Intermolecular Contact between Globular N-terminal Fold and C-terminal Domain of ApoA-I Stabilizes Its Lipid-bound Conformation

STUDIES EMPLOYING CHEMICAL CROSS-LINKING AND MASS SPECTROMETRY

Received for publication, May 9, 2005, and in revised form, June 14, 2005 Published, JBC Papers in Press, June 22, 2005, DOI 10.1074/jbc.M505081200

Shaila Bhat,* Mary G. Sorci-Thomas,‡§, Eric T. Alexander,§, Michael P. Samuel,§ and Michael J. Thomas§

From the Departments of Pathology and Biochemistry, Wake Forest University Health Sciences, Winston-Salem, North Carolina 27157

The structure of apolipoprotein A-I (apoA-I) has been intensely investigated in efforts to understand its highly significant role in protecting against coronary heart disease in humans (1–3). ApoA-I is abundantly found in plasma high density lipoproteins (HDL) and functions as the main carrier of excess cholesterol to the liver in a process termed "reverse cholesterol transport" (4–6). ApoA-I also plays a significant role in mediating anti-inflammatory/antioxidative processes (7–12) intervening in the escalation of damage to the artery wall. As with many proteins, the functional roles played by apoA-I are tightly coupled to the structure of the apoprotein. Thus, the lack of a detailed lipid-bound apoA-I x-ray crystal structure has seriously hindered our understanding of this apoprotein’s unique features (13, 14) in its biologically active lipid-bound form. A major advance occurred when the x-ray crystal structure of lipid-free Δ43 apoA-I was reported (15). This report suggested that apoA-I adopts an antiparallel “belt-like” conformation when bound to a lipid surface. Unfortunately, lipid-bound apoA-I has not yielded crystals of the quality needed to solve its three-dimensional conformation. Instead, a number of alternative and highly innovative approaches have been used to probe the conformation suggested by the lipid-free crystal structure (16–27). In addition, computer modeling studies suggest that the two molecules of apoA-I wrap in an extended belt completely around the edge of a lipid bilayer, maximizing intermolecular salt bridges, which act to stabilize the protein conformation in an antiparallel arrangement (23). Although all of these studies support the concept of a “belt” model of apoA-I, their inability to distinguish between an “extended belt” or a “hairpin” belt conformation have lead to the need for alternative techniques to explore the tertiary structure of lipid-bound apoA-I.

Chemical cross-linking combined with mass spectrometry is a powerful new technique that has been utilized to yield structural information on protein folding (28–34). Based on the cross-linker’s specificity and spacer arm length, low resolution distance constraints can be obtained to assist in the construction of a three-dimensional structure of a protein. Recently, the lipid-free (35) and lipid-bound (36) structure of apoA-I have been investigated using these approaches. However, a large number of intramolecular and intermolecular cross-links obtained in these studies prevented an unambiguous identification of the preferred lipid-bound conformation for apoA-I.

In the present studies, we report the use of a cross-link, mass spectrometry approach, which yielded two dimeric forms of lipid-bound apoA-I that were separated using 12% SDS-PAGE and then subjected to in-gel trypsin digestion and mass spectrometry. Utilizing MS/MS analysis, identification of cross-linked peptides was performed, and the two dimeric forms were found to be enriched in linkages in different domains of the protein. The most structurally informative of these cross-links existed between lysine 118 and lysine 140, indicating a helix 4 to helix 5 intermolecular bond found exclusively in the 80-kDa product, whereas another cross-link involving the N-terminal domain of lipid-bound apoA-I was predominately in the 53-kDa apoA-I dimeric product. Overall, these data provide direct experimental evidence validating the extended belt model of apoA-I over a hairpin type conformation and provide structural information suggesting that the flexible globular N-terminal domain of apoA-I associates with the C-terminal domain stabilizing lipid-protein interaction on 96-Å rHDL complexes.
**EXPERIMENTAL PROCEDURES**

1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and Me2SO were obtained from Sigma. Dithiobis(succinimidylpropionan) (DSP) was from Pierce. RapiGest SE™ was obtained from Waters Inc. Sequencing grade modified trypsin and restriction enzymes were from Promega. Sodium deoxycholate, optima grade methanol, chloroform, acetonitrile, and glacial acetic acid were purchased from Fisher. Formic acid was from Aldrich.

Oligonucleotides were synthesized by International DNA Technologies. Plasmid DNA was purified using the Wizard™ purification systems from Promega Inc. Deoxyribonuclease I was purchased from Worthington. The expression vector pTYB12, Escherichia coli strain ER2566, and chitin matrix were purchased from New England Biolabs.

Restriction enzymes, T4 DNA ligase, DH5α—competent cells, and isopropyl-β-D-thiogalactopyranoside and Mark 12 molecular weight standards and SimplyBlue™ SafeStain were from Invitrogen. The mini-EDTA-free protease inhibitor tablets were from Roche Applied Science. Q-Sepharose Fast Flow matrix and the high molecular weight standards were from Amersham Biosciences. Ultrafree-15 centrifugal units and Biomax™10K membranes were from Millipore Corp.

**Preparation and Purification of 96-Å Human ApoA-I-containing rHDL**—ApoA-I was purified from human plasma using standard procedures (37) and found to be greater than 99% pure by mass spectrometry. Homogenous reconstituted HDL particles with an approximate diameter of 96 Å were prepared by adding 1 mol of apoA-I to 80 mol of POPC as previously described (38). Briefly, POPC dissolved in chloroform was first dried on the side of a glass tube, and all traces of solvent were removed under vacuum. POPC was suspended in a solution containing sodium deoxycholate at a molar ratio of 2 cholate to 1 POPC by incubation at 37 °C in a shaker water bath for 1 h and then vortexed on a standard multitupe vortexer (VWR Scientific) for 1 h at room temperature. Sodium deoxycholate was removed by exhaustive dialysis at room temperature against a buffer containing 10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 1.5 mM sodium azide, and 0.25 mM EDTA.

The crude rHDL was further purified by application to three Superdex 200 HR 10/30 columns linked in tandem and run at 0.5 ml/min (25, 26) with 0.9% NaCl, 1.5 mM sodium azide, and 0.3 mM EDTA, pH 7.4. Fractions corresponding to the rHDL peak were combined, concentrated using an Amicon Ultra-15, 10,000 molecular weight cut-off filtration units, and then assayed for protein and phospholipids with the day of the experiment, DSP was dissolved in Me2SO to make a 5 μg/μl stock solution and then added to lipid-free apoA-I at a 5:1 molar ratio (apoA-I:1.5 μg/μl final concentration), whereas DSP was added to lipid-bound apoA-I at a 2:1, 10:1, 20:1, and 200:1 molar ratio (apoA-I final concentration of 0.4 μg/μl) in a 10 mM sodium phosphate, pH 7.4, buffer. Once the DSP was added, incubations were carried out at 37 °C for 5 min. Cross-linking conditions were used that kept Me2SO concentration less than 2% in the reaction. After the short incubation, the reaction was immediately quenched by the addition of Tris, pH 7.4, giving a final concentration of 50 mM Tris. The samples were then immediately dialyzed against several changes of 10 mM ammonium bicarbonate, pH 7.4, at 4 °C and then kept at −80 °C.

12% SDS Polyacrylamide Gel Electrophoresis—Following treatment of lipid-free or lipid-bound rHDL apoA-I with DSP, the cross-linked products were separated by 12% SDS-PAGE under nonreducing conditions as described previously (42). The gels were washed to remove SDS and stained with SimplyBlue. In some experiments, cysteine-containing lipid-free apoA-I homodimers were also run on 12% SDS-PAGE under nonreducing conditions using the same reagents (42). In these instances, the molecular size of products obtained from 12% SDS-PAGE were compared with the molecular size obtained from nonreducing 6 M urea, 12% SDS-PAGE (43, 44). In these experiments, all buffers except the stacking buffer were adjusted to a final concentration of 6 M urea. Prior to loading the gel, the gel was equilibrated for 1 h at 35 mM before loading the samples. All protein samples were boiled for 5 min in nonreducing SDS-PAGE loading buffer with a final concentration of 6 M urea and loaded immediately onto the gel.

**In-gel Trypsin Digest**—Bands corresponding to cross-linked products were removed and minced to give 1-mm3 sized pieces, which were then soaked overnight at room temperature in wash solution (methanol/acetic acid/water (v/v/v), 50:5:45). After removing the wash solution, the gel pieces were dehydrated by suspending them in 400 μl of acetone for 5 min at room temperature. The dehydation step was repeated as many times as needed to obtain white, opaque gel pieces (45). After removing the acetone, the gel pieces were dried in vacuo for 3 min. Rehydration was accomplished using the gel pieces with 400 μl of 100 mM ammonium bicarbonate, pH 7.8, for 10 min. After the last dehydation step, the samples were rehydrated by adding trypsin at a mass ratio of 1 trypsin to 20 apoA-I using a freshly prepared trypsin solution at 20 ng/μl in 10 mM ammonium bicarbonate, pH 7.8, 1 mM CaCl2, and 0.1% (w/v) RapiGest SF. After rehydrating in the trypsin solution on ice for 10 min, any trypsin solution not absorbed by the gel pieces was removed, and the samples were incubated for 1–18 h at 37 °C. The next morning, the gel pieces were mixed with 120 μl of 100 mM ammonium bicarbonate, pH 7.8, at room temperature for 10 min with occasional, gentle vortexing. To extract the peptides from the gel pieces, 200 μl of extraction solution (acetonitrile/formic acid/water (v/v/v), 50:5:45) was added. After 10 min, the extraction solution containing peptides was transferred to a fresh tube and saved, and a second aliquot of extraction solution was added to the gel pieces. The alquots of extraction solution were combined and then acidified by adding 500 mM HCl to give a final (v/v) ratio of 1 HCl:10 apoA-I. After incubating at 37 °C for 35 min, the samples were centrifuged at 13,000 rpm for 10 min at room temperature, and the supernatant was transferred to a fresh tube and processed immediately for mass spectrometry.

**In-solution Trypsin Digest**—In-solution digest of lipid-free and lipid-bound apoA-I was carried out essentially as described with slight mod-
ifficitation (45). One vial of RapiGest SF (1 mg) was dissolved in 999 µl of 100 mM ammonium bicarbonate, pH 7.8, to which 1 µl of 1 N CaCl₂ was added, giving a final concentration of 0.1% and 1 mM, for RapiGest SF and CaCl₂, respectively. One vial of trypsin (20 µg) was dissolved in the entire 1 ml of 0.1% RapiGest SF/CaCl₂ and then placed on ice. Trypsin was then added to the samples at a mass ratio of 1 trypsin:20 apoA-I and allowed to incubate for 1–18 h at 37 °C. The samples were then acidified by adjusting with 500 mM HCl, giving a final (v/v) ratio of 1 HCl:10 apoA-I. After incubating at 37 °C for 35 min, the samples were centrifuged at 13,000 rpm for 10 min at room temperature, and supernatant was transferred to fresh tubes and processed immediately for mass spectrometry.

**ES/Q3 Mass Spectrometry**—Acidified peptides were concentrated in vacuo but not taken to dryness, and then they were diluted to a final volume of 20 µl with 1% acetic acid. Peptides were separated on a 2.1-mm diameter × 15-cm Polymer Laboratories, Inc. PLRP-S column packed with 3-µm diameter particles having 100-Å pores. Peptides were separated using a gradient starting at 90% solvent A (25 mM formic acid in water) and 10% solvent B (20 mM formic acid in 1:1 acetonitrile/water) and ending at 10% solvent A and 90% solvent B. The gradient profile had a 5-min hold at 90% A, 10% B, with a linear gradient to 100% B in 50 min followed by a hold at 100% B for 10 min. The flow rate was 0.2 ml/min. The eluant from the column was split, with 15% directed to the electrospray interface of a Quattro II triple quadrupole mass spectrometer running MassLynx™ 3.5 software. Positive ion electrospray spectra were recorded in the centroided mode using a scan width of 200–2000 m/z with a scan time of 2.5 s. Cone and capillary voltages were set to 35 V and 3.5 kV, respectively. The desolvation temperature was 200 °C, and the source temperature was 80 °C. Total ion chromatograms were searched for all possible cross-links based on cross-link tables constructed in Microsoft Excel. The criteria for selection as a possible cross-linked peptide were the presence of 2–3 sequential protonated ions for a predicted cross-linked peptide with an experimental mass that was within 0.5 Da of the calculated mass.

**ES-Q/TOF Mass Spectrometry**—Peptide sequencing was carried out using a Waters Q-TOF API-TOF US mass spectrometer running MassLynx™ 4.0 software and equipped with a Waters CapLC. Peptides were separated on a 100-µm × 5-cm PLRP-S column packed with 3.0-µm diameter particles having 100-Å pores. Peptides were loaded onto the column in water/acetonitrile/formic acid (97:3:0.2) at 500 nl/min. Peptides were eluted using a gradient that started with solvent A (25 mM formic acid in 97% water and 3% acetonitrile) and ended at 50% solvent B (25 mM formic acid in 3% water 97% acetonitrile). The gradient profile was 12 min at 100% A followed by a linear gradient to 50% B in 40 min with a hold at 50% B for 20 min before recycling to the initial conditions. The flow rate was 470 nl/min. Positive ion electrospray spectra were recorded in the continuum mode using a scan window from 400 to 1600 m/z with an accumulation time of 2 s. Product ion scans were acquired in the continuum mode from 50 to 1800 m/z using charge state selective collision energies and a 2-s accumulation time. Cone and capillary voltages were 55 V and 4 kV, respectively. The source temperature was 80 °C.

**Protein Explorer Software**—Inter- and intramolecular distances between apoA-I lysine-lysine pairs were determined using Protein Explorer version 1.982 (available on the World Wide Web at protein-explorer.org) and the x-ray crystal structure (Protein Data Bank number 1av1) of lipid-free A43 apoA-I (15). Inclusion criteria were based on the maximum combined distance of DSP linker arm and both lysine side chains (total ~25 Å) (32), although it has been reported that linker arms may be significantly shorter than that reported by the manufacturer (46).

**RESULTS**

Cross-linking of ApoA-I-containing rHDL—POPC rHDL were prepared using human plasma apoA-I at a starting molar ratio of 80 POPC:1 apoA-I. The crude mixture was purified by gel filtration and found to contain 73 molecules of POPC to 1 molecule of apoA-I with an approximate diameter of 96 Å, shown in Fig. 1. The pure rHDL particles were then treated with DSP, a thiol-cleavable lysine-specific cross-linker with a 12-Å spacer arm, using the standard protocol described under “Experimental Procedures.” In initial experiments, the DSP to apoA-I molar ratio was varied from 2:1 to 200:1, keeping the concentration of apoA-I low (0.4 µg/µl) and the incubation time short (5 min at 37 °C) to prevent inter-rHDL particle cross-linking. Fig. 2 shows the

![FIGURE 1. 4–30% nondenaturing gradient gel electrophoresis. POPC rHDL were prepared using the sodium cholate dialysis method and purified by fast protein liquid chromatography as described under “Experimental Procedures.” The gels were fixed and then stained with Coomassie G-250 as described under “Experimental Procedures.” Lane 1, high molecular weight markers; lane 2, purified 96-Å POPC rHDL-containing human apoA-I; lane 3, apoA-I, lane 4, 96-Å HDL apol-A-I cross-linked at a molar ratio of 2 DSP:1 apoA-I; lane 5, 96-Å rHDL apoA-I cross-linked at a molar ratio of 20 DSP:1 apoA-I.](image1)

![FIGURE 2. 12% nondenaturing SDS-PAGE of DSP cross-linked, 96-Å diameter rHDL apoA-I. Purified 96-Å diameter POPC rHDL-containing human apoA-I was cross-linked with DSP at varying molar ratios of cross-linker to protein for 5 min at 37 °C and then quenched by the addition of Tris, pH 7.4, as described under “Experimental Procedures.” Cross-linked products were separated by nondenaturing SDS-PAGE and visualized using Simply Blue™. Lane 1, molecular mass standards; lane 2, lipid-free apoA-I cross-linked at a molar ratio of 5 DSP:1 lipid-free apoA-I; lane 3, 96-Å rHDL apoA-I cross-linked at a molar ratio of 2 DSP:1 apoA-I; lane 4, 96-Å HDL apoA-I cross-linked at a molar ratio of 20 DSP:1 apoA-I; lane 5, 96-Å rHDL apoA-I cross-linked at a molar ratio of 200 DSP:1 apoA-I. Approximately 6 µg of apoA-I protein were loaded in lanes 3–5, whereas 20 µg of protein was loaded in lane 2 in order to emphasize the higher order cross-linked products.](image2)
N-Terminal Fold of ApoA-I Stabilizes Lipid-bound Conformation

**FIGURE 3.** Effects of 6 M urea on cysteine-containing apoA-I homodimers and cross-linked rHDL apoA-I. A, shows lipid-free cysteine-containing apoA-I homodimers separated by nonreducing 12% SDS-PAGE as described under “Experimental Procedures.” Lane 1, Q5C apoA-I; lane 2, Q109C apoA-I; lane 3, Q132C apoA-I; lane 4, A154C apoA-I; lane 5, wild-type apoA-I. The actual or true molecular mass of all cysteine-containing apoA-I homodimers was verified by mass spectrometry. Approximately 3 μg of protein were loaded per lane. B, lipid-free cysteine-containing apoA-I homodimers separated by 6 M urea, 12% SDS-PAGE under nonreducing conditions (same order as in A) described under “Experimental Procedures.” Lane 1, Q5C apoA-I; lane 2, Q109C apoA-I; lane 3, Q132C apoA-I; lane 4, A154C apoA-I; lane 5, wild-type apoA-I. Approximately 6 μg of protein were loaded per lane. C, DSP cross-linked rHDL apoA-I separated by nonreducing 12% SDS-PAGE. Lane 1, 10 DSP:1 apoA-I; lane 2, Q109C apoA-I homodimer; lane 3, wild-type apoA-I. Approximately 6 μg of protein were loaded per lane. D, DSP cross-linked rHDL apoA-I separated using 6 M urea, 12% SDS-PAGE under nonreducing conditions. Lane 1, 10 DSP:1 apoA-I; lane 2, Q109C apoA-I homodimer; lane 3, wild-type apoA-I.

Distribution of cross-linked products obtained after separation on 12% SDS-PAGE under nonreducing conditions. The results show that when rHDL apoA-Is were cross-linked at a molar ratio of 2:1, two major products were obtained, corresponding to sizes of ~53 and ~80 kDa, as shown in Fig. 2, lane 3. As the molar ratio of DSP increased to 20:1, more products appeared to run at intermediate sizes between ~53 and ~80 kDa, as seen in Fig. 2, lane 4. When DSP was used at a 200:1 molar ratio, these products merged to form a band that migrated close to the theoretical size of dimeric apoA-I, ~56 kDa, as seen in Fig. 2, lane 5. When lipid-free apoA-I was cross-linked at a low DSP/protein ratio (5:1) but at a high concentration (>1 μg/μl), the ~53- and ~80-kDa products were seen (Fig. 2, lane 2). However, in addition to these products, a number of additional higher molecular mass products were seen, probably corresponding to higher order oligomeric forms of cross-linked apoA-I. All cross-linked apoA-I products were completely reversible by treatment with dithiothreitol prior to loading on SDS-PAGE (data not shown).

**SDS-PAGE Migration and the Molecular Mass of ApoA-I Cysteine Homodimers**—In order to investigate the basis for the two distinctly sized cross-linked products, the migration of monomeric and dimeric cysteine-containing mutant apoA-I was investigated by 12% SDS-PAGE. The cysteine-containing mutant apoA-I proteins were constructed by replacing a single solvent-exposed hydrophilic amino acid with a cysteine residue, as described previously (26, 39). Following expression and purification, Q5C, Q109C, Q132C, and A154C apoA-I were analyzed by mass spectrometry and found to be ~85% in the homodimeric form with molecular masses corresponding to 56,248, 56,272, 56,464, and 56,364 Da, respectively (data not shown). When the apoA-I proteins were treated with dithiothreitol prior to mass spectrometric analysis, the monomeric form of the apoA-I protein predominated (99%), corresponding to 28,124, 28,136, 28,232, and 28,182 Da, respectively (data not shown), matching their appropriate theoretical molecular masses. These same four cysteine-containing apoA-I homodimeric proteins were then separated using 12% nonreducing SDS-PAGE, as shown in Fig. 3A. Interestingly, the Q5C and A154C apoA-I homodimers migrated with an apparent molecular mass of ~53 kDa (Fig. 3A, lanes 1 and 4), with their monomer forms migrating at ~28 kDa. In contrast, Q109C and Q132C apoA-I homodimers migrated at ~80 kDa (Fig. 3A, lanes 2 and 3) on 12% SDS-PAGE. These results suggest that when cross-links form centrally near residues 100–140, the apoA-I dimer migrates with an apparent mass of ~80 kDa, whereas if the cross-links form within the N- or C-terminal ends of apoA-I, the homodimer migrates at a molecular size of ~53 kDa. When disulfide cross-linked apoA-I was treated with dithiothreitol prior to 12% SDS-PAGE, only a single band migrating at ~28 kDa was apparent, consistent with the reduction of the apoA-I dimer to a monomer form (Fig. 3A, lane 5).

In order to investigate whether the basis for alternate SDS-polyacrylamide gel migration of apoA-I cysteine homodimer was a result of SDS-resistant protein structure, the lipid-free cysteine-containing apoA-I homodimers were separated by 6 M urea on 12% SDS-PAGE under nonreducing conditions shown in Fig. 3B. In Fig. 3B, the lanes show the same order of proteins loaded as described for Fig. 3A, suggesting that denaturation by urea resolved the conformational resistance to SDS unfolding. In addition, Fig. 3C shows DSP cross-linked rHDL apoA-I separated by nonreducing 12% SDS-PAGE for 10 DSP:1 apoA-I (lane 1), Q109C apoA-I homodimer (lane 2), and wild-type apoA-I (lane 3). For comparison, Fig. 3D shows DSP cross-linked rHDL apoA-I separated using 6 M urea, 12% SDS-PAGE under nonreducing conditions. Lane 1,
In-solution Versus In-gel Trypsin Digestion—In theory, the complete digestion of apoA-I with trypsin should yield 37 peptides; however, only 27 of these peptides have protonated molecular weights greater than a mass/charge ratio (m/z) of 400, the lower scan limit employed for these analyses. In order to test our ability to identify all 27 apoA-I peptides generated from in-gel trypsin digestion, we compared the results of in-solution with in-gel digestion of apoA-I with trypsin. In-solution and in-gel digest of lipid-free apoA-I was carried out as described by Kinter et al. (45) with slight modifications as outlined under “Experimental Procedures.” Comparison of the two methods showed that the in-gel digestion, as shown in TABLE ONE, gave better coverage of apoA-I as well as a higher ion intensity for the individual tryptic fragments than in-solution digest (data not shown). The total-ion chromatogram from LC/MS showed that the highest coverage and intensity of tryptic peptides was obtained from an 18-h in-gel trypsin digestion (TABLE ONE) when compared with an 18-h in-solution trypsin digestion. Tryptic fragments of cysteine homodimers were also tested to ensure that large peptide fragments were efficiently extracted from the gel slices. No difference was noted between in-solution and in-gel tryptic digestion based on the intensity of these peptides containing the cysteine cross-link when analyzed by LC/MS.

### TABLE ONE

| Tryptic Fragments  | 1 hour | 2 hour | 4 hour | 18 hour |
|--------------------|--------|--------|--------|---------|
| T1                 | 32,434 | 42,391 | 249,483| 54,495  |
| T3                 | 4,569  | 18,137 | 28,037 | 34,292  |
| T4                 | 32,434 | 42,391 | 249,483| 54,495  |
| T5                 | 2,197  | 4,690  | 84,162 | 44,885  |
| T6                 | 4,604  | 5,507  | 90,042 | 41,449  |
| T7                 | -      | 72,575 | 43,372 | 61,908  |
| T9                 | -      | -      | -      | 17,698  |
| T10                | -      | -      | -      | -       |
| T11                | -      | -      | -      | -       |
| T12                | 1,027  | -      | -      | -       |
| T14                | 9,772  | 12,221 | 82,227 | 45,578  |
| T16                | 11,444 | 19,436 | 116,062| 66,886  |
| T18                | 32,434 | 42,391 | 249,483| 54,495  |
| T19                | 1,507  | -      | -      | -       |
| T21                | -      | -      | -      | 53,089  |
| T22                | 17,670 | 19,814 | 229,790| 67,703  |
| T25                | 5,537  | 17,701 | 215,729| 53,089  |
| T26                | -      | -      | -      | 54,495  |
| T28                | -      | -      | 187,408| 54,495  |
| T29                | 257,403| -      | 30,186 |
| T30                | -      | -      | -      | -       |
| T31                | 17,395 | 32,085 | 14,822 | 19,383  |
| T32                | 41,635 | 37,543 | 92,127 | 77,176  |
| T33                | -      | -      | -      | 6,379   |
| T34                | -      | 3,527  | 65,490 | 7,884   |
| T35                | -      | -      | 15,617 | 9,147   |
| T37                | -      | -      | 15,617 | 9,147   |

**Total Tryptic Fragments**

| Helix 1 | Helix 2 | Helix 3 | Helix 4 |
|---------|---------|---------|---------|
| 14      | 14      | 15      | 24      |

* A total of 37 possible T-fragments are generated from the treatment of apoA-I with trypsin. Ten of these T-fragments (2, 8, 13, 15, 17, 20, 23, 24, 27, and 36) represent 1–2 amino acid peptides and have mass to charge (m/z) < 400. Breaks in the solid black line indicate fragments that were not detected, whereas solid black lines indicate detected fragments (>95% coverage), and red lines represent fragments that were not detected but had m/z > 400. Stars indicate position of lysine residues. Different colored sequence corresponds to the 10 amphipathic α-helical domains in apoA-I. The 1–43 amino acid region represents the N-terminal globular domain of apoA-I.

### TABLE TWO

| DSP cross-links having similar masses |
|--------------------------------------|
| **D** | **Theoretical mass of cross-linked products** | **Lysines involved** | **Tryptic fragments** | **ApoA-I helices** |
|-------|-----------------------------------------------|---------------------|----------------------|-------------------|
| 2338.1120 | 133–140 | T20-21 to 22 | 3, 4 |
| 2338.0861 | 94–96 | T12-13 to 14 | 5, 6 |
| 2337.1800 | 133–208 | T20-21 to T33 | 5, 8 |

* Lists the three theoretical cross-links that are similar to the observed cross-link of 2338.0833 Da found in purified 96-Å rHDL apoA-I treated with DSP at 37 °C for 5 min. Missed cut sites, T20-21 and T12-13 occur, since trypsin cannot cut after a DSP-modified lysine.

* ApoA-I helices correspond to the sequence shown at the bottom of Table I, denoted by differently colored rectangles. Cross-linked products were separated by 12% SDS-PAGE, digested with trypsin, and then analyzed by mass spectrometry, as described under “Experimental Procedures.”

**Mass Spectrometry of DSP Cross-linked Tryptic Peptides**—The 96-Å rHDLs containing human apoA-I were treated with DSP at three different molar ratios, and the product bands corresponding to the monomer and each of the apoA-I dimer products (i.e. D53, D80, and D59) were separated by 12% SDS-PAGE (Fig. 2), excised, digested, and then analyzed by LC-electrospray mass spectrometry. Non-DSP-treated homodimeric Q109C apoA-I was also analyzed in an identical manner.
and used as a control. The criteria for selection as a potential cross-linked peptide were the presence of 2–3 sequential protonated ions for the cross-linked peptide with an experimental mass that was within 0.5 Da of the theoretical mass for each.

For example, a peptide with an observed mass of 2,338.08 Da was identified in the total ion chromatogram that was similar to the theoretical mass of two different potential intramolecular cross-links, listed in TABLE TWO. In addition to these two potential cross-link sites, a potential intermolecular cross-linked peptide, T20-21 to T33, had a theoretical mass of 2,337.1800, also close to this observed mass. In order to unambiguously identify these cross-links, all candidate peptides identified from the Quattro II analyses were sequenced, since the distinction among these three structural conformations listed in TABLE TWO would greatly impact the final interpretation of all the structural information.

MS/MS of the m/z 780.39 ion (MH$_3^+$) gave a y series sequence, shown in Fig. 4, identical to that found in the T22 peptide and nowhere else in the apoA-I sequence. This was conclusive evidence that the 2,338.1257-Da peptide corresponded to the tryptic fragment T20-22 with an intramolecular cross-link between lysines 133 and 140, as listed in TABLE THREE. Capillary LC separated a second cross-linked peptide with a calculated mass of 2,338.0833 Da, whereas its sequence corresponded to the T12-14 peptide, indicating an intramolecular cross-link between lysines 94 and 96 (TABLE THREE). Several peptides found in the digests of lipid-bound apoA-I had masses very similar to the theoretical masses of other cross-linked peptides, making structural prediction nearly impossible. Thus, within the mass accuracy of the Quattro II, it was not possible to confirm the suspected identity of unknown peptides.

Another example illustrating the ambiguities of using only the m/z for peptide identification was found with the peptides of masses 2,950.45 and 2,933.43 Da that could be assigned to the peptide T17-18 and T14-15 to T14-15 with a cross-link between lysines 118 and 140 and the peptide T14-15 to T14-15 with a cross-link between lysines 118 and 118, respectively. Sequencing the MH$_3^+$ ion, m/z 984.89, from the 2,950.45-Da peptide gave MS/MS y series sequences that were identical to those of T18 and T22, suggesting a cross-link between lysines 118 and 140, as shown in Fig. 5. Sequencing the MH$_3^+$ ion, m/z 978.80, from the 2,933.43-Da peptide gave MS/MS y series sequences that were also identical to those of T18 and T22, suggesting that the 2,933.43-Da peptide was a modified form of the intermolecular cross-linked peptide T17-18 to T21-22, with a mass of 2,950.45 Da, shown in Fig. 5 and not the T14-15 to T14-15 cross-linked peptide. A 17-Da difference between the two tryptic peptides and the identification of glutamine 138 in the b series sequence suggests that glutamine 117 in the T17-18 component of the intermolecular cross-linked peptide had been converted to pyroglutamic acid. Another prominent intermolecular cross-link peptide with a mass of 2,772.34 Da was analyzed and found to contain the fragments T2-3 and T29-30, suggesting a cross-link between the N-terminal lysine 40 and the helix 10 lysine 239, as shown in Fig. 6.
Although the mass accuracy of the Q-TOF was important for distinguishing among cross-linked peptides and between other adventitious peptides, sequencing became the final arbiter of cross-link identity. The Q-TOF mass spectrometer was operated under conditions where the mass accuracy of survey scans was $\pm 30$ ppm, and suspect peptides were sequenced with a mass accuracy of $\pm 10$ ppm. Complete analysis of ~30 potential cross-links identified with the Quattro resulted in only a total of eight unique cross-links from peptide digests of lipid-bound DSP.

### TABLE THREE

**DSP cross-linked peptides identified for ApoA-I in 96-Å rHDL**

Purified 96-Å rHDL apoA-I's were treated with DSP at 37 °C for 5 min. Products were separated by 12% SDS PAGE, digested with trypsin, and then analyzed by mass spectrometry as described under “Experimental Procedures.”

| Lysines involved in cross-link | Theoretical mass of cross-linked peptide | Observed mass of cross-linked peptide | Error | Tryptic fragments involved in cross-link | ApoA-I helices involved | Cross-link peptide assigned by MS/MS analysis |
|-------------------------------|------------------------------------------|--------------------------------------|-------|------------------------------------------|-------------------------|---------------------------------------------|
| Intramolecular cross-links    |                                           |                                      |       |                                          |                         |                                             |
| $\alpha$-NH$_2$-12            | 2861.3569                                 | 2861.3789                            | −7.7  | T1 to T2-3                               | N-terminal, N-terminal | D (1–12) K-V (11–23) K                      |
| 12–23                         | 2051.0068                                 | 2051.0081                            | −0.6  | T2 to T4                                 | N-terminal             | V (11–27) R                                |
| 88–94                         | 1707.7518                                 | 1707.7528                            | −0.6  | T11 to T13                               | 3                      | Q (84–96) K                                |
| 94–96                         | 2338.0861                                 | 2338.0833                            | 1.2   | T12 to T14                               | 3                      | D (89–106) K                               |
| 133–140                       | 2338.1120                                 | 2338.1257                            | −5.9  | T20 to T22                               | 5                      | Q (132–149) R                               |
| Intermolecular cross-links    |                                           |                                      |       |                                          |                         |                                             |
| 12–182                        | 2792.4404                                 | 2792.4290                            | 4.1   | T2-3 to T29-30                           | N-terminal, 7          | V (11–23) R-L (178–188) R                  |
| 40–239                        | 2772.3417                                 | 2772.3472                            | −2.0  | T36-37 to T5-6                           | N-terminal, 10         | K (239–243) Q-D (28–45) K                  |
| 118–140                       | 2950.4666                                 | 2950.4524                            | 4.8   | T17-18 to T21-22$^d$                     | 4, 5                    | Q (117–123) R-L (134–149) R                |

$^a$ Since trypsin does not cut after a DSP-modified lysine, the column shows tryptic fragments with one missed cut site where the cross-linker connects the internal lysine in the two tryptic fragments.

$^b$ Extended peptide T2-T5 also sequenced.

$^c$ Extended, methionine sulfoxide form (T10-T13) was also sequenced.

$^d$ Pyroglutamic acid derivative also sequenced.

**FIGURE 5. Nanoelectrospray ionization-Q/TOF-MS/MS spectrum of intermolecular cross-linked apoA-I tryptic peptide: lysines 118 and 140.** The top panel shows the product ion spectrum for the 2,950.45-Da fragment isolated from the 80-kDa cross-linked product after tryptic digestion (TABLE THREE), as described under “Experimental Procedures.” The MS/MS spectrum was obtained from the triply charged ion $m/z$ 738.86. The sequence of the peptide is shown using single-letter abbreviations. A $y$ series for the QKVEPLR and both $y$ and $b$ series for LHELQKSPGLEEMR were identified for the peptide constituents that were cross-linked by DSP. The lower panel lists $m/z$ for experimental and theoretical product ions.
treated apoA-I on 96-Å rHDL. Of these, five were identified as intramo-
lecular cross-links, since they were found in monomer and dimer prod-
ucts, and the other three cross-links were designated as intermolecular,
since they were found predominately in apoA-I dimers. Relevant to
determining structural constraints for a model of lipid-bound apoA-I,
we noted that the intermolecular cross-link involving lysines 118–140
was found mainly in the D80 product isolated from SDS-PAGE, whereas
the cross-link involving the N-terminal lysine 40 and the helix 10 lysine
239 was found mainly in the D53 product.

Consideration was also given to the number of times a DSP cross-link
was found experimentally. Therefore, TABLE THREE only includes
cross-linked peptides that first met all of the criteria outlined under
“Experimental Procedures,” were found predominately in either dimer
or monomer products from multiple cross-link experiments (n > 3),
and finally were sequenced by MS/MS analysis. In general, the intensity
of an ion from a tryptic digest of apoA-I does not reflect the quantity of
cross-linked apoA-I peptides. Ion intensity depends upon how “readily”
the peptide is protonated and its size. Cross-linked peptides are gener-
ally of greater molecular weight compared with most of the noncross-
linked tryptic peptides from apoA-I. Thus, there is a discrimination
against peptides of greater molecular weight. In addition, there appears
to be some reduction in the extraction efficiency of the cross-linked
peptides that results in reduced ion intensity.

DISCUSSION

The current studies were designed to identify the spatial arrangement
of apoA-I when bound to 96-Å rHDL. Since the publication of the x-ray
crystal structure for lipid-free N-terminal truncated Δ43 apoA-I (15)
little progress has been made using this same approach for lipid-bound
full-length wild-type apoA-I. Although a number of other methods used
to deduce the lipid-bound conformation generally support the con-
formation suggested by the crystal structure, these studies have not
been able to distinguish between an extended belt and a hairpin
conformation (16–27).

Utilizing the lysine-reactive cross-linker, DSP, and mass spectrome-
try, we have identified and validated five intramolecular and three inter-
molecular cross-links for lipid-bound apoA-I on 96-Å rHDL. Based
on these data, we have constructed a molecular model using distance con-
straints, as shown in Fig. 7. The most striking finding from these data
and the resulting model relates to the magnitude and placement of the
cross-links compared with the number of predicted cross-links based on
the x-ray crystal structure of lipid-free Δ43 apoA-I. Theoretically, 13
intramolecular and 22 intramolecular cross-links are within a distance
that could yield cross-links, listed in TABLES FOUR and FIVE, respec-
tively (15). Even with the N-terminal 43 residues missing, predictions
based on the x-ray crystal structure compared with our observed num-
er of lysine cross-links suggest that the lipid-bound apoA-I conforma-
tion does not precisely follow the x-ray crystal structure. In fact, two of
the three intermolecular cross-links identified in these studies involve
the N-terminal region of apoA-I, absent from the x-ray crystal structure
of lipid-free Δ43 apoA-I.

Thus, if the in-solution conformation of lipid-bound apoA-I was
identical to the conformation seen in the x-ray crystal structure, then
most of the cross-links listed in TABLES FOUR and FIVE should have
been identified, with the caveat that each lysine had equal reactivity.
There is good evidence that the reactivity of lysine with chemical cross-
linkers depends on the local environment of each lysine and that there is
considerable variation in the reactivity of lysines at various positions on
a protein. However, the rather small number of confirmed cross-links in
TABLE THREE suggests that the orientation of apoA-I lysines on rHDL

FIGURE 6. Nanoelectrospray ionization-Q/TOF-MS/MS spectrum of intermolecular cross-linked apoA-I tryptic peptide: lysine 40 to lysine 239. The top panel shows the product ion spectrum for the 2,772.34-Da fragment isolated from the 53-kDa dimer after tryptic digestion (TABLE THREE) using conditions as described under “Experimental Procedures.” The MS/MS spectrum was obtained from the triply charged ion m/z 935.19. The peptide sequence is shown using the single-letter abbreviations. A y series was identified for each peptide constituent. The lower panel lists m/z for experimental and theoretical product ions.
are significantly different from those found in the crystal structure of lipid-free Δ43 apoA-I.

Previous studies from this laboratory employing FRET (25, 26) suggested that lipid-bound apoA-I exists as either an antiparallel belt or a hairpin conformation. In the current studies, a head to head or head to tail hairpin conformation could not accommodate the combination of the central cross-link, Lys118–Lys140, and the two intermolecular cross-links located at the N- and C-terminal ends of the molecule. Given the structural constraints dictated by these intermolecular cross-links identified, it appears that the highly amphipathic and α-helical central domain (residues 44–186) of apoA-I wraps around the lipid bilayer, in a manner consistent with a “belt” conformation, whereas the globular N-terminal domain of apoA-I folds in a “hairpin” or loops back on itself, maximizing interaction with the C-terminal domain. Protein-protein interactions may stabilize the weak lipid-binding globular N terminus of apoA-I through interaction with the highly hydrophobic C-terminal domain, functioning as a “belt buckle” to stabilize binding of apoA-I to HDL complexes.

The accuracy of the three-dimensional information derived from chemical cross-linking/mass spectrometry studies has been addressed by comparing the distance constraints deduced from cross-linking with various homobifunctional, lysine-reactive cross-linkers compared with the structure determined by x-ray crystallography. In studies of fibroblast growth factor-2 (29) and human serum albumin (30), data from chemical cross-linking/mass spectrometry reproduced the correct stereochemical relationships deduced from both NMR and x-ray crystallography.

The only G* class domain of ApoA-I (47), residues 1–43, may share some similarity to the G* domain in apoE that encompasses residues 1–160, which includes most of the N-terminal four-helix bundle as determined by x-ray crystallography (48) and which contains the low density lipoprotein receptor binding domain (residues 136–150). The G* domain in apoE has weak lipid binding affinity, but its principle function is to bind to the low density lipoprotein receptor. The interaction between the N terminus of apoE and the low density lipoprotein receptor has been shown to depend on salt bridge formation exemplified by the apoE2 isoform in which Arg158 is mutated to cysteine, thereby disrupting a network of salt bridges (49) and altering apoE and low density lipoprotein receptor binding.

Interestingly, the function of the N terminus of apoA-I has also been linked to hereditary amyloidosis (also called familial amyloidotic polyneuropathy) which is a late onset autosomal dominant disease characterized by the deposition of extracellular proteinaceous material and varying degrees of neuropathy, nephropathy, and cardiomyopathy. The most common protein found in amyloid deposits is a variant of transthyretin, also referred to as prealbumin. Presumably due to its high content of β-conformation, it readily forms amyloid fibrils. A new subtype of hereditary amyloidosis was described in an Iowa kindred that was
N-Terminal Fold of ApoA-I Stabilizes Lipid-bound Conformation

characterized by lower limb neuropathy, peptic ulcers, and renal failure. Amyloid fibrils isolated from the liver and spleen of a patient who died with the Iowa type familial amyloidotic polyneuropathy was found to contain the amino-terminal fragment (residues 1–83) of a mutated form of apoA-I, called apoA-I Iowa, with an arginine for glycine substitution at position 26. The amino-terminal fragment of apoA-I Iowa was the major constituent of the amyloid deposit, with no evidence of a transthyretin component present. Seven other apoA-I mutations have been reported to be associated with hereditary amyloidosis and low circulating levels of plasma HDL. Most of these mutations are localized around the amino terminus of apoA-I (50), suggesting that mutations in the N-terminal domain may cause misfolding and/or aggregation of apoA-I.

Studies by Rogers et al. (51) first suggested that the N- and C-terminal regions of lipid-free apoA-I exist in close proximity, representing its preferred conformation in the absence of lipid. Since then, this idea has been refined and extended in more recent studies (52–57) and consistent with bioinformatics studies showing that essentially all proteins known to fold kinetically in a two-state manner have their N- and C-terminal regions in contact (58). It is generally believed that the N-terminal truncation of apoA-I (∆43 apoA-I) causes apoA-I to assume a conformation resembling its lipid-bound structure, and thus the N terminus of apoA-I is critical for the solution structure and stability of apoA-I, whereas the C terminus of apoA-I functions as a “lipid-trigger” (56) that is activated in the presence of lipid. However, a number of other reports suggest that both the N- and C-terminal domains of apoA-I are important in initiating the lipid-binding process together (14, 57, 59–61) and interact with each other, allowing stabilization of the lipid-free solution conformation.

To minimize rHDL particle to particle cross-linking, dilute solutions of lipid-bound apoA-I were cross-linked at low molar ratios of DSP to apoA-I for a very short period of time. These studies showed that DSP-treated lipid-bound apoA-I yielded two distinct intermolecular cross-linked dimeric products. Comparing the unique migration of the cysteine-containing apoA-I homodimers to the dimers generated by the treatment of apoA-I with low molar ratios of DSP suggested that there were two conformationally distinct dimers of apoA-I. Using a panel of cysteine-containing apoA-I homodimers, we compared their migration on nonreducing 12% SDS-PAGE and found that when cysteine cross-links existed near the N- or C-terminal ends of the protein (i.e. S231C or Q5C), the apoA-I homodimer migrated at ~53 kDa, whereas apoA-I homodimers with the cross-links at the center (i.e. Q109C or Q132C) migrated with an apparent mass of ~80 kDa. Heterodimers consisting of radiolabeled S231C dimerized to Q132C apoA-I were found to migrate at ~80 kDa, suggesting that the conformational constraints dictated by cross-links at a central region dominate, giving rise to a slower electrophoretic mobility. Further studies showed that the conformational constraint imposed by centrally located apoA-I cross-links was overcome by denaturation with 6 M urea, suggesting that the mechanism for the difference in apoA-I dimer migration may be related to strong hydrophobic protein-protein interactions present when apoA-I is cross-linked or dimerized at its center. Thus, the cysteine homodimer migration was consistent with the identification and location of intermolecular apoA-I cross-links found on 96-Å rHDL.

Considering the 53- and 80-kDa apoA-I cysteine homodimer migration pattern, we compared this to the DSP-generated cross-links found in each of these product bands from SDS-PAGE. Only one intermolecular cross-link, Lys118–Lys140, was found exclusively in the 80-kDa dimer band upon SDS-PAGE. Based on the migration of apoA-I cysteine homodimers, an internal cross-link in the central region would migrate at 80 kDa. The ~53-kDa band gave cross-links corresponding to Lys12–Lys182 and Lys80–Lys239. The position of both of these cross-links is consistent with migration at 53 kDa as demonstrated by the

### TABLE FOUR

| Lysine residues involved | Helix location | Distance between α-carbons of lysine pair in apoA-I Å |
|-------------------------|---------------|---------------------------------------------------|
| 45–59                   | 1–1           | 22.711                                            |
| 59–77                   | 1–2           | 22.751                                            |
| 77–88                   | 2–3           | 16.263                                            |
| 88–94                   | 3–3           | 9.957                                             |
| 88–96                   | 3–3           | 12.820                                            |
| 94–96                   | 3–3           | 5.681                                             |
| 94–106                  | 3–4           | 17.331                                            |
| 94–107                  | 3–4           | 18.860                                            |
| 96–106                  | 3–4           | 16.992                                            |
| 96–107                  | 3–4           | 18.951                                            |
| 106–107                 | 4–4           | 3.7821                                            |
| 106–118                 | 4–4           | 19.210                                            |
| 107–118                 | 4–4           | 17.951                                            |
| 118–133                 | 4–5           | 24.728                                            |
| 133–140                 | 5–5           | 10.313                                            |
| 182–195                 | 7–8           | 19.943                                            |
| 195–206                 | 8–8           | 16.360                                            |
| 195–208                 | 8–8           | 22.087                                            |
| 206–208                 | 8–8           | 5.300                                             |
| 226–238                 | 10–10         | 17.427                                            |
| 226–239                 | 10–10         | 20.012                                            |
| 238–239                 | 10–10         | 3.811                                             |

* Distance between α-carbons of the cross-linked lysine pair as calculated from x-ray crystallography of lipid-free ∆43 apoA-I (Protein Data Bank code 1av1) (15). Inclusion criteria were based on the maximum combined distance of DSP linker arm and lysine side chains (total of 25 Å) (32), although the linker arm may be significantly shorter than the reported 12 Å (46).

### TABLE FIVE

| Lysine residues involved | Helix location | Distance between α-carbons of lysine pair in apoA-I Å |
|-------------------------|---------------|---------------------------------------------------|
| 45–208                  | 1–10          | 20.008                                            |
| 59–195                  | 1–8           | 17.058                                            |
| 59–206                  | 1–8           | 9.199                                             |
| 59–208                  | 1–8           | 14.351                                            |
| 77–182                  | 2–7           | 14.161                                            |
| 7–195                   | 2–8           | 18.534                                            |
| 88–182                  | 3–7           | 21.057                                            |
| 118–133                 | 4–5           | 18.169                                            |
| 118–140                 | 4–5           | 12.254                                            |
| 133–133                 | 5–5           | 13.702                                            |
| 133–140                 | 5–5           | 22.669                                            |
| 226–238                 | 10–10         | 18.177                                            |
| 226–239                 | 10–10         | 14.679                                            |

* Distance between α-carbons of the cross-linked lysine pair as calculated from x-ray crystallography of lipid-free ∆43 apoA-I (Protein Data Bank code 1av1) (15). Inclusion criteria were based on the maximum combined distance of DSP linker arm and lysine side chains (total of 25 Å) (32), although the linker arm may be significantly shorter than the reported 12 Å (46).
apoA-I cysteine homodimers. The identification of these two intermolecular cross-links also demonstrated interaction between the N- and C-terminal domains, as depicted in Fig. 7. Compared with another study of lipid-bound apoA-I using the cross-linking mass spectrometry approach (36), Lys<sup>230</sup>–Lys<sup>40</sup> was identified as an intramolecular link, whereas the Lys<sup>12</sup>–Lys<sup>82</sup> was not found. It is possible that the lack of agreement between the two studies results from differences in the length of cross-linking incubation time and/or product resolution. Additional studies will be required to resolve these issues.

Overall, these data provide direct experimental evidence validating the “belt model” of apoA-I over the “hairpin” conformation, although these studies suggest that elements of an N-terminal fold or hairpin do exist, making contact with the C terminus and possibly acting to stabilize the lipid-protein interactions on 96 Å rHDL complexes.

Addendum—Relevant to the current studies, a related paper (62) was published during the manuscript review process.

REFERENCES

1. Boden, W. E. (2000) Am. J. Cardiol. 86, 191–211.
2. Harper, C. R., and Jacobson, T. A. (1999) Arch. Intern. Med. 159, 1049–1057.
3. Libby, P. (2000) Am. J. Cardiol. 88, 3N–8N.
4. Trigatti, B. L., Rigotti, A., and Braun, A. (2000) Biochim. Biophys. Acta 1529, 276–286.
5. Trigatti, B., Rigotti, A., and Krieger, M. (2000) Cur. Opin. Lipid. 11, 123–131.
6. Yancey, P. G., Bortnick, A. E., Kellner-Weibel, G., De La Llera-Moya, M., Phillips, M. C., and Rothblat, G. H. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 712–719.
7. Libby, P. (2000) Am. J. Cardiol. 88, 3N–8N.
8. Steinberg, D. (2002).
9. Maiorano, J. N., Jandacek, R. J., Horace, E. M., and Davidson, W. S. (2004).
10. Pennathur, S., Bergt, C., Shao, B., Byun, J., Kassim, S. Y., Singh, P., Green, P. S., Getz, G. S. (2002) J. Biol. Chem. 277, 17374–17380.
11. Chait, A., Han, C. Y., Oram, J. F., and Heinecke, J. W. (2005).
12. Zheng, L., Settle, M., Brubaker, G., Schmitt, D., Hazen, S. L., Smith, J. D., and Kinter, M. V. (2005) J. Biol. Chem. 280, 31755–31758.
13. Niessen, R., van den Heuvel, M., Blokland, A. J., van Boeijen, M. H., Roskams, T., Feron, V. (2005) J. Biol. Chem. 280, 31759–31764.
14. Saito, Y., Price, R. W., Rottenberg, D. A., Fox, J. J., Su, T. L., Watanabe, K. A., and Gribble, F. M. (1999) Science 286, 1709–1712.
15. Chait, A., Han, C. Y., Oram, J. F., and Heinecke, J. W. (2005) J. Biol. Chem. 280, 389–403.
16. Zheng, L., Settle, M., Brubaker, G., Schmitt, D., Hazen, S. L., Smith, J. D., and Kinter, M. V. (2005) J. Biol. Chem. 280, 38–47.
17. Marcel, Y. L., and Kiss, R. S. (2003) Curr. Opin. Lipidol. 14, 151–157.
18. Saito, Y., Price, R. W., Rottenberg, D. A., Fox, J. J., Su, T. L., Watanabe, K. A., and Phillips, F. S. (1982) Science 217, 1115–1153.
19. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12291–12296.
20. Wang, G. (2002) FEBS Lett. 529, 157–161.
21. Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellingler, M., De Loof, H., and Harvey, S. C. (1999) J. Biol. Chem. 274, 31755–31758.
22. Klon, A. E., Segrest, J. P., and Harvey, S. C. (2002) J. Mol. Biol. 324, 703–721.
23. Koppaka, V., Silvestro, L., Engler, J. A., Brouillette, C. G., and Axelsson, P. H. (1999) J. Biol. Chem. 274, 61565–61574.
24. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12291–12296.
25. Wang, G. (2002) FEBS Lett. 529, 157–161.
26. Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellingler, M., De Loof, H., and Harvey, S. C. (1999) J. Biol. Chem. 274, 31755–31758.
27. Klon, A. E., Segrest, J. P., and Harvey, S. C. (2002) J. Mol. Biol. 324, 703–721.
28. Koppaka, V., Silvestro, L., Engler, J. A., Brouillette, C. G., and Axelsson, P. H. (1999) J. Biol. Chem. 274, 61565–61574.
29. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12291–12296.
30. Wang, G. (2002) FEBS Lett. 529, 157–161.
31. Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellingler, M., De Loof, H., and Harvey, S. C. (1999) J. Biol. Chem. 274, 31755–31758.
32. Klon, A. E., Segrest, J. P., and Harvey, S. C. (2002) J. Mol. Biol. 324, 703–721.
33. Koppaka, V., Silvestro, L., Engler, J. A., Brouillette, C. G., and Axelsson, P. H. (1999) J. Biol. Chem. 274, 61565–61574.
34. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12291–12296.
35. Wang, G. (2002) FEBS Lett. 529, 157–161.
36. Segrest, J. P., Jones, M. K., Klon, A. E., Sheldahl, C. J., Hellingler, M., De Loof, H., and Harvey, S. C. (1999) J. Biol. Chem. 274, 31755–31758.
37. Klon, A. E., Segrest, J. P., and Harvey, S. C. (2002) J. Mol. Biol. 324, 703–721.
38. Koppaka, V., Silvestro, L., Engler, J. A., Brouillette, C. G., and Axelsson, P. H. (1999) J. Biol. Chem. 274, 61565–61574.
39. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12291–12296.