an endothermic process). In a previous ITC-based analysis of the interaction between Plg K4 and *t*-AMCHA (*36*), a stoichiometry of 0.87 at pH 7.4 was determined. No trend in the values determined was found to correlate with the variation of concentration of Plg K4, but the stoichiometry determined for the reaction varied with pH, being closer to unity at both higher and lower pH values. However, the Plg K4 used in that study was less homogeneous as it was prepared by partial digestion of human Plg purified from plasma, and measurements were performed in a buffer of low-ionic strength. This may well alter the pH profile of the putative oligomerization.

The titration analysis of the single-residue mutant Plg K4 derivatives and *t*-AMCHA showed that three of the mutants, R32A, K35I, and K58A, bind with increased apparent affinities ($K'_A = 9.2 \times 10^4$, 12.4×10^4, and 8.9×10^4 M^{-1}, respectively). Two of the mutants, D55N and R71Q, bind *t*-AMCHA with significantly lower apparent affinities ($K'_A = 0.5 \times 10^4$ and 0.4×10^4 M^{-1}, respectively), whereas no binding of the mutant D57N was detectable (Table 3). Overall, the K'_As agree with values determined previously for *t*-AMCHA binding (*4*, *5*), although the mutant protein K35I exhibited an increased affinity for *t*-AMCHA. The mutant D55N has not previously been expressed, mainly due to technical obstacles (*5*). Although structural analysis has proposed that D55 and D57 could contribute to the binding of 6-AHA with equal strength, our analysis shows that D57N is the more important residue for binding of *t*-AMCHA. This finding may also extend to binding of 6-AHA because the D57N mutant does not bind to lysine-Sepharose, whereas rPlg K4 D55N does bind (not shown).

DISCUSSION

Three charged amino acid residues, K148, E150, and D165, in the calcium-free form of the TN CRD, have been identified as being important for specific interaction with Plg K4 (*17*). This interaction, like many other interactions between kringle domains and various proteins, is sensitive to lysine, thereby implying the so-called kringle lysine-binding pocket in the binding of TN. The present study clearly demonstrates that the charged residues, D55, D57,

Table 2: Binding Parameters for the Interaction between rPlg K4 and *t*-Amcha

pH	8.2	8.2	8.2	8.0	8.0
$[K4]_{Chamber}$ (mM)	0.22	0.11	0.054	0.11	0.054
$[t\text{-AMCHA}]_{Syringe}$ (mM)	3.0	1.5	0.75	1.5	0.75
stoichiometry	0.75 ± 0.01	0.85 ± 0.02	0.92 ± 0.02	1.03 ± 0.01	1.16 ± 0.02
K'_A (10^4 M^{-1})	5.62 ± 0.19	6.01 ± 0.13	6.60 ± 0.52	7.63 ± 0.25	8.98 ± 0.95
$\Delta G°$ (kJ/mol)	−27.1 ± 0.1	−27.3 ± 0.1	−27.5 ± 0.2	−27.9 ± 0.1	−28.3 ± 0.3
$-T\Delta S°$ (kJ/mol)	−7.4 ± 0.2	−7.3 ± 0.2	−6.5 ± 0.7	−12.3 ± 0.1	−11.1 ± 0.6
ΔH (kJ/mol)	−19.7 ± 0.2	−20.0 ± 0.2	−21.0 ± 0.7	−15.6 ± 0.1	−17.2 ± 0.Y

Table 3: Binding Parameters for Interaction between rPlg K4 Mutants and *t*-Amcha

	r32A	K35I	D55N		D57N	K58A	R71Q	
$[K4]_{Chamber}$ (mM)	0.11	0.11	0.11	0.054	0.11	0.11	0.11	0.11
$[t\text{-AMCHA}]_{Syringe}$ (mM)	1.5	1.5	1.5	1.5	2.5	1.5	1.5	2.5
stoichiometry	1.00 ± 0.01	1.06 ± 0.01	1.11 ± 0.06	1.52 ± 0.15	no binding	1.09 ± 0.01	1.03 ± 0.1	1.04 ± 0.11
K'_A (10^4 M^{-1})	9.2 ± 0.6	12.4 ± 0.4	0.49 ± 0.02	0.64 ± 0.05	detected	8.9 ± 0.5	0.40 ± 0.03	0.48 ± 0.03
$\Delta G°$ (kJ/mol)	−28.3 ± 0.2	−29.1 ± 0.1	−21.1 ± 0.1	−21.7 ± 0.2		−28.2 ± 0.1	−20.6 ± 0.2	−21.0 ± 0.2
$-T\Delta S°$ (kJ/mol)	−7.1 ± 0.4	−9.7 ± 0.1	4.0 ± 1.9	−4.6 ± 2.1		−8.1 ± 0.2	9.7 ± 3.2	5.2 ± 3.2
ΔH (kJ/mol)	−21.2 ± 0.4	−19.4 ± 0.1	−25.1 ± 1.9	−17.1 ± 2.1		−20.1 ± 0.2	−30.3 ± 3.2	−26.2 ± 3.2

and R71, previously identified as being important in ω-amino acid binding, are also important for TN binding. It also demonstrates that at least one residue, R32, located outside the lysine-binding pocket is involved in this interaction.

Quantification of the strength of the interaction is hampered by a competitive reaction revealing itself as a large endothermic heat signal obtained upon diluting Plg K4 into buffer, here interpreted as a possible self-association of Plg K4 wild-type. This process was not observed for any of the single-residue mutant derivatives that do not bind rTN3 and not to the same extent for rPlg K4 K35I and K58A. It was not possible to fit these data to a simplistic equation corresponding to dissociation of Plg K4 oligomers into monomers. To calculate the apparent binding constant for the interaction between rTN3 and rPlg K4, appropriate blank titration data were subtracted from each of the binding isotherms before fitting to a one-site model. This approximation will generate results where the K'_A determined for the complex is too low due to putative competition between oligomerization and TN binding. That competition could take place can be seen from the fact that none of the mutants which binds TN3 generate this characteristic blank titration thermogram. Nevertheless, whether rPlg K4 forms oligomers or not does not affect the overall conclusions shown in this study, with regard to the role of the individual amino acid residues in interacting with *t*-AMCHA or TN, since the effect of substituting amino acid residues appear to be at least an order of magnitude larger than the effect from the putative multimerisation, as judged from the *t*-AMCHA and rTN3 affinities of rPlg K35I and K58A relative to rPlg K4.

The importance of Plg K4 amino acid residues D55 and D57 in binding to *t*-AMCHA are opposite to those found for Plg kringle 1, where the mutation D54N led to disruption of the binding of *t*-AMCHA, and the mutation D56N led to a measurable, albeit more than 1000 times lower, K'_A (*7*). For 6-AHA, the difference in affinity between the two ligands was smaller (*7*). Tissue-type plasminogen activator kringle 2 has also been mutated and analyzed for binding to *t*-AMCHA. Here, the mutation D59E exhibited an approximately 6 times stronger effect in diminishing the binding of *t*-AMCHA and 6-AHA compared to D57E (*11*). This might indicate a general property of the double acid motif, that the amino acid residues corresponding to D55 and D57 in Plg K4 are not of equal importance in binding different lysine analogues.

For the three mutants rPlg K4 R32A, K35I, and K58A, slightly stronger binding to *t*-AMCHA was observed relative to that of the wild-type. The reason for the increase in affinity of K35I for *t*-AMCHA in comparison to the previous studies (*4*, *5*) may be the use of a different buffer with a considerably higher ionic strength, which leads to stabilization of hydrophobic interactions. K35 is located close to the binding site in the structure of the wild-type K4 (*3*), so it is possible that the Ile side chain may contribute to hydrophobic interaction with *t*-AMCHA and thereby stabilize the complex in higher ionic strength buffers. Another explanation for the generally higher affinity of these mutants for *t*-AMCHA may be that rPlg K4 R32A does not form oligomers and rPlg K4 K35I and K58A exhibit significantly smaller blank titration signals than do rPlg K4, indicating smaller tendency to form oligomers.

R32, D55, D57, and R71 are of pivotal importance for the interaction with rTN3, while K35 and K58 are of minor importance, if any. Notably, K35, which in the crystal structure of the K4−6-AHA complex interacts with 6-AHA (*3*), are not of importance in binding neither *t*-AMCHA nor rTN3 in solution. The K'_A determined for rPlg K4 is two times higher than that reported in a previously study (*17*), but a different buffer was used in the present study, which might reduce the tendency of rPlg K4 (wt) to form oligomers, and, in turn, lead to estimation of a higher affinity.

Mutations in rPlg K4 that lower the affinity for *t*-AMCHA disrupt binding to TN, indicating that the Plg K4-binding site of TN involves the entire lysine-binding site of Plg K4. However, the TN-binding site of rPlg K4 involves at least one amino acid residue not involved in lysine binding, namely R32. The interactions between lysine analogues and kringles have been described in great detail, but the protein interactions of kringles have not yet received the same attention, so whether this is a general feature of kringle−protein interactions, or is unique for the interaction between TN and Plg K4, remains to be seen. However the nonlysine-binding kringle 1 of hepatocyte growth factor has been shown to bind the c-Met receptor trough a site partly overlapping the nonfunctional lysine binding pocket and involving an E that aligns with R 32 of Plg K4 (*37*).

The binding of protein ligands to a kringle has been examined with respect to the intermolecular interaction between the N-terminal peptide of Plg and kringle 5 (*38*). The effect of mutating the residue corresponding to D57 in Plg kringle 4 was studied, and found to result in loss of intramolecular interaction. This result is in accord with our findings for the rPlg K4 D57N mutant.

TN binds apo-lipoprotein (a), presumably through one or more of its kringle 4-type domains. Our results from the studies of the binding between Plg K4 and TN show that conservation of the four residues R32, D55, D57, and R71 is crucial. Inspection of the different kringle types of apo-lipoprotein (a) searching for conservation of these four residues is suggestive of kringle 4 type 10 as the most likely site for the interaction with TN.

The present and an earlier study identifying residues in TN involved in binding Plg (*17*) show that a few charged residues are involved in the interaction, namely R32, D55, D57, and R71 in Plg K4, and K148, E150, and D165 in TN. These residues are probably responsible for most of the binding free energy.

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