# Comparative Analyses of the Lysine Binding Site Properties of Apolipoprotein(a) Kringle IV Types 7 and 10[†]

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ABSTRACT: Apolipoprotein(a) [apo(a)] shares extensive sequence similarity with plasminogen and consists of multiple tandem repeats of domains similar to plasminogen kringle IV (KIV), followed by domains homologous to the plasminogen KV and protease domains. The apo(a) KIV domains can be classified into 10 types on the basis of amino acid sequence ($KIV_1$−$KIV_{10}$) of which $KIV_{10}$ contains a canonical lysine binding site (LBS); $KIV_{10}$ mediates the lysine-dependent interaction of Lp(a) with certain biological substrates. Molecular modeling studies indicated the presence of weak LBS in each of $KIV_5$−$KIV_8$, and subsequent biochemical studies have revealed contributions of these kringles to lysine-mediated interactions involving apo(a). The present study describes the direct demonstration of a weak LBS within $KIV_7$, as well as the first characterization of the ligand specificity of an LBS outside that of $KIV_{10}$. We have expressed both $KIV_7$ and $KIV_{10}$ from bacterial cells and purified them to homogeneity from cell lysates. Equilibrium binding analyses of the $KIV_7$ LBS using intrinsic fluorescence revealed an affinity for L-lysine and its analogues ~10-fold weaker ($K_D = 230 \pm 42$ $\mu$M for $\epsilon$-aminocaproic acid) than that of $KIV_{10}$ ($K_D = 33 \pm 4$ $\mu$M for $\epsilon$-aminocaproic acid). Moreover, we demonstrated differences in specificity of the LBS of $KIV_7$ in comparison with $KIV_{10}$ in that $KIV_7$ preferentially bound L-proline. Both kringles bind 4-aminobutyric acid with similar affinities albeit with apparently different mechanisms. Key $Phe^{62} \rightarrow Tyr$ and $Asp^{56} \rightarrow Glu$ substitutions in the $KIV_7$ LBS result in alterations in the size of the LBS and in the spatial relationship between the cationic and anionic centers in the LBS and thus account for the differences in the binding properties of $KIV_7$ and $KIV_{10}$.

Elevated plasma concentrations of lipoprotein(a)[1] [Lp(a)] have been identified as a significant risk factor for the development of a variety of atherosclerotic disorders (reviewed in refs *1* and *2*). Structurally, Lp(a) resembles low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoprotein B-100 (apoB-100) but is distinguished from LDL by the presence of a unique glycoprotein, apolipoprotein(a) [apo(a)], which is covalently linked to apoB-100 by a single disulfide bond (*3*−*5*). Apo(a) shares a high degree of sequence identity with the fibrinolytic proenzyme plasminogen and contains tandemly repeated copies of sequences that closely resemble plasminogen kringle IV (KIV), followed by sequences which are homologous to the kringle V and protease domains of plasminogen (*6*). The plasminogen KIV-like domains of apo(a) can be classified into 10 types on the basis of amino acid sequence which, with the exception of KIV type 2 ($KIV_2$), are each present in single copy in all individuals (*7*, *8*). The $KIV_2$ domain is present in differing numbers of identically repeated copies which forms the basis of the apo(a) isoform size heterogeneity observed within the human population (*1*, *7*−*10*).

While several different mechanisms have been proposed to account for the association between elevated Lp(a) and the development of atherosclerosis (reviewed in refs *1* and *2*), the true pathophysiological role of this lipoprotein remains unclear. However, it is likely that a key determinant of the pathogenic effects of Lp(a) resides in its ability to bind to specific components present in the arterial wall (*11*−*15*). Numerous studies have demonstrated that the apo(a) component of Lp(a) binds to a variety of biological substrates via both lysine-dependent and lysine-independent mechanisms. The lysine-dependent interactions are mediated through lysine binding sites (LBS) present in some of the apo(a) KIV types. For example, apo(a) KIV type 10 ($KIV_{10}$) contains a canonical LBS similar to that in plasminogen kringle IV (*16*−*19*); the LBS in $KIV_{10}$ has been proposed to mediate the binding of Lp(a) to biological substrates such as fibrin (*17*, *20*−*22*) although one study provided evidence for a fibrin binding site outside of $KIV_{10}$ (*23*). Defects in the LBS of apo(a) $KIV_{10}$ have been associated with a diminished capacity for vascular accumulation of the corresponding apo(a)/Lp(a) and a reduction in the extent of development of atherosclerosis in transgenic mouse models (*21*, *22*). In addition, molecular modeling studies (*16*) have predicted that, despite substitution of some critical residues, apo(a) KIV types 5−8 may retain the capacity to bind to lysine and other amino acids. In this context, recent studies

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[1] Abbreviations: Lp(a), lipoprotein(a); LDL, low-density lipoprotein; apoB-100, apolipoprotein B-100; apo(a), apolipoprotein(a); KIV, kringle IV; LBS, lysine binding site(s); IPTG, isopropyl $\beta$-D-thiogalactopyranoside; DTT, dithiothreitol; $\epsilon$-ACA, $\epsilon$-aminocaproic acid; 4-ABA, 4-aminobutyric acid; r-apo(a), recombinant apo(a).

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have shown that apo(a) KIV types 6−8 (in particular, $KIV_7$ and $KIV_8$) contribute to the initial noncovalent interactions between apo(a) and apoB that precede specific disulfide bond formation (*24*). It has also been shown that the noncovalent association of apo(a) and apoB-100 can be inhibited by lysine, lysine analogues, phenylalanine, proline, and arginine (*24*−*27*), which may reflect the ability of these ligands to bind to the putative LBS in KIV types 6, 7, and 8 (*24*) although this has not been shown directly to date.

In the present study we have expressed apo(a) $KIV_7$ and $KIV_{10}$ in bacteria and purified these proteins to homogeneity in order to characterize the LBS in $KIV_7$ and to compare its properties to that of $KIV_{10}$. On the basis of our experimental results, in conjunction with known structural information, we were able to gain substantial insight into the ligand specificity of $KIV_7$ as well as its overall lysine affinity relative to that of $KIV_{10}$. The properties of the LBS in $KIV_7$ are consistent with a role for this kringle in fibrin binding and Lp(a) assembly.

## EXPERIMENTAL PROCEDURES

*Expression of $KIV_7$ and $KIV_{10}$ in Escherichia coli.* $KIV_7$ and $KIV_{10}$ were expressed in the *E. coli* strain BL21(DE3) using the pET expression system. Construction of $KIV_7$-pET16b and $KIV_{10}$-pET16b expression plasmids and conditions for protein expression have been described previously (*28*). Kringles, which contained His tags, were purified using His-Bind resin (Novagen) as previously described (*28*).

In all cases, protein concentrations were determined by measurement of the absorbance at 280 nm. Extinction coefficients for each recombinant protein were previously determined for $KIV_7$ (W. S. Sangrar and M. L. Koschinsky, unpublished results) and $KIV_{10}$ (*29*) using the tyrosine difference spectral method (*30*). The extinction coefficients and molecular weights used for calculating protein concentrations were as follows: $KIV_7$ [MW = 18052; $\epsilon_{0.1\%}$(280 nm) = 2.12]; $KIV_{10}$ [MW = 17821; $\epsilon_{0.1\%}$(280 nm) = 1.27].

*Determination of the Lysine−Sepharose Binding Properties of $KIV_7$.* Lysine−Sepharose CL-4B (Pharmacia) columns (250 $\mu$L) were prepared and equilibrated with 10 column volumes of 20 mM Tris-HCl, pH 7.9. Protein (125 $\mu$g) was allowed to bind the column in a volume of 500 $\mu$L over a period of ~1 h with occasional resuspension of the resin. Unbound protein was collected in the flow-through, and the column was subsequently washed with 20 mM Tris-HCl, pH 7.9, until no protein was detected by Bio-Rad protein assay (Bio-Rad Laboratories) of 10 $\mu$L of each 250 $\mu$L fraction. The column was then washed with 20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl to remove weakly bound protein until protein was no longer detectable as described above. In some experiments, the washes were performed with 20 mM Tris-HCl, pH 7.9, containing 0.15 M NaCl. Specifically bound protein was eluted by the addition of $\epsilon$-ACA (either 200 mM in 20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl or a 20−200 mM gradient in 20 mM Tris-HCl, pH 7.9). All column fractions were analyzed by SDS−PAGE on 15% polyacrylamide gels followed by silver staining.

*Analysis of the Binding of Amino Acids to $KIV_7$ and $KIV_{10}$ by Measurement of Intrinsic Fluorescence.* Fluorescence measurements of $KIV_7$ or $KIV_{10}$ were made using an LS 50B luminescence spectrometer (Perkin-Elmer). Kringles were titrated with L-arginine, L-phenylalanine, L-proline, L-lysine, and a variety of lysine analogues. Titrations were performed using 1 $\mu$M protein in 20 mM Tris-HCl, pH 7.9, containing 0.1% Tween-20 in a quartz cuvette which had been conditioned for 1 h in this buffer. Proteins were excited using a wavelength of 280 nm and a slit width of 2.5 nm. Intrinsic fluorescence was measured at an emission wavelength of 340 nm for $KIV_7$ and 335 nm for $KIV_{10}$, respectively, and a slit width of 5.0 nm, with a cutoff filter of 290 nm placed in the emission beam. Ligand solutions contained a 1 $\mu$M amount of the respective kringles to eliminate dilution effects upon ligand addition. Titration curves were subjected to nonlinear regression analysis (SigmaPlot version 4.0, Jandel Scientific) to estimate apparent $K_D$ and $\Delta I_{max}$ values. The data were modeled according to the equation:

$$\Delta I = I_0 + \Delta I_{max}[\text{ligand}]/(K_D + [\text{ligand}]) \quad (1)$$

where $\Delta I$ is the change in relative fluorescence, $I_0$ is initial relative fluorescence, $\Delta I_{max}$ is the maximal change in relative fluorescence at saturation, and $K_D$ is the dissociation constant.

In an analogous fashion, the ability of L-proline to compete with $\epsilon$-ACA for binding to $KIV_7$ and $KIV_{10}$ and the ability of 4-aminobutyric acid (4-ABA) to compete with $\epsilon$-ACA for binding to $KIV_{10}$ were determined. Briefly, each protein was titrated with $\epsilon$-ACA, as described above, until saturation. Subsequently, L-proline or 4-ABA was titrated, and changes in intrinsic fluorescence were monitored. Kringle concentration was maintained at 2 $\mu$M throughout the assay. The dilution of $\epsilon$-ACA during the proline titration was accounted for in the data analyses. The data were modeled according to the equation:

$$\Delta I = (\partial i/2)\left\{\left([\text{Kr}]_0 + [\text{E}]_0 + K_D + \frac{K_D[\text{L}]_0}{K_I}\right) - \left[\left([\text{Kr}]_0 + [\text{E}]_0 + K_D + \frac{K_D[\text{L}]_0}{K_I}\right)^2 - 4[\text{Kr}]_0[\text{E}]_0\right]^{1/2}\right\} \quad (2)$$

where $\Delta I$ is the absolute change in fluorescence, $\partial i$ is the difference between the respective fluorescence coefficients for the free kringle and $\epsilon$-ACA-bound kringle (the fluorescence coefficient for the proline-bound kringle is identical to that of the free kringle), $K_D$ and $K_I$ are the dissociation constants for $\epsilon$-ACA and L-proline, respectively, and $[\text{Kr}]_0$, $[\text{E}]_0$, and $[\text{L}]_0$ are the respective total concentrations of kringle, $\epsilon$-ACA, and competing ligand (L-proline or 4-ABA).

*Molecular Modeling of $KIV_7$ in Complex with L-Proline.* Modeling studies of the binding site of $KIV_7$ were based on the crystal structure of $KIV_7$ (*31*). Modeling and structural analyses were carried out using Sybyl, version 5.3 (Tripos Inc., St. Louis, MO). Following removal of water molecules from the $KIV_7$ crystal structure, L-proline was approximately positioned in the LBS manually. The docking position of L-proline was optimized using the "flexible docking" routine in Sybyl; further energy minimization of the resulting model was performed using the energy gradient determination method employed in Sybyl. Diagrams were generated using MOLSCRIPT (*32*).

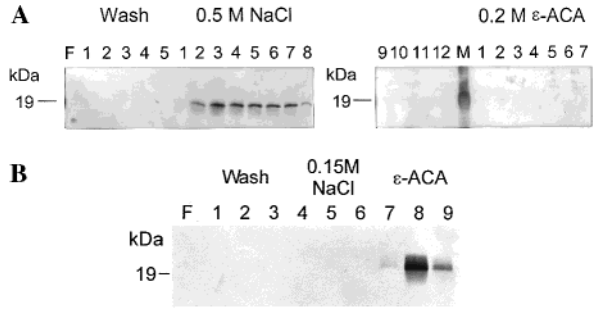


FIGURE 1: Binding of recombinant $KIV_7$ to lysine−Sepharose. Panel A: Purified recombinant $KIV_7$ was incubated with lysine−Sepharose for 1 h and the flowthrough (F) collected. The resin was washed with 20 mM Tris-HCl, pH 7.9, to collect unbound protein (wash fractions). Weakly bound protein was eluted with 20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl (0.5 M NaCl fractions). Protein specifically bound to the resin was eluted with 20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 0.2 M $\epsilon$-ACA (0.2 M $\epsilon$-ACA fractions). Fractions were analyzed for the presence of protein by SDS−PAGE analysis on a 15% polyacrylamide gel (15 $\mu$L/fraction, 30 $\mu$L flowthrough) followed by silver staining. Panel B: An experiment similar to that in panel A was performed except that the column was washed with 20 mM Tris-HCl, pH 7.9 (wash fractions), and then with 20 mM Tris-HCl, pH 7.9, containing 0.15 M NaCl prior to elution with a gradient of $\epsilon$-ACA in 20 mM Tris-HCl, pH 7.9. The gradient consisted of 20 mM steps such that fractions 7−9 correspond to 20, 40, and 60 mM $\epsilon$-ACA, respectively. The fractions were analyzed as described for panel A.

## RESULTS

*Analysis of Binding of $KIV_7$ to Lysine−Sepharose.* To characterize the lysine binding site (LBS) in apo(a) $KIV_7$, in comparison with that in apo(a) $KIV_{10}$ which has been described previously by LoGrasso and colleagues (*17*), we engineered constructs encoding these kringles in the pET16b vector for expression in *E. coli*. We have previously described the use of this expression system for the similar production of single apo(a) KIV domains in bacteria (*28*, *31*, *33*). To determine the lysine binding properties of $KIV_7$, in comparison with those of $KIV_{10}$, we initially assessed the ability of bacterially expressed $KIV_7$ to bind to a lysine−Sepharose column. Analysis of the elution fractions by SDS−PAGE revealed the absence of $KIV_7$ in both the flow-through and the "unbound" fractions obtained by washing the column with 20 mM Tris, pH 7.9 (i.e., containing neither NaCl nor $\epsilon$-ACA), indicating that $KIV_7$ was bound to the resin (Figure 1A). It should be noted that kringles that patently lack a lysine binding site (i.e., $KIV_2$ and mutants of $KIV_{10}$ that abolish the LBS) do not bind at all to this resin and appear in the flow-through and unbound fractions (ref *28*; M. N. Rahman and M. L. Koschinsky, unpublished results). All of the $KIV_7$ appeared to be removed from the lysine−Sepharose column with Tris buffer containing 0.5 M NaCl (Figure 1A) in contrast to $KIV_{10}$ expressed in this system, which can only be eluted by the addition of $\epsilon$-ACA (*28*). Thus, these data imply the presence of an LBS in $KIV_7$ but suggest that it exhibits a weaker lysine binding capacity relative to that of $KIV_{10}$. Indeed, $KIV_7$ could not be eluted from the column by Tris buffer containing 0.15 M NaCl (Figure 1B); subsequent addition of Tris buffer containing 20 mM $\epsilon$-ACA resulted in the elution of most of the $KIV_7$ from the column (Figure 1B).

*Measurement of Binding of Amino Acids to $KIV_7$ Compared to $KIV_{10}$.* Previous studies in our laboratory have demonstrated that a variety of amino acids can inhibit noncovalent interactions between apo(a) and LDL (*24*). We found that lysine, the lysine analogue $\epsilon$-ACA, arginine, phenylalanine, and proline inhibited the binding of a 17-kringle form of recombinant apo(a) [17K r-apo(a)] to immobilized LDL, an interaction mediated, in part, by $KIV_7$. Thus, we investigated the ability of these amino acids, as well as a variety of lysine analogues, to bind to this kringle using intrinsic fluorescence measurements. Purified $KIV_7$ was titrated with each of the respective amino acids and binding quantified from the observed changes in the intrinsic fluorescence of the protein. Specific binding of amino acids was associated with a saturable increase in the fluorescence intensity. The respective equilibrium dissociation constants were estimated by modeling the fluorescence data using nonlinear regression analysis according to an equation describing equilibrium binding (eq 1). As a comparison, parallel analyses were performed on purified $KIV_{10}$, which contains a strong LBS, as it has been characterized previously in a similar manner (*17*). Representative values are reported in the Table 1. L-Phenylalanine exhibited significant fluorescence at the wavelength utilized and, thus, could not be used for the characterization since the increase in protein fluorescence in the presence of phenylalanine was similar to the increase of signal achieved with phenylalanine alone (data not shown).

Table 1: Binding of Amino Acids to $KIV_7$ and $KIV_{10}$[a]

| amino acid | $KIV_7$ | | $KIV_{10}$ | |
|---|---|---|---|---|
| | $K_D$ | $\Delta I_{max}$ | $K_D$ | $\Delta I_{max}$ |
| L-lysine | $2.6 \pm 0.4$ mM | $0.45 \pm 0.02$ | $170 \pm 29$ $\mu$M | $0.44 \pm 0.02$ |
| $\epsilon$-ACA | $230 \pm 42$ $\mu$M | $0.18 \pm 0.09$ | $33 \pm 4$ $\mu$M | $0.66 \pm 0.02$ |
| $N_\alpha$-acetyl-L-lysine | $390 \pm 44$ $\mu$M | $0.43 \pm 0.02$ | $52 \pm 7$ $\mu$M | $0.59 \pm 0.02$ |
| $N_\epsilon$-acetyl-L-lysine | NC[b] | NC | NC | NC |
| 4-amino butyric acid | $260 \pm 13$ $\mu$M | $0.30 \pm 0.004$ | $150 \pm 22$ $\mu$M[c] | N/A[d] |
| L-arginine | $6.7 \pm 1.1$ mM | $0.54 \pm 0.02$ | >25 mM | >0.2 |
| L-proline | $4.5 \pm 0.5$ mM[c] | N/A | $54 \pm 5$ mM[c] | N/A |

[a] Kringles were titrated with the indicated amino acids, and the protein intrinsic fluorescence was recorded. $K_D$ and $\Delta I_{max}$ values were obtained as fit parameters for nonlinear regression of the fluorescence data to eq 1 (see Experimental Procedures); values given are from one representative experiment of $n$ trials ($n = 5$ for $KIV_7$; $n = 2$ for $KIV_{10}$) and are given $\pm$ the standard errors of the estimates provided by the regression algorithm. [b] No significant change in fluorescence observed. [c] Determined by competition for binding with $\epsilon$-ACA. [d] Not applicable.

In general, the binding affinities of $KIV_7$ for lysine and its analogues were found to be ~10-fold weaker than those determined for $KIV_{10}$ (Table 1); L-lysine was found to bind relatively weakly to $KIV_7$ with a $K_D$ of $2.6 \pm 0.4$ mM in comparison with $170 \pm 29$ $\mu$M for binding to $KIV_{10}$ (Figure 2). Removal or modification of the α-amino group of lysine increases its affinity for $KIV_7$ and $KIV_{10}$ by ~10-fold; the lysine analogues $\epsilon$-ACA and $N_\alpha$-acetyl-L-lysine were each found to be relatively good ligands for $KIV_7$ with $K_D$ values of $230 \pm 42$ $\mu$M and $390 \pm 44$ $\mu$M, respectively (Table 1). A change in the position of the acetyl group from $N_\alpha$-acetyl-L-lysine to $N_\epsilon$-acetyl-L-lysine resulted in the abolishment of binding to both $KIV_7$ and $KIV_{10}$.

In addition to the generally weaker affinity of the $KIV_7$ LBS relative to that of $KIV_{10}$, several differences in ligand preference were observed. Similar to L-lysine, L-arginine was

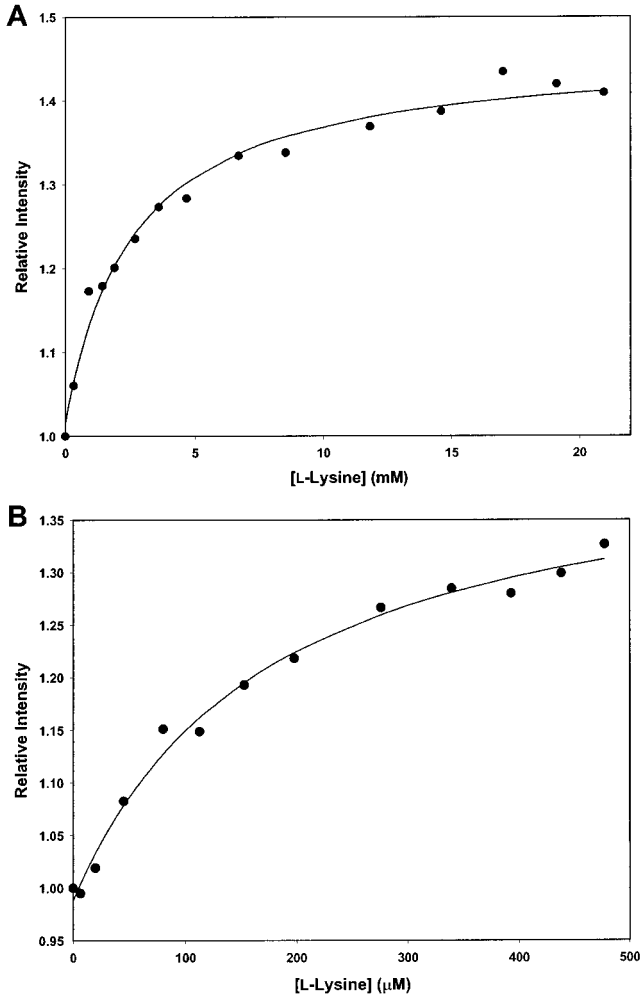


FIGURE 2: Binding of L-lysine to $KIV_7$ and $KIV_{10}$. $KIV_7$ (panel A) or $KIV_{10}$ (panel B) was titrated with lysine and the intrinsic protein fluorescence measured (symbols). The data were subjected to nonlinear regression to an equation describing equilibrium binding (eq 1; see Experimental Procedures) to obtain values for $K_D$ and $\Delta I_{max}$. The solid lines are regression lines calculated from the fit parameters. Titrations illustrated are representative of *n* independent experiments ($n = 5$ for $KIV_7$; $n = 2$ for $KIV_{10}$).

found to bind weakly to $KIV_7$ with a $K_D$ of 6.7 ± 1.1 mM (Table 1). However, L-arginine was found to be an even poorer ligand for $KIV_{10}$; saturation of the fluorescence signal was not observed at ligand concentrations of up to 25 mM. Additionally, 4-ABA was found to bind $KIV_7$ with an affinity ($K_D = 260 \pm 13$ $\mu$M) similar to that of $\epsilon$-ACA and $N_\alpha$-acetyl-L-lysine (Table 1). In contrast, titration of 4-ABA resulted in no significant change in the intrinsic fluorescence of $KIV_{10}$ at ligand concentrations up to 16 mM.

The addition of L-proline also resulted in no significant change in the intrinsic fluorescence of either $KIV_7$ or $KIV_{10}$ (data not shown). However, we have demonstrated that L-proline can interfere with the binding of $KIV_7$, but not of $KIV_{10}$, to plasmin-modified fibrinogen (*28*). These observations suggest that proline may bind to the $KIV_7$ LBS but in a manner which does not affect the tryptophan environment. To investigate this possibility, we determined the ability of L-proline to compete for binding of $\epsilon$-ACA to $KIV_7$ and $KIV_{10}$. The respective kringles were titrated with $\epsilon$-ACA until saturation of the signal was achieved; subsequently, increasing amounts of L-proline were added, and the resultant decrease in the intrinsic fluorescence was monitored (Figure 3). Equilibrium dissociation constants for L-proline values were estimated by modeling the fluorescence data according to eq 2 (see Experimental Procedures). In this manner, L-proline was found to bind $KIV_7$ and $KIV_{10}$ with $K_I$ values of 4.5 ± 0.5 mM and 54 ± 5 mM, respectively (Table 1).

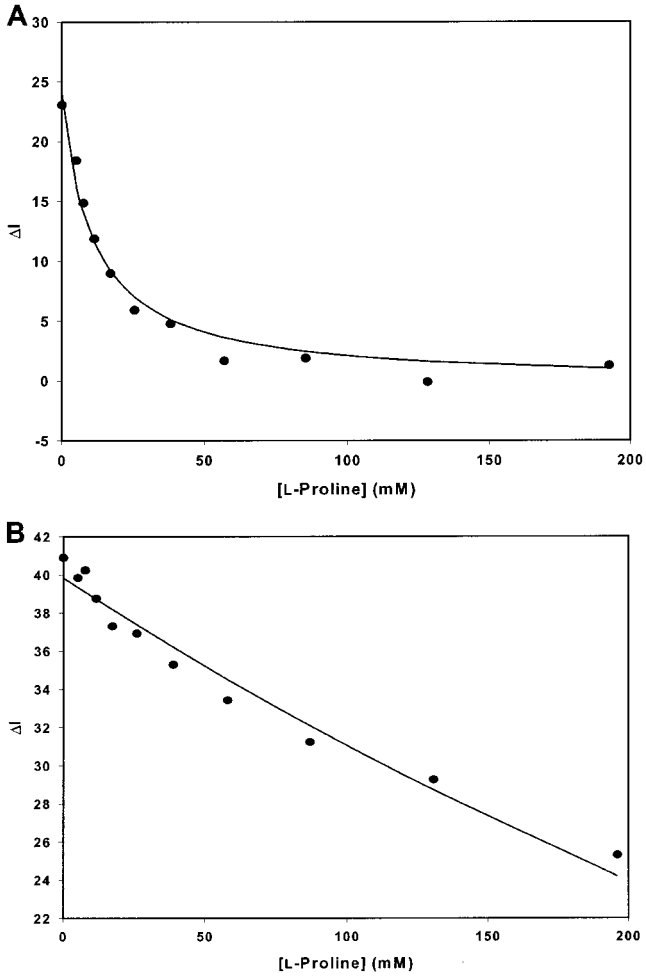


FIGURE 3: Binding of L-proline to $KIV_7$ and $KIV_{10}$. $KIV_7$ (panel A) and $KIV_{10}$ (panel B) were titrated with $\epsilon$-ACA until the intrinsic fluorescence of the protein reached saturation. Increasing amounts of L-proline were then added while maintaining the protein concentration at 2 $\mu$M, and the intrinsic fluorescence of the protein was recorded (symbols). The data were subjected to nonlinear regression analysis using an equation describing competitive binding equilibria (eq 2; see Experimental Procedures) to obtain the inhibition constant (effective dissociation constant) for L-proline. The solid lines are regression lines calculated from the fit parameters. Titrations illustrated are representative of three independent experiments.

Similarly, we used this competition strategy to assess the ability of 4-ABA to bind to $KIV_{10}$. A $K_I$ value of 150 ± 22 $\mu$M was found, which is comparable to the $K_D$ for binding of this ligand to $KIV_7$ (Table 1).

## DISCUSSION

Using the pET bacterial expression system, we have expressed recombinant apo(a) $KIV_7$ and purified it to homogeneity. The structural integrity of the resulting protein was recently confirmed by our successful crystallization of $KIV_7$ and solving of its structure to a resolution of 1.45 Å (*31*). In the present study, using this recombinant $KIV_7$ protein, we have presented direct evidence of the existence

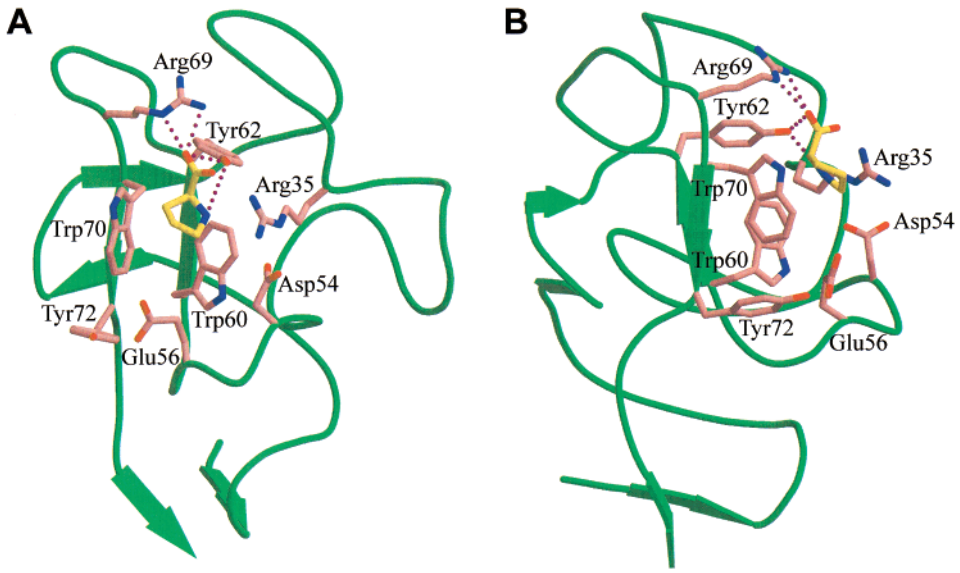


FIGURE 4: Model of apo(a) $KIV_7$ complexed with L-proline. The molecular model is based on the coordinates of the $KIV_7$ crystal structure (*33*). The $KIV_7$ backbone is shown in green, $KIV_7$ amino acid side chains are shown in pink, and L-proline is shown in yellow. Potential hydrogen bond interactions between the ligand and $KIV_7$ are indicated as dotted lines. Panels A and B are views rotated approximately 90° to each other. The diagram was prepared using MOLSCRIPT.

of a weak LBS in this kringle. The presence of an intrinsic lysine binding ability was initially demonstrated by the ability of $KIV_7$ to bind lysine−Sepharose (Figure 1). Comparison of the binding properties of the $KIV_7$ LBS with those of $KIV_{10}$ revealed that, unlike $KIV_{10}$, $KIV_7$ could be eluted from lysine−Sepharose by the addition of Tris buffer containing 0.5 M NaCl; elution of $KIV_{10}$ from this resin required the addition of the lysine analogue $\epsilon$-ACA (*28*). $KIV_7$ did indeed bind specifically to this resin, as evidenced by the inability of a 0.15 M NaCl wash to elute the kringle but the ability of a 20 mM $\epsilon$-ACA to elute the kringle. In contrast, $KIV_2$ expressed in this bacterial system was found not to bind lysine−Sepharose, being present both in the flow-through and in fractions resulting from washing the resin with Tris buffer in the absence of NaCl and $\epsilon$-ACA (data not shown), which is consistent with molecular modeling studies that predict the absence of a lysine binding capacity of this kringle domain (*16*). Taken together, these initial results suggested that $KIV_7$ contains a functional LBS which exhibits a weaker lysine binding capacity than that of $KIV_{10}$.

To characterize further the LBS of $KIV_7$ with respect to ligand specificity and affinity, equilibrium binding analyses were performed in which ligand binding was detected as a change in the intrinsic fluorescence of the protein. These analyses (Figure 2, Table 1) provided direct evidence that $KIV_7$ is capable of binding lysine and lysine analogues. Upon comparison with results obtained in parallel for $KIV_{10}$ it was revealed that, in general, while the relative preferences of $KIV_7$ for lysine and its analogues were comparable to those of $KIV_{10}$ (i.e., $\epsilon$-ACA $\approx$ $N_\alpha$-acetyl-L-lysine $>$ L-lysine, no binding to $N_\epsilon$-acetyl-L-lysine), the binding affinities of $KIV_7$ for these ligands were much weaker, with $K_D$ values ~10-fold greater for L-lysine, $\epsilon$-ACA, and $N_\alpha$-acetyl-L-lysine relative to $KIV_{10}$ (Table 1). As expected from the known properties of the $KIV_{10}$ LBS (*17*), lysine was found to be a poorer ligand than $\epsilon$-ACA; the affinity of the $KIV_7$ LBS for $\epsilon$-ACA was comparable to that observed for $N_\alpha$-acetyl-L-lysine (230 $\pm$ 42 $\mu$M and 390 $\pm$ 44 $\mu$M, respectively, Table 1), indicating a potential repulsive interaction associated with the $\alpha$-amino group of L-lysine. Interestingly, the presence of the acetyl group at the $N_\epsilon$-position of lysine resulted in the apparent loss of binding to the LBS of both $KIV_7$ and $KIV_{10}$, indicating the importance of this position in forming stabilizing interactions with the respective LBS.

Information derived from the crystal structure of the $KIV_7$ protein (*31*) provides a structural rationale for the differences in both affinity and ligand specificity between $KIV_7$ and $KIV_{10}$ that we have observed in this study. The LBS of $KIV_{10}$ consists of a hydrophobic trough, lined by $Trp^{60}$, $Phe^{62}$, and $Trp^{70}$, which acts to stabilize the aliphatic backbone of $\omega$-amino acids such as lysine. Anionic ($Asp^{54}$ and $Asp^{56}$) and cationic ($Arg^{35}$ and $Arg^{69}$) charge pairs on either side of the trough act to stabilize the amino and carboxyl groups, respectively, of the zwitterionic ligand (*18*, *19*; reviewed in *34*). The $KIV_7$ LBS contains two substitutions of the residues critical for lysine interaction in $KIV_{10}$: $Phe^{62}$ → Tyr and $Asp^{56}$ → Glu. The substitution of $Tyr^{62}$ in $KIV_7$, in conjunction with the presence of $Arg^{35}$, results in the formation of a unique network of hydrogen bonds and electrostatic interactions which act to restrict the accessibility of the $KIV_7$ LBS as well as to diminish the flexibility of key residues within the LBS to, subsequently, reduce their adaptability in accommodating ligands. Furthermore, this interaction network eliminates critical lysine-stabilizing interactions involving $Asp^{54}$ and the ligand amino group. The presence of the hydroxyl group of $Tyr^{62}$ may also contribute to the weaker affinity of the $KIV_7$ LBS by steric hindrance with the ligand as well as by decreasing the hydrophobicity of the hydrophobic trough (*31*).

The ligand L-arginine has been shown to inhibit binding of both $KIV_{10}$ and $KIV_7$ to plasmin-modified fibrinogen surfaces at concentrations of 0.2 M amino acid (*33*). The present study confirms that this ligand can interact directly with the LBS of $KIV_7$. It should be noted that for $KIV_{10}$, while a change in intrinsic fluorescence was observed upon addition of L-arginine, saturation was not achieved at ligand concentrations $>$25 mM. This is in contrast to studies by LoGrasso and colleagues in which a $K_D$ of 4.6 mM was

reported for the binding of L-arginine to bacterially expressed $KIV_{10}$ (*17*). The reasons for this discrepancy are unclear at present. However, while the recombinant kringles produced in our study contained the core kringles flanked by the respective N- and C-terminal interkringle sequences in their entirety, kringles used in the study reported by LoGrasso et al. (*17*) contained minimal sequences outside the core kringle domain (three and seven residues preceding and following the first and last cysteine residues of the $KIV_{10}$ sequence, respectively). Our high-resolution crystallographic studies on $KIV_7$, which include the entire interkringle domain, revealed that these sequences are flexible (*31*). This finding suggests that intramolecular interactions involving the $KIV_{10}$ interkringle regions may have affected arginine binding in our study. Such interactions would not be observed in the context of intact apo(a), however.

We have recently observed that the binding of $KIV_7$ to plasmin-modified fibrinogen is inhibited in the presence of proline, yet this ligand shows no effect on the binding $KIV_{10}$ to these surfaces (*28*). In the present study, we have demonstrated the ability of L-proline to compete with $\epsilon$-ACA for the LBS of $KIV_7$ and, to a lesser extent, $KIV_{10}$, observed as a decrease in the fluorescence of the $\epsilon$-ACA-saturated protein upon addition of L-proline (Figure 3). These analyses, taken together, suggest that the $KIV_7$ LBS may be capable of binding proline and led to the development of a structural model by which this may occur (Figure 4). The structure of this amino acid is unique, exhibiting no structural similarity with the other ligands for which experimental $K_D$ values could be obtained. Notably, L-proline is too short to interact with both of the active, charged centers of the binding pocket simultaneously (i.e., $Asp^{54}$/$Glu^{56}$ and $Tyr^{62}$/$Arg^{69}$). The ligand carboxyl group would presumably play a major stabilizing role as it bears significantly more charge than the nitrogen in the pentagonal ring. Modeling revealed that it is possible to orient L-proline such that both of its charged centers are involved in hydrogen bond interactions with $KIV_7$: the carboxyl group forms two with $Arg^{69}$ and one with $Tyr^{62}$ while the ring nitrogen forms one with $Tyr^{62}$ (Figure 4). The hydrophobic trough (lined by $Trp^{60}$, $Tyr^{62}$, and $Trp^{70}$) and the anionic center ($Asp^{54}$/$Glu^{56}$) are not predicted to be involved in stabilizing interactions with the amino acid, which accounts for the relatively weak binding of proline to $KIV_7$. Substitution of Phe in $KIV_{10}$ for the corresponding $Tyr^{62}$ of $KIV_7$ would result in the loss of two potential hydrogen bond interactions to significantly reduce the stabilization of this ligand ($K_D$ = 54 mM versus 4.5 mM for $KIV_7$; see Table 1).

The binding of lysine (and its analogues) occurs by insertion of a hydrophobic segment into the binding pocket which causes a substantial increase in the local hydrophobicity surrounding the tryptophan residues ($Trp^{60}$ and $Trp^{70}$) within the hydrophobic trough. The resultant increase in fluorescence intensity observed upon ligand binding is, thus, attributable to a subsequent increase in the fluorescence quantum yield of these tryptophan residues. Our model suggests that proline binds $KIV_7$ in a manner which would cause minimal interference with the tryptophan residues of the hydrophobic trough, thus accounting for the inability of L-proline to alter the intrinsic fluorescence of the kringle.

Interestingly, binding of 4-ABA to $KIV_7$ increased the intrinsic fluorescence of the kringle, but binding of this ligand to $KIV_{10}$, while comparable in affinity, did not result in a fluorescence change. These observations may be accounted for by consideration of the differences in the sizes of the two binding pockets as described for proline above. Therefore, we suggest that the smaller pocket of $KIV_7$, in concert with the additional ligand contact made available by the presence of $Tyr^{62}$ rather than Phe, allows 4-ABA to bind to this kringle in a manner analogous to that of lysine. As such, the fluorescence of $Trp^{60}$ and $Trp^{70}$ is quenched. Conversely, the presence of $Phe^{62}$, and thus the absence of the hydroxyl group, and the shorter side chain of $Asp^{56}$ eliminate two points of contact for 4-ABA in $KIV_{10}$, and so this ligand may bind to $KIV_{10}$ outside of the LBS in a manner analogous to that of proline, producing no detectable fluorescence change.

Although studies have shown that the LBS of $KIV_7$ is masked in the context of the Lp(a) particle (*26*), there is evidence of the existence of uncomplexed apo(a), both intact and fragmented, in the plasma [~3−10% of total apo(a) (reviewed in ref *35*)] as well as in atherosclerotic lesions [~50% of total apo(a) (*36*, *37*)]. Moreover, it is feasible that the noncovalent interactions between apo(a) and apoB-100 do not result in the LBS of $KIV_6$−$KIV_8$ being permanently apposed to the LDL surface, especially given the weak affinity of the $KIV_7$ LBS and, presumably, the other LBS of this region (*16*). As with all reversible binding interactions, an equilibrium between bound and unbound states may exist in vivo to result in limited accessibility of the $KIV_7$ domain. Thus, there is potential for the $KIV_7$ domain to mediate interactions in these contexts. Indeed, lysine- and proline-sensitive interactions with plasmin-modified fibrinogen have been described for Lp(a) containing a defective LBS in $KIV_{10}$ (*23*) as well as for $KIV_7$ (*28*). Using truncated r-apo(a) derivatives, the $KIV_7$ domain has also been implicated in mediating proline-sensitive cell surface binding to CHO cells (*38*). In summary, the unique properties of the $KIV_7$ LBS are compatible with a role in mediating noncovalent association of apo(a) with apoB-100 in Lp(a) assembly, as well as in mediating interactions of uncomplexed apo(a) or apo(a) fragments with biological substrates such as fibrin(ogen).

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