Quantitative Evaluation of the Contribution of Weak Lysine-binding Sites Present within Apolipoprotein(a) Kringle IV Types 6–8 to Lipoprotein(a) Assembly*  

Lev Becker, P. Michael Cook, Theodore G. Wright, and Marlys L. Koschinsky†  
From the Department of Biochemistry, Queen’s University, Kingston, Ontario K7L 3N6, Canada  

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During lipoprotein(a) (Lp(a)) assembly, non-covalent interactions between apolipoprotein(a) (apo(a)) and low density lipoprotein precede specific disulfide bond formation. Studies have shown that the non-covalent step involves an interaction between the weak lysine-binding sites (WLBS) present within each of apo(a) kringle IV types 6, 7, and 8 (KIV<sub>6–8</sub>) and two lysine residues (Lys<sup>680</sup> and Lys<sup>690</sup>) within the NH<sub>2</sub> terminus of the apolipoprotein B-100 (apoB) component of low density lipoprotein. In the present study, we introduced single point mutations (E56G) into each of the WLBS present in apo(a) KIV<sub>6–8</sub> and expressed these mutations in the context of a 17-kringle (17K) recombinant apo(a) variant. Single mutations that disrupt the WLBS in KIV<sub>6</sub> apo(a), KIV<sub>7</sub>, or both KIV<sub>7</sub> and KIV<sub>8</sub>, were assessed for their ability to form non-covalent and covalent Lp(a) complexes. Our results demonstrate that both apo(a) KIV<sub>7</sub> and KIV<sub>8</sub> but not KIV<sub>6</sub> are required for maximally efficient non-covalent and covalent Lp(a) assembly. Single mutations in the WLBS of KIV<sub>6</sub> or KIV<sub>8</sub> resulted in a 3-fold decrease in the affinity of 17K recombinant apo(a) for apoB, and a 20% reduction in the rate of covalent Lp(a) formation. Tandem mutations in the WLBS in both KIV<sub>7</sub> and KIV<sub>8</sub> resulted in a 19-fold reduction in the binding affinity between apo(a) and apoB, and a 75% reduction in the rate of the covalent step of Lp(a) formation. We also showed that KIV<sub>6</sub> and KIV<sub>8</sub> specifically bind with high affinity to apoB-derived peptides containing Lys<sup>680</sup> or Lys<sup>690</sup>, respectively. Taken together, our data demonstrate that specific interactions between apo(a) KIV<sub>7</sub> and KIV<sub>8</sub> and Lys<sup>680</sup> and Lys<sup>690</sup> in apoB mediate a high affinity non-covalent interaction between apo(a) and low density lipoprotein, which dictates the efficiency of covalent Lp(a) formation.

Lipoprotein(a) (Lp(a))<sup>†</sup> contains a low density lipoprotein (LDL)-like moiety that is attached to the glycoprotein apolipoprotein(a) (apo(a)) by a single disulfide bond (1, 2). The LDL moiety of Lp(a) is similar to plasma-derived LDL both in lipid composition as well as in the presence of apolipoprotein B-100 (apoB). As such, the apo(a) component likely confers the unique structural and functional properties that have been attributed to Lp(a). Apo(a) contains tandem repeats of a sequence that closely resembles plasminogen kringle IV, followed by sequences that are highly homologous to the kringle V and protease domains of plasminogen (3). Based on amino acid sequence, apo(a) contains 10 distinct subclasses of plasminogen kringle IV-like domains (3); the kringle IV type 2 domain (KIV<sub>2</sub>) is present in a variable number of identically repeated copies, which is the molecular basis for the observed isofrom size heterogeneity of Lp(a) (4, 5). An unpaired cysteine residue in apo(a) KIV<sub>7</sub> (Cys<sup>165</sup>) is involved in disulfide linkage with apoB to form covalent Lp(a) particles (1, 2).

Numerous epidemiological studies (both case-control and prospective designs) have identified elevated plasma Lp(a) concentrations as a risk factor for the development of atherosclerotic disorders including coronary heart disease (reviewed in Refs. 6 and 7). Conventional therapies such as lifestyle changes and statin therapy, which are highly effective in lowering plasma LDL concentrations, have been relatively unsuccessful in reducing Lp(a) concentrations (7). As such, alternative strategies for lowering plasma Lp(a) levels have been suggested, including inhibition of the assembly of Lp(a) particles (8–10). It is generally accepted that Lp(a) assembly is a two-step process in which initial non-covalent interactions between apo(a) and apoB precede disulfide bond formation (10, 11), which occurs between cysteine residues located within the carboxyl-terminal ends of the respective proteins (1, 2, 12, 13). Although previous data indicate that the efficiency of the covalent step of Lp(a) assembly is dictated by the affinity of the non-covalent interaction between apo(a) and apoB (11, 14), we have recently demonstrated that the formation of apo(a) is also an important determinant of the rate of covalent Lp(a) particle formation (15). Apo(a) adopts a closed conformation that inhibits covalent Lp(a) assembly, possibly by restricting access to the free sulphhydryl group in apo(a) KIV<sub>2</sub> (15). In this regard, we have previously shown that the addition of the lysine analog ε-aminocaproic acid (ε-ACA) elicits a conformational change in apo(a) to a more open structure, which results in a 6-fold enhancement in its ability to form covalent Lp(a) particles.

Sequences have been identified within both the NH<sub>2</sub> and COOH termini of apoB that mediate its non-covalent interaction with apo(a) (9, 16, 17). Previous data from our group underscore a role for sequences within the NH<sub>2</sub>-terminal 18% of apoB (apoB-18) in mediating its lysine-dependent, non-covalent association with apo(a) (16). More recently, using a combination of citraconic anhydride modification and site-directed mutagenesis, we have identified two lysine residues (Lys<sup>680</sup> and Lys<sup>690</sup>) within apoB-18 that are required for non-covalent binding to apo(a) (18); mutation of Lys<sup>680</sup> was found to completely abolish non-covalent binding to apo(a), whereas mu-

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† Career Investigator of the Heart and Stroke Foundation of Ontario.
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tation of Lys^690 significantly diminished the affinity of this interaction.

With respect to sequence requirements in apo(a) that mediate its non-covalent interaction with apoB, several groups have shown that the weak lysine-binding sites (WLBS) present within apo(a) KIV5–8 are likely involved in mediating this interaction (11, 14, 19–21). Studies performed by several investigators have underscored the importance of KIV6 (10, 14, 20, 21) and possibly KIV7 (10, 14, 21) in this process. Previous data from our group also suggest that the WLBS in apo(a) KIV8 plays a major role in non-covalent binding to LDL (14); all mutants in both KIV6 and KIV7, or KIV7 and KIV8; all of these were then inserted, using a number of steps, into the 17K r-apo(a) expression plasmid corresponding to the 17KKIV6E56G variant.

### Construction and Expression of Recombinant Apo(a) Variants in Mammalian Cells

Mammalian Cells — The plasmid encoding the 6K r-apo(a) derivative (pRK5ha6 plasmid) (11) was used as a PCR template in the construction of an expression plasmid encoding the 17K KIV6E56G variant. Fragments generated by PCR using primer pair 5 and 6 and pair 7 and 4 were used (see Table I) to create the mutation. Fragments containing the mutation were cloned into pRK5ha17 to generate the expression plasmid designated pRK5ha17KIV6E56G.

Human embryonic kidney (293) cells stably expressing each of the r-apo(a) derivatives were generated as previously described (22). Briefly, these cells were transfected by the calcium phosphate co-precipitation method using 10 μg of each r-apo(a) plasmid and 1 μg of a plasmid encoding the neomycin resistance gene per 100-mm plate. Stable transformants were selected by culturing the cells in the presence of 0.8 mg/ml G418 (Invitrogen) until foci developed. At this time, the foci were picked and screened for apo(a) expression by Western blot analysis.

### Purification of Recombinant Apo(a) Variants

Purification of LDL— LDL was purified from the plasma of a normolipidemic volunteer as previously described (18). The concentration of purified LDL was determined using a modified Bradford assay, and the lipoprotein was stored at 4°C in the presence of 1 mM phenylmethyl-sulfonyl fluoride and 1 mM EDTA for a maximum of 5 days prior to use.

**Table 1**  
Primer sequences used for generation of recombinant apo(a) variants by PCR

| Primer | Sequence* |
|--------|-----------|
| 1 (18-mer) | 5'-CCACAGGTGTCACCTCCC-3' |
| 2 (27-mer) | 5'-AGGACTAATGCCGCGATTGGATTCTCT-3' |
| 3 (27-mer) | 5'-AACCTGACATTGCCGCCTGCTTCCTGG-3' |
| 4 (20-mer) | 5'-CCTGGACGCCAGTGCGTGGTTT-3' |
| 5 (22-mer) | 5'-GGACGGCGCTGTTCTTGAAACA-3' |
| 6 (24-mer) | 5'-CCAGATGCAGGACCTAACCTGGTTATAT-3' |
| 7 (30-mer) | 5'-CCAGATGCAGGACCTAACCTGGTTATAT-3' |
| 8 (30-mer) | 5'-CCAGATGCAGGACCTAACCTGGTTATAT-3' |
| 9 (17-mer) | 5'-TAACCTATTATAAGCTGC-3' |
| 10 (33-mer) | 5'-GGAGTCAGATATTGGACCAACACGGGAGAAGCC-3' |
| 11 (33-mer) | 5'-GGAGTCAGATATTGGACCAACACGGGAGAAGCC-3' |

* Restriction sites are underlined; engineered mutations are in italics.

### EXPERIMENTAL PROCEDURES

**Construction and Expression of Recombinant Apo(a) Variants**

The plasmid encoding the 6K r-apo(a) derivative (pRK5ha6 plasmid) (11) was used as a PCR template in the construction of an expression plasmid encoding the 17K KIV6E56G variant. Fragments generated by PCR using primer pair 5 and 6 and pair 7 and 4 were used (see Table I) to create the mutation. Fragments containing the mutation were cloned into pRK5ha17 to generate the expression plasmid designated pRK5ha17KIV6E56G.

### Purification of LDL— LDL was purified from the plasma of a normolipidemic volunteer as previously described (18). The concentration of purified LDL was determined using a modified Bradford assay, and the lipoprotein was stored at 4°C in the presence of 1 mM phenylmethyl-sulfonyl fluoride and 1 mM EDTA for a maximum of 5 days prior to use.

### Purification of Recombinant Apo(a) Variants—All r-apo(a) variants used in this study, with the exception of KIV6 and KIV8, were purified from the conditioned medium of stably expressing 293 cell lines using lysine-Sepharose chromatography as previously described (24). Protein concentrations for all r-apo(a) derivatives were obtained by absorbance measurements at 280 nm (corrected for Rayleigh scattering) using previously determined extinction coefficients (25). Protein integrity and purity were assessed by SDS-PAGE under non-reducing and reducing conditions followed by staining with Coomassie Blue.

Single kringle constructs encoding KIV7 or KIV6 were used to transform BL21 (DE3) cells (Novagen). LB containing ampicillin was inoculated with 1 ml M, and cultures were grown for an additional 3 h at 37°C.

### Role of Apo(a) Weak Lysine-binding Sites in Lp(a) Assembly

With respect to sequence requirements in apo(a) that mediate its non-covalent interaction with apoB, several groups have shown that the weak lysine-binding sites (WLBS) present within apo(a) KIV5–8 are likely involved in mediating this interaction (11, 14, 19–21). Studies performed by several investigators have underscored the importance of KIV6 (10, 14, 20, 21) and possibly KIV7 (10, 14, 21) in this process. Previous data from our group also suggest that the WLBS in apo(a) KIV8 plays a major role in non-covalent binding to LDL (14); all studies to date have demonstrated no role for apo(a) KIV5. In the above studies, domains in apo(a) involved in non-covalent binding to apoB were identified by sequential truncation of full-length apo(a), followed by assessment of binding to immobilized LDL. Using this approach, however, neither the individual contribution of each WLBS to the non-covalent interaction with apoB in the context of full-length apo(a) nor the quantitative relationship between apo(a)/LDL non-covalent interactions and covalent Lp(a) assembly can be assessed. Instead, based on our recent studies, the truncated derivatives previously used would lack the closed conformation that is adopted by full-length apo(a).2

In the present study, we generated mutations in the WLBS of apo(a) KIV6, KIV7, and KIV8, as well as tandem WLBS mutants in both KIV6 and KIV7, or KIV7 and KIV8; all of these mutations were made in the context of a 17-kringle-containing apo(a) variant (17K; Ref. 22), which corresponds to a physiologically relevant apo(a) isoform (4, 5). The apo(a) variants were assessed for their ability to associate with LDL in solution and for the efficiency with which they form covalent Lp(a) particles. We further determined whether lysine residues (Lys^680 and Lys^690), within apoB-18 are important for binding to the WLBS in apo(a) by measuring solution phase interactions between bacterially expressed apo(a) KIV7 and KIV6 and synthetic peptides containing apoB Lys^680 and Lys^690. Taken together, our data identify a critical role for the WLBS in both apo(a) KIV7 and KIV8 in mediating non-covalent association with the Lys^680 and Lys^690 residues in apoB, respectively, and demonstrate that these interactions are required for the covalent step of Lp(a) formation.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of Recombinant Apo(a) Variants**

Construction of expression plasmids for KIV6 and KIV8 was achieved using site-directed mutagenesis of the pRK5ha6 plasmid (14) and the pRK5haKIV6-P plasmid (11) used as PCR templates in the construction of an expression plasmid encoding the 17K KIV6E56G variant; primer pair 1 and 6 and pair 7 and 4 were used (see Table I) to create the mutation. Fragments containing the mutation were cloned into pRK5ha17 to generate the expression plasmid designated pRK5ha17KIV6E56G.

The pRK5haKIV6-P plasmid (14) and the pRK5haKIV6-P plasmid (11) were used as PCR templates in the construction of an expression plasmid encoding the 17K KIV6E56G variant; primer pair 8 and 9, respectively, were used for the PCR reaction (see Table I). Mutated fragments were cloned into pRK5ha17 to generate the expression vector pRK5ha17KIV6E56G. Constructs encoding the 17K KIV6E56G and 17K KIV7E56G were generated via a number of cloning steps making use of fragments derived from the plasmids encoding single mutations in the WLBS of KIV6, KIV7, and KIV8, described above.

### Purification of LDL— LDL was purified from the plasma of a normolipidemic volunteer as previously described (18). The concentration of purified LDL was determined using a modified Bradford assay, and the lipoprotein was stored at 4°C in the presence of 1 mM phenylmethyl-sulfonyl fluoride and 1 mM EDTA for a maximum of 5 days prior to use.

**Purification of Recombinant Apo(a) Variants—All r-apo(a) variants used in this study, with the exception of KIV6 and KIV8, were purified from the conditioned medium of stably expressing 293 cell lines using lysine-Sepharose chromatography as previously described (24). Protein concentrations for all r-apo(a) derivatives were obtained by absorbance measurements at 280 nm (corrected for Rayleigh scattering) using previously determined extinction coefficients (25). Protein integrity and purity were assessed by SDS-PAGE under non-reducing and reducing conditions followed by staining with Coomassie Blue.

Single kringle constructs encoding KIV7 or KIV6 were used to transform BL21 (DE3) cells (Novagen). LB containing ampicillin was inoculated with an overnight culture of transformed BL21 (DE3) cells and grown to an A_{600} of ~0.8. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM, and cultures were grown for an additional 3 h at 37°C. Cells were harvested by centrifugation, and the cell pellets were lysed by sonication. Following centrifugation, the insoluble pellet was then solubilized by the addition of a buffer containing 6 M guanidine HCl. The purifica-
tion of the single kringles was performed essentially as we have previously described for KIV6, using TFA-Sepharose chromatography and gel filtration (23), with a few minor modifications. Following gel filtration, proteins were dialyzed against 20 mM Tris-HCl pH 7.9, 1 mM EDTA for 2 h, and were then dialyzed against double-distilled H2O. Purified proteins were concentrated by lyophilization; before use, protein pellets were dissolved in 20 mM Tris-HCl, pH 7.9, and protein concentrations were determined by measurement of absorbance at 280 nm (corrected for Rayleigh scattering), using calculated extinction coefficients (ε280 = 2.12 and 2.24 for KIV6 and KIV8, respectively) (25). Protein integrity and purity were assessed by SDS-PAGE under non-reducing and reducing conditions, followed by staining with Coomassie Blue.

**Analytical Ultracentrifugation of Apo(a)—**Sedimentation velocity experiments on the 17K and 17K variants were performed using a Beckman XL-I analytical ultracentrifuge as previously described (15).

**Modification of LDL**—LDL was labeled with [35S]iodoacetamidofluorescein as previously described (15). The concentration of fluorescein-labeled LDL (flu-LDL) was determined using a modified Bradford assay, and the modified lipoprotein was stored at 4°C for no longer than 3 days prior to use.

**Binding of Recombinant Apo(a) to Flu-LDL**—Apo(a) binding to flu-LDL was performed as previously described (15). Briefly, fluorescein fluorescence measurements of flu-LDL were performed using an LS50B Luminescence Spectrometer (PerkinElmer Life Sciences). Flu-LDL (50 nM) was titrated with 17K, 17KIV7 or 17KIV8, respectively, at concentrations of KIV7 or KIV8 to eliminate dilution effects, were added to buffer. The fluorescence values obtained from this titration were used in the determination of dissociation constant values for the interactions between apo(a) and LDL, respectively. LDL was labeled with [35S]cysteine as previously described (15). The corresponding LDL sample was incubated with [35S]cysteine-labeled LDL (50 nM) was incubated with conditioned medium, followed by staining with Coomassie Blue and purified to homogeneity as determined by SDS-PAGE under non-reducing conditions, followed by staining with Coomassie Blue.

**Role of Apo(a) Weak Lysine-binding Sites in Lp(a) Assembly**—We have recently demonstrated that apo(a) is maintained in a closed conformation that can be disrupted by the addition of the lysine analog e-ACA (15). Importantly, data from our previous study predicts that the WLBS in apo(a) KIV5–7 are stably expressed in 293 cells and metabolically labeled with [35S]cysteine as previously described. Following electrophoresis, gels were placed in fixing solution (25% methanol, 12.5% acetic acid in double-distilled H2O) for 20 min, followed by incubation in Amplify solution (Amersham Life Sciences) for 20 min. The gels were then dried and exposed to a PhosphorImager screen (Bio-Rad) for 16 h. The screen was developed using a Bio-Rad Molecular Imager FX, and the bands were quantified using Quantity One 4.0.1 densitometry software. The extent of covalent apo(a) particle formation was quantified according to the following formula: %Lp(a) = [Lp(a) - (Lp(a))]/(Lp(a)) × 100. The data were fit according to an exponential rise to maximum formula given in Equation 2, which was derived from first principles assuming that covalent apo(a) formation occurs as a two-step process.

\[ %Lp(a) = 100 \times \left(1 - e^{-k(t - t_0)} \right) \]  
(Eq. 2)

Where %Lp(a) is the amount of Lp(a) formed at time t, t is the time of reaction, and k is a constant is described by the following relationship.

\[ a = \frac{B_l}{[B_l]_0 + K_d} \]  
(Eq. 3)

Where [B_l]_0 is the total concentration of LDL, K_d is the dissociation constant for the interaction between apo(a) and LDL, and k is the rate constant for disulfide bond formation.

**ApoB Peptide Synthesis and Purification**—ApoB peptides corresponding to amino acids 675–689, apoB675–689 (apoB685–699) in the primary sequence of apoB were synthesized by the Alberta Peptide Institute (Edmonton, Alberta, Canada). Both of the peptides were purified using high performance liquid chromatography, lyophilized, and stored at −20°C. Peptides were reconstituted in 20 mM Tris base, and the solution was adjusted to pH 7.9 by the dropwise addition of 1 N HCl. Reconstituted peptides were stored at −70°C prior to use.

**Measurement of the Binding of e-ACA and ApoB Peptides to KIV7 and KIV8 Using Intrinsic Fluorescence**—Intrinsic fluorescence experiments were conducted using an LS50B Luminescence Spectrometer (PerkinElmer Life Sciences). Apo(a) KIV7 and KIV8 were each titrated with e-ACA, apoB675–689, or apoB685–699. Titrations were performed in 20 mM Tris-HCl, pH 7.9, containing 0.1% Tween 20 in a quartz cuvette, which had been conditioned with this buffer prior to use. Tryptophan residues were excited at a wavelength of 280 nm and a slit width of 2.5 nm, whereas tryptophan emission was detected at a wavelength of 340 nm and a slit width of 5 nm with a 290-nm cut-off filter placed in the emission beam. Ligand solutions, containing equimolar concentrations of KIV7 or KIV8, to eliminate dilution effects, were added in a stepwise manner until saturation of the fluorescence change was attained. To determine K_d values for the interactions, titration curves were subjected to non-linear regression analysis using hyperbolic equations for binding to e-ACA, and quadratic equations (see Equation 1) for binding to the apoB-derived peptides. For titrations with e-ACA, a final concentration of 2 μM KIV7 or KIV8 was used, whereas titrations with the apoB-derived peptides were performed using 200 nM KIV7 or KIV8. Because apoB685–699 contains a tryptophan residue, an additional control experiment was performed in which identical concentrations of the peptide to those used in the titrations of KIV7 and KIV8 were added to buffer. The fluorescence values obtained from this titration were subjected to linear regression; the slope from this fit (λ_s) was used to correct for the direct contribution of apoB685–699 to the fluorescence signal obtained from the titrations of KIV7 and KIV8, respectively. The formula used to obtain K_d values for the interactions between apoB685–699 and KIV7 or KIV8 is as follows.

\[ ΔI = i_b[B_l]_0 + 0.5 \times dI \times ([K_d] + [A_l]) - [B_l]_0 \times ([K_d] + [A_l]) - [B_l]_0 - 4[A_l][B_l]_0 \]  
(Eq. 4)

Where ΔI is the fluorescence change, i_b is the fluorescence coefficient for free apoB685–699, [B_l]_0 and [A_l] are the total concentrations of apoB685–699 and KIV7 or KIV8, respectively, dI is the difference between the fluorescence coefficients for LDL in the free and apo(a)-bound states, K_d is the dissociation constant, and [A_l] and [B_l] are the total concentrations of apo(a) and flu-LDL, respectively.

**Transient and Metabolic Labeling of 17K and Recombinant Apo(a) Variants with [35S]Cysteine—**Plasmids encoding 17K, 17KIV7 or 17KIV8, respectively, were transiently transfected into 293 cells and metabolically labeled with [35S]cysteine as previously described (15). Labeled conditioned medium was harvested and stored at −70°C prior to use.

**Covariant Lp(a) Assembly Assays—**In vitro covariant Lp(a) assembly assays were performed essentially as previously described (15). Briefly, purified native LDL (50 nM) was incubated with conditioned medium containing 2 nM 17K or each of the 17K variants at 37°C in a total volume of 500 μL. At selected time points (0–8 h), a 30-μL aliquot was removed, added to an equal volume of 2% Laemmli sample buffer (26) in the absence of a reducing agent, and heated at 95°C for 5 min. Samples were then subjected to SDS-PAGE on 4% polyacrylamide gels. Following electrophoresis, gels were placed in fixing solution (25% methanol, 12.5% acetic acid in double-distilled H2O) for 20 min, followed by incubation in Amplify solution (Amersham Life Sciences) for 30 min. The gels were then dried and exposed to a PhosphorImager screen (Bio-Rad) for 16 h. The screen was developed using a Bio-Rad Molecular Imager FX, and the bands were quantified using Quantity One 4.0.1 densitometry software. The extent of covalent apo(a) particle formation was quantified according to the following formula: %Lp(a) = [Lp(a)]/[Lp(a)] + [apo(a)] × 100. The data were fit according to an exponential rise to maximum formula given in Equation 2, which was derived from first principles assuming that covariant apo(a) formation occurs as a two-step process.

\[ %Lp(a) = 100 \times \left(1 - e^{-k(t - t_0)} \right) \]  
(Eq. 2)

Where %Lp(a) is the amount of Lp(a) formed at time t, t is the time of reaction, and k is a constant is described by the following relationship.

\[ a = \frac{B_l}{[B_l]_0 + K_d} \]  
(Eq. 3)

Where [B_l]_0 is the total concentration of LDL, K_d is the dissociation constant for the interaction between apo(a) and LDL, and k is the rate constant for disulfide bond formation.
Role of Apo(a) Weak Lysine-binding Sites in Lp(a) Assembly

**Fig. 1.** Recombinant apo(a) variants used in this study. Panel A, recombinant apo(a) variants corresponding to 17K, 17KIV6E56G, 17KIV7E56G, and 17KIV8E56G were constructed in the pRK5 vector and expressed in human embryonic kidney (293) cells as outlined under “Experimental Procedures.” Each mutant apo(a) variant contains an amino acid substitution at position 56 (denoted by an asterisk) within one or more of the WLBS, where position 1 corresponds to the first cysteine residue in the kringle. Sequences corresponding to either KIV7 or KIV8 were assembled in the pET16b vector and expressed in E. coli, as described under “Experimental Procedures.” Panel B, 17K, 17KIV6E56G, 17KIV7E56G, 17KIV8E56G, 17KIV6/7E56G, and 17KIV7/8E56G r-apo(a) derivatives were purified using lysine-Sepharose chromatography (24). To assess protein integrity and purity, purified r-apo(a) derivatives (5 μg) corresponding to 17K (1), 17KIV6E56G (2), 17KIV7E56G (3), 17KIV8E56G (4), 17KIV6/7E56G (5), and 17KIV7/8E56G (6) were subjected to SDS-PAGE under non-reducing (NR) and reducing (R) and stained with Coomassie Blue. Panel C, apo(a) KIV8 was purified using a similar procedure to that previously described for apo(a) KIV7 (23). To assess protein integrity and purity, purified apo(a) KIV8 (10 μg) was subjected to SDS-PAGE under non-reducing (NR) and reducing (R) and stained with Coomassie Blue.

**TABLE II**

| Analysis of the affect of mutating the WLBS on the conformational status of 17K |
|-------------------------------|-----------------|
| r-apo(a) variant            | s20,w (M)       |
|-------------------------------|-----------------|
| 17K                           | 9.21            |
| 17KIV6E56G                    | 9.20            |
| 17KIV7E56G                    | 9.25            |
| 17KIV8E56G                    | 9.22            |
| 17KIV6/7E56G                  | 9.21            |
| 17KIV7/8E56G                  | 9.26            |

* The r-apo(a) variants used are shown schematically in Fig. 1.  
+ s20,w values were obtained using analytical ultracentrifugation.  
* Data were taken from Ref. 15.

Taken together, these data clearly demonstrate that mutation of the WLBS in 17K r-apo(a) does not alter the conformational status of variants, nor does it appreciably alter the ability of e-ACA to convert the 17K variants to the open conformation.

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**Fig. 1.**

**A**

| 17K | 17KIV6E56G | 17KIV7E56G | 17KIV8E56G | 17KIV6/7E56G | 17KIV7/8E56G |
|-----|------------|------------|------------|--------------|--------------|
| 1   | 1          | 1          | 2          | 1            | 5            |
| 2   | 2          | 2          | 2          | 2            | 2            |
| 3   | 2          | 2          | 2          | 2            | 2            |
| 4   | 2          | 2          | 2          | 2            | 2            |
| 5   | 2          | 2          | 2          | 2            | 2            |
| 6   | 2          | 2          | 2          | 2            | 2            |

**B**

**C**

200 kDa

**TABLE II**

| Analysis of the affect of mutating the WLBS on the conformational status of 17K |
|-------------------------------|-----------------|
| r-apo(a) variant            | s20,w (M)       |
|-------------------------------|-----------------|
| 17K                           | 9.21            |
| 17KIV6E56G                    | 9.20            |
| 17KIV7E56G                    | 9.25            |
| 17KIV8E56G                    | 9.22            |
| 17KIV6/7E56G                  | 9.21            |
| 17KIV7/8E56G                  | 9.26            |

* The r-apo(a) variants used are shown schematically in Fig. 1.  
+ s20,w values were obtained using analytical ultracentrifugation.  
* Data were taken from Ref. 15.
Binding of Apo(a) Derivatives to Fluorescently Labeled LDL—To monitor the non-covalent association between the r-apo(a) variants and LDL, we utilized a solution phase, fluorescence-based system in which LDL is modified with fluorescein (15, 18). To measure the contribution of each of the WLBS in apo(a) KIV6–8 to the non-covalent association between 17K r-apo(a) and LDL, we titrated flu-LDL with 17K r-apo(a) and 17K r-apo(a) variants containing WLBS mutations (see Fig. 1). Fluorescence changes were observed for all of the 17K r-apo(a) variants (Fig. 3 and data not shown), and thus the affinity of each mutant for LDL could be assessed by modeling these fluorescence changes according to Equation 1 (Table III). The 17K r-apo(a) bound LDL with a $K_D$ value of 21.7 nM, a dissociation constant that is consistent with our previous findings ($K_D = 23.9$ nM; Ref. 15). The 17KKIV6E56G bound to LDL with a similar affinity ($K_D = 11.4 \pm 1.0$ nM) to 17K, indicating that KIV6 does not contribute to the non-covalent association with LDL. Interestingly, titration of flu-LDL with 17KKIV7E56G or 17KKIV8E56G yielded $K_D$ values of 54.6 ± 7.1 and 68.7 ± 5.0 nM, respectively, suggesting that both KIV7 and KIV8 are required for maximally high affinity binding of apo(a) to LDL. The 17KKIV6E56G variant bound LDL with a similar affinity ($K_D = 47.0 \pm 6.1$ nM) to 17KKIV7E56G, reaffirming that the WLBS in KIV6 is not involved in non-covalent Lp(a) assembly. Tandem mutation of the WLBS in both KIV7 and KIV8 (17KKIV7/8E56G) resulted in the largest decrease in affinity, corresponding to a $K_D$ of 283.2 ± 89.1 nM. Taken together, these data indicate that apo(a) KIV7 and KIV8, but not KIV6, contribute to the non-covalent step of Lp(a) assembly.

Covalent Lp(a) Assembly Assays—We assessed the ability of the 17K r-apo(a) WLBS mutants to form covalent Lp(a) particles using in vitro covalent Lp(a) assembly assays. The kinetics of covalent Lp(a) assembly were studied over an 8-h time period. Covalent Lp(a) particles were separated from free apo(a) by SDS-PAGE, and the percentage of Lp(a) formed was determined for each 17K r-apo(a) variant (Fig. 4A). The data obtained were modeled with respect to time according to the exponential rise to maximum relationship described by Equation 2 (Fig. 4B). Importantly, the values obtained for the constant $a$ from the fit of the data to this equation (Table III) reflect the efficiency with which each r-apo(a) variant forms covalent Lp(a) particles. The constant $a$ depends on the concentration of LDL ($[B]_0$), the affinity of the interaction between apo(a) and LDL ($K_D$), as well as the rate constant for the
process of disulfide bond formation (k) (see Equation 3). The 17K r-apo(a) formed covalent Lp(a) particles with an a value of 0.050 ± 0.004 h⁻¹. Mutation in KIV₆ (17KKIV₆E56G) did not appreciably alter efficiency of covalent Lp(a) assembly (a = 0.055 ± 0.002 h⁻¹), whereas mutation of the WLBS in KIV₇ (17KKIV₇E56G) or KIV₈ (17KKIV₈E56G) reduced the a value by ~20% (a = 0.043 ± 0.001 and 0.040 ± 0.001 h⁻¹, respectively). Tandem mutation of the WLBS in these kringles (17KKIV₇–KIV₈E56G) resulted in an ~75% reduction in the efficiency of covalent Lp(a) assembly (a = 0.014 ± 0.001 h⁻¹). Tandem mutation of the WLBS in KIV₆ and KIV₇ (17KKIV₆/KIV₇E56G) resulted in the formation of covalent Lp(a) particles with a similar efficiency (a = 0.044 ± 0.001 h⁻¹) to that observed for 17KKIV₈E56G, confirming that the WLBS in apo(a) KIV₆ does not affect non-covalent or covalent Lp(a) assembly. Taken together, these data indicate that the WLBS in apo(a) KIV₇ and KIV₈, but not KIV₆, significantly contribute to covalent Lp(a) particle formation.

Qualitatively, the efficiency of covalent Lp(a) assembly (a) appears to coincide with the affinity of the various apo(a) derivatives for LDL (Kₚ) (Table III). As previously mentioned, however, the Kₚ is dependent upon both the Kᵦ for the interaction between apo(a) and LDL, as well as the rate constant (k) for disulfide bond formation. To ensure that the observed decreases in a can be fully explained at the level of Kᵦ (and not k), we quantitatively compared the values obtained for a in the covalent assay to the values obtained for Kᵦ in the non-covalent assay using Equation 3. The variables a and Kᵦ exhibited an inverse relationship, as predicted by Equation 3 (Fig. 5), indicating that mutation of the WLBS in apo(a) does not alter the rate constant (k) for disulfide bond formation. In fact, the rate constant obtained from the regression of the data (k = 0.0613 ± 0.0027 h⁻¹) is in very good agreement with our previously reported value for 17K r-apo(a) (k = 0.0497 h⁻¹) (15). As such, the changes observed in the efficiency of covalent Lp(a) particle formation for the 17K WLBS mutants can be fully explained by alterations in the affinity of these proteins for LDL (Kᵦ).

**Table III**

| r-apo(a) variant | Kᵦ (nM) | a (h⁻¹) |
|------------------|---------|---------|
| 17K              | 21.7 ± 4.0 | 0.050 ± 0.004 |
| 17K/KIV₇E56G     | 11.4 ± 1.0 | 0.055 ± 0.002 |
| 17K/KIV₈E56G     | 54.6 ± 7.1 | 0.043 ± 0.001 |
| 17K/KIV₇E56G     | 68.7 ± 5.0 | 0.040 ± 0.001 |
| 17K/KIV₈E56G     | 47.0 ± 6.1 | 0.044 ± 0.001 |
| 17K/KIV₁₀E56G    | 283.2 ± 89.1 | 0.014 ± 0.001 |

* The recombinant apo(a) variants used are shown schematically in Fig. 1.
* Dissociation constants (Kᵦ) were determined using a solution-phase, fluorescence-based binding assay in which LDL was labeled with 5’-iodoacetomidofluorescein.
* The efficiency of covalent Lp(a) particle formation (a) was determined using in vitro covalent Lp(a) assembly assays as described under "Experimental Procedures."

Initially, we examined the ability of bacterially expressed apo(a) KIV₇ and KIV₈ to bind to e-ACA using intrinsic fluorescence as previously described (23). Titration of KIV₇ with e-ACA resulted in an increase in fluorescence, whereas titration of KIV₈ yielded a decrease in fluorescence (Fig. 6). The observed changes in fluorescence (ΔI) were modeled with respect to the concentration of e-ACA according to a simple hyperbolic relationship to obtain affinities (Kₐ) for the interaction between apo(a) KIV₇ or KIV₈ and e-ACA. The results indicated that apo(a) KIV₇ and KIV₈ bound e-ACA with comparable affinities (Kₐ = 217 μM, Kᵦ = 800 μM, respectively); the value obtained for KIV₇ is comparable with what we have previously reported (23). The affinity of the respective kringles containing the WLBS for e-ACA is substantially less than the affinity of KIV₁₀ containing a strong LBS, for this ligand (33 μM; Ref. 23). We used intrinsic fluorescence to investigate the ability of apo(a) KIV₇ and KIV₈ to bind apoB675–689 and apoB685–699. Titration of KIV₈ with apoB675–689 yielded a decrease in fluorescence that was subsequently modeled to obtain a Kᵦ of 167 ± 46 nM (Fig. 7A). This corresponds to a 4000-fold increase in affinity of KIV₈ for apoB675–689 compared with e-ACA. Interestingly, titration of KIV₇, with apoB675–689 did not elicit a change in intrinsic fluorescence (Fig. 7A), suggesting that KIV₇ does not bind to apoB675–689. In contrast, however, titration of KIV₇ with apoB685–699 elicited a non-linear increase in intrinsic fluorescence (Fig. 7B). Subsequent modeling of the fluorescence change (ΔI) with respect to the concentration of the apoB685–699 peptide according to Equation 4 yielded a Kᵦ of 1.2 ± 0.18 μM, indicating that KIV₇ interacts with this peptide with high affinity compared with e-ACA. Titration of KIV₈ with apoB685–699 did not elicit a detectable change in fluorescence (Fig. 7B), suggesting that this peptide does not interact with KIV₈.

**Discussion**

Lp(a) assembly is a two-step process in which non-covalent interactions between apo(a) and the apoB component of LDL precede the formation of a single disulfide bond; the affinity of these non-covalent interactions is thought to be a determinant of the rate of covalent Lp(a) assembly. We have previously established that the initial non-covalent interaction is mediated by the interaction of lysine residues in the NH₁ terminus of apoB (11) with WLBS present in apo(a) KIV₆–₈, (14). To quantitatively define a role for each of the WLBS in apo(a) KIV₆–₈, we created single point mutations in each of the WLBS in these kringle domains (either separately or pairwise) in the context of 17K r-apo(a), and studied non-covalent and covalent binding to apoB in LDL. Our study is the first to demonstrate unequivocally that the affinity of the apo(a)/LDL non-covalent interactions determines the rate of covalent Lp(a) assembly and constitutes the first direct, quantitative evidence for a role of the WLBS in apo(a) KIV₇ and KIV₈ in these non-covalent interactions.

We have recently shown that the conformational status of apo(a) is an important determinant of the efficiency covalent of Lp(a) assembly (15). Apo(a) adopts a closed conformation that can be converted to an open form by the addition of the lysine analog e-ACA. Importantly, the conformational status of apo(a) does not alter its affinity for LDL (Kᵦ), but rather influences the rate constant for disulfide bond formation (k), possibly by restricting access of LDL to the free cysteine in apo(a) KIV₈. We have also recently demonstrated that the closed conformation of apo(a) is maintained by an intramolecular interaction between the strong lysine-binding site in KIV₁₀ (located in the COOH-terminal half of the molecule) and sequences within the
amino-terminal half of the molecule. As such, the closed conformation of apo(a) is disrupted when truncated apo(a) variants are used. This is important in that the studies used to identify domains in apo(a) that are required for Lp(a) assembly have been conducted using truncated apo(a) variants (11, 14, 20, 21). In light of our recent discovery of the role of the conformational status of apo(a), a quantitative re-evaluation of the role of the respective WLBS is required. As we have previously shown, although the conformational status of apo(a) does not directly affect the measurement of non-covalent binding between apo(a) and free apo(a) at a given time (t). A representative autoradiograph for 17K_KIV7/8_E56G and 17K is shown. Panel B, the percentage of Lp(a) formed using metabolically labeled r-apo(a) (17K (●), 17K_KIV7/8_E56G (▲), 17K_KIV7_E56G (■), 17K_KIV8_E56G (▼), 17KKIV7/8_E56G, (♦) and 17K_KIV7/8E56G (○)) was modeled with respect to t using Equation 2 (solid lines) to obtain a constant (a) that represents the efficiency of covalent Lp(a) assembly.

**FIG. 4.** The effect of mutation of the weak lysine-binding sites in KIV6 and KIV7 in 17K on the covalent step of Lp(a) assembly. In vitro covalent Lp(a) assembly assays were used to study the kinetics of covalent Lp(a) particle formation over an 8-h time course. Panel A, Lp(a) was separated from free apo(a) by SDS-PAGE on a 4% gel and visualized by autoradiography. The percentage of Lp(a) formed was determined using densitometry to measure the amount of Lp(a) and free apo(a) at a given time (t). A representative autoradiograph for 17K_KIV7/8_E56G and 17K is shown. Panel B, the percentage of Lp(a) formed using metabolically labeled r-apo(a) (17K (●), 17K_KIV7/8_E56G (▲), 17K_KIV7_E56G (■), 17K_KIV8_E56G (▼), 17KKIV7/8_E56G, (♦) and 17K_KIV7/8E56G (○)) was modeled with respect to t using Equation 2 (solid lines) to obtain a constant (a) that represents the efficiency of covalent Lp(a) assembly.

Elucidation of the molecular structures of apo(a) KIV6 by NMR (27) and apo(a) KIV7 by x-ray crystallography (28) has provided a rationale for the use of site-directed mutagenesis to disrupt the lysine-binding properties of the WLBS as opposed to an approach that involves deleting entire WLBS-containing kringles domains. The lysine-binding pockets in each of apo(a) KIV6-8 contains three centers that coordinate e-ACA: a cationic center (Arg54 and Arg69), which coordinates the carboxylate group; an anionic center (Arg54 and Glu65), which coordinates the amino group; and a hydrophobic trough (Trp60, Tyr62, and Trp70), which stabilizes the methylene groups (28). Together, these three centers account for the interaction of the WLBS with free lysine, lysine analogs, and lysyl residues on physio-
logical binding partners such as apoB. Using site-directed mutagenesis, we introduced E56G point mutations into 17K r-apo(a) to generate mutant variants with diminished lysine-binding properties in KIV6 (17KKIV6E56G), KIV7 (17KKIV7E56G), KIV8 (17KKIV8E56G), and 17KKIV6E56G (K0) were regressed as a function of the efficiency with which these recombinant variants form covalent Lp(a) particles (corresponding to the term a) using Equation 3 (line). The fit obtained was used to calculate the rate constant for disulfide bond formation (k) for all of the 17K r-apo(a) species used.

Because the mutant r-apo(a) variants were expressed in the context of the 17K r-apo(a), we were able to assess the role of each of the WLBS in both the non-covalent and covalent steps of Lp(a) assembly. The data clearly indicate that the WLBS in KIV7 and KIV8, but not KIV6, contribute to the initial non-covalent association between apo(a) and LDL (Fig. 3B, Table III). Mutation of either KIV7 or KIV8 resulted in a 4-fold reduction in the affinity of apo(a) for LDL, whereas tandem mutation of both WLBS led to a 13-fold decline in the affinity. We further demonstrated that the WLBS in KIV7 and KIV8, but not KIV6, are required for covalent Lp(a) particle formation. Importantly, the efficiency of covalent Lp(a) particle formation (a value) for the wild-type and mutant 17K r-apo(a) variants exhibited an inverse relationship with the efficiency for LDL (K0) obtained in the non-covalent assays (Fig. 5). This relationship agrees with the model that we have derived from first principles based on the assumption that Lp(a) assembly is a two-step process (see Equations 2 and 3). Our observation that the data obtained in both the non-covalent and covalent assays follow this relationship implies that the KD values obtained in the non-covalent assay describe productive binding interactions between apo(a) and LDL (i.e. interactions that result in disulfide bond formation). Furthermore, the relationship between KD and a that is predicted by Equation 3 demonstrates clearly that mutation of the WLBS in apo(a) KIV7 and KIV8 alters the efficiency of Lp(a) assembly at the level of KD and not k. This result is consistent with data obtained from analytical ultracentrifugation, which shows that mutation of the WLBS in apo(a) does not alter the s20,w of 17K r-apo(a). In addition, the k value (0.0613 h⁻¹) obtained from the fit of KD versus a is in excellent agreement with the k value (0.0497 h⁻¹) previously measured for 17K r-apo(a) (15).

Although the involvement of the WLBS in the non-covalent interaction between apo(a) and LDL has been demonstrated by numerous groups (14, 19–21), the data from the present study represent the first quantitative assessment of the role of each of the WLBS present in apo(a) KIV6–8 to both the non-covalent and covalent steps of Lp(a) assembly. Our findings are not in agreement with previous studies, which suggested that apo(a) KIV6 mediates the non-covalent interaction with LDL (14, 20,
It is important to note, however, that in all of these studies roles for apo(a) KIV6–KIV8 in apo(a)-LDL interactions were identified using truncated variants in which entire kringle domains containing the WLBS were removed. Our present observation that two WLBS present in each of apo(a) KIV7 and KIV8 mediate non-covalent Lp(a) assembly is in good agreement with our previous report suggesting that two lysine residues (Lys680 and Lys690) within the NH2-terminal 18% of apoB (apoB-18) are required for binding to WLBS present within apo(a) KIV5–KIV8 (18). Studies from another group, however, suggest that lysine residue(s) within the carboxyl-terminal end of apoB mediate non-covalent binding to apo(a). In this context, a recent study demonstrated that a lysine-rich, apoB-derived peptide corresponding to amino acids 4372–4392 (apoB4372–4392) binds apo(a) non-covalently and inhibits covalent Lp(a) particle formation with a higher potency than ε-ACA (9). Taken together, these studies suggest that several regions of apoB may be involved in non-covalent association with apo(a).

Evaluation of the relative contribution of these sequences should be addressed in future studies; the current study is an extension of our previous work and thus focuses on the lysine-dependent non-covalent interaction between apo(a) KIV6–KIV8 and sequences within the amino-terminal 18% of apoB (16, 18).

To investigate whether the WLBS in KIV7 and KIV8 are specific for Lys680 and Lys690 in LDL, we measured the affinity of these kringle domains for ε-ACA, as well as peptides spanning Lys680 (apoB675–689) or Lys690 (apoB685–699). Both KIV7 and KIV8 bound ε-ACA with comparable affinities (Fig. 6; $K_D = 217$ and 800 μM, respectively) that are in keeping with previously reported values for the interaction between ε-ACA and the WLBS in apo(a) (15, 23, 27). Analysis of the primary sequence alignment of apo(a) KIV7 and KIV8 reveals that both of these kringle domains contain all of the crucial amino acid residues that form the lysine-binding pocket, an observation that explains their similar affinity for ε-ACA (3). Interestingly, KIV8 bound apoB675–689 with a 4000-fold higher affinity (Fig. 7A; $K_D = 167$ nM) than ε-ACA, suggesting that flanking sequences surrounding Lys680 are required for high affinity binding. Moreover, KIV7 did not interact with apoB675–689, suggesting that sequences flanking Lys680 also mediate specificity of this pep-
tide for KIV\textsubscript{7}. When binding of KIV\textsubscript{7} and KIV\textsubscript{8} to apoB685–699 was investigated, the opposite result was obtained; KIV\textsubscript{7} bound apoB685–699 with a 200-fold higher affinity than \textepsilon\-ACA (Fig. 7B; \(K_D\) = 1.2 \(\mu\)M), whereas KIV\textsubscript{8} did not interact with this peptide. Once again, this suggests that sequences flanking Lys\textsubscript{690} mediate the affinity and specificity that characterize the interaction between KIV\textsubscript{7} and apoB685–699. These results are interesting in the context of our previous study (18), in which we demonstrated that mutation of the Lys\textsubscript{680} residue reduced the apo(a)-apoB interaction to a much greater extent than was observed upon mutation of the Lys\textsubscript{690} residue. This is in good agreement with the data in the present study in which the interaction of apo(a) KIV\textsubscript{7} with the peptide containing apoB Lys\textsubscript{680} is weaker than the interaction of apo(a) KIV\textsubscript{8} with the peptide containing Lys\textsubscript{680}. Furthermore, we have previously demonstrated that an apoB-derived peptide containing Lys\textsubscript{680} and Lys\textsubscript{690} (apoB680–704) bound 17K r-apo(a) with a \(K_D\) of 83.4 nM (18), which corresponds to a higher affinity than either apoB685 or apoB680.

Given the extraordinarily high degree of primary sequence identity between KIV\textsubscript{7} and KIV\textsubscript{8} (88%; Ref. 3), the observed specificity of apoB675–689 for KIV\textsubscript{8} is somewhat surprising. With the exception of the sequences flanking Arg\textsubscript{35} (Thr\textsubscript{37} and Glu\textsubscript{38} in KIV\textsubscript{7} and Pro\textsubscript{37} and Leu\textsubscript{38} in KIV\textsubscript{8}), the amino acid differences between KIV\textsubscript{7} and KIV\textsubscript{8} are conservative substitutions. Understanding the nature of the specificity of KIV\textsubscript{7} and KIV\textsubscript{8} for sequences in apoB must be validated experimentally using high resolution methodologies such as x-ray crystallography and/or NMR. In this regard, preliminary \(^{1}H\)-\(^{15}N\) NMR analyses of KIV\textsubscript{7} and KIV\textsubscript{8} suggest that the backbone conformation of KIV\textsubscript{7} is significantly different from that of KIV\textsubscript{8} for a subset of residues,\(^4\) indicating that these two kringle domains may adopt unique structures in some regions.

We have demonstrated that the initial non-covalent interaction between apo(a) and apoB is mediated by a highly specific interaction between two lysine residues (Lys\textsubscript{680} and Lys\textsubscript{690}) in apoB and two WLBS (KIV\textsubscript{7} and KIV\textsubscript{8}) in apo(a). Taken together, our data are consistent with the hypothesis that this highly specific interaction does not merely anchor apo(a) to apoB, but also plays an important role in orienting the two proteins in a manner that facilitates a secondary non-covalent interaction and/or disulfide bond formation. On a molecular level, our data suggest that a productive orientation between apo(a) and LDL would be provided for by the observed specificity of KIV\textsubscript{7} for Lys\textsubscript{680} and KIV\textsubscript{8} for Lys\textsubscript{680}; this specificity would provide the appropriate directionality to the initial non-covalent interaction, thereby ensuring the efficiency and fidelity of the process of covalent apo(a) assembly.

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