

## $\alpha$ -Helix Formation Is Required for High Affinity Binding of Human Apolipoprotein A-I to Lipids\*

Received for publication, February 24, 2004, and in revised form, March 11, 2004  
Published, JBC Papers in Press, March 12, 2004, DOI 10.1074/jbc.M402043200

Hiroyuki Saito<sup>‡</sup>, Padmaja Dhanasekaran<sup>‡</sup>, David Nguyen<sup>‡</sup>, Els Derudder<sup>§</sup>, Paul Holvoet<sup>§</sup>,  
Sissel Lund-Katz<sup>‡</sup>, and Michael C. Phillips<sup>‡</sup>¶

From the <sup>‡</sup>Division of Gastroenterology and Nutrition, The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-4318 and the <sup>§</sup>Center for Experimental Surgery and Anesthesiology, University of Leuven, B-3000 Leuven, Belgium

Apolipoprotein (apo) A-I is thought to undergo a conformational change during lipid association that results in the transition of random coil to  $\alpha$ -helix. Using a series of deletion mutants lacking different regions along the molecule, we examined the contribution of  $\alpha$ -helix formation in apoA-I to the binding to egg phosphatidylcholine (PC) small unilamellar vesicles (SUV). Binding isotherms determined by gel filtration showed that apoA-I binds to SUV with high affinity and deletions in the C-terminal region markedly decrease the affinity. Circular dichroism measurements demonstrated that binding to SUV led to an increase in  $\alpha$ -helix content, but the helix content was somewhat less than in reconstituted discoidal PC-apoA-I complexes for all apoA-I variants, suggesting that the helical structure of apoA-I on SUV is different from that in discs. Isothermal titration calorimetry showed that the binding of apoA-I to SUV is accompanied by a large exothermic heat and deletions in the C-terminal regions greatly decrease the heat. Analysis of the rate of release of heat on binding, as well as the kinetics of quenching of tryptophan fluorescence by brominated PC, indicated that the opening of the N-terminal helix bundle is a rate-limiting step in apoA-I binding to the SUV surface. Significantly, the correlation of thermodynamic parameters of binding with the increase in the number of helical residues revealed that the contribution of  $\alpha$ -helix formation upon lipid binding to the enthalpy and the free energy of the binding of apoA-I is  $-1.1$  and  $-0.04$  kcal/mol per residue, respectively. These results indicate that  $\alpha$ -helix formation, especially in the C-terminal regions, provides the energetic source for high affinity binding of apoA-I to lipids.

HDL against coronary artery disease is due in part to the role of apoA-I in mediating cholesterol transport from peripheral cells to sites of catabolism and activating lecithin-cholesterol acyltransferase in the reverse cholesterol transport process (1–3). Recent studies have shown that the lipid-free form of apoA-I also promotes cholesterol efflux from cells via ATP-binding cassette transporter A1 (ABCA1) (4, 5). In contrast to the passive diffusion of cholesterol to HDL, the release of cellular lipids to lipid-free apoA-I via ABCA1 is an active process (6, 7).

The 243-amino-acid polypeptide chain of apoA-I is organized into characteristic 22- and 11-residue tandem repeats that are predicted to form amphipathic  $\alpha$ -helices (8). Although the tertiary structure of lipid-free apoA-I has not been resolved at high resolution, a large number of studies using deletion and site-directed mutagenesis techniques have provided important insights into the lipid-free structure of apoA-I (for reviews, see Refs. 9 and 10). Proteolysis analysis (11) and deletion mutagenesis studies (12, 13) have suggested that the lipid-free apoA-I molecule is organized into two structural domains. The N-terminal and central parts form a helix bundle, whereas the C-terminal  $\alpha$ -helices form a separate, less organized structure. The helix bundle organization in the N-terminal domain is also supported by fluorescence studies of single Trp mutants of human (14) and chicken apoA-I (15). In addition, recent studies using more specific mutations in the C-terminal region (16) and site-directed spin-label electron paramagnetic resonance spectroscopy (17) indicated that there is a stable helical structure in the extreme C terminus of apoA-I.

Amphipathic  $\alpha$ -helical segments in the apoA-I molecule are responsible for the strong lipid binding properties of this protein, and the C-terminal domain of apoA-I is critical for lipid binding (12, 13, 18, 19). In agreement with this, the 22-residue amphipathic peptides representing the first and last repeat helices in the intact apoA-I molecule have the greatest lipid affinities (20). Importantly, studies investigating apoA-I-mediated cholesterol efflux from cells via ABCA1 have shown that the C-terminal  $\alpha$ -helix in apoA-I plays a key role in efflux to this protein (21–25). However, the molecular mechanism for the interaction between apoA-I and the cell surface remains to be elucidated; two models in which apoA-I interacts directly with either ABCA1 (26–28) or adjacent membrane lipid domains (23, 25, 29) have been proposed.

The conformational transition from random coil to  $\alpha$ -helix upon lipid binding is thought to provide the energetic source to drive the lipid interaction of apolipoproteins (17, 30, 31), but this has not been demonstrated directly by quantitative analysis of the thermodynamics involved. In the present study, we examined the energetics of  $\alpha$ -helix formation in apoA-I upon binding to small unilamellar vesicles (SUV) using six deletion

Apolipoprotein (apo)<sup>1</sup> A-I, the major protein component of high density lipoproteins (HDL), plays an important role in the stability and function of HDL particles. A protective effect of

\* This work was supported by National Institutes of Health Grant HL22633 and the Belgian Interuniversitaire Attractiepolen Programma (Grant P05/02). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: The Children's Hospital of Philadelphia, Abramson Research Ctr., Ste. 1102, 3615 Civic Center Blvd., Philadelphia, PA 19104-4318. Tel.: 215-590-0587; Fax: 215-590-0583; E-mail: phillipsmi@email.chop.edu.

<sup>1</sup> The abbreviations used are: apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A1; 6,7-diBrPC, 1-palmitoyl-2-stearoyl-(6-7)-dibromo-phosphatidylcholine; HDL, high density lipoprotein; ITC, isothermal titration calorimetry; PC, phosphatidylcholine; POPC, 1-palmitoyl,2-oleoyl phosphatidylcholine; SUV, small unilamellar vesicles; WT, wild type.

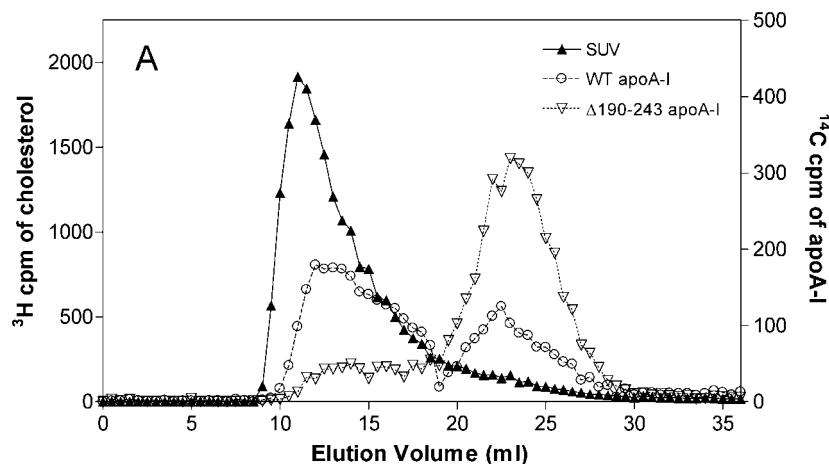
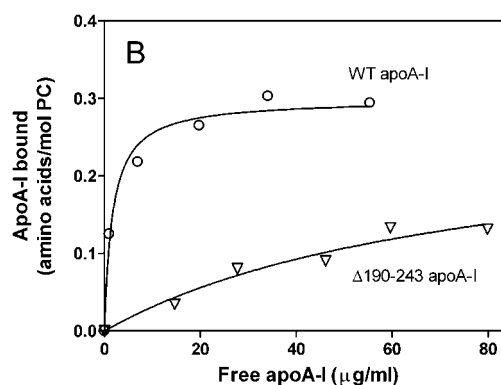


FIG. 1. Binding of apoA-I to egg PC SUV measured by gel filtration. A, elution profiles of 1 mg/ml egg PC SUV after incubation with apoA-I variants (60  $\mu$ g/ml) separated by Sepharose CL-6B. B, binding isotherms of WT and  $\Delta$ 190–243 apoA-I to egg PC SUV.



mutants of apoA-I lacking N-terminal ( $\Delta$ 1–43,  $\Delta$ 44–65, and  $\Delta$ 44–126), central ( $\Delta$ 123–166), or C-terminal ( $\Delta$ 190–243 and  $\Delta$ 223–243) regions along the molecule. In the N-terminal deletion mutants,  $\Delta$ 1–43 lacks the first 43 residues, which are encoded by exon 3 and are suggested to stabilize the lipid-free structure of protein (32),  $\Delta$ 44–65 lacks the first amphipathic  $\alpha$ -helical repeat, which has high lipid affinity (20), and  $\Delta$ 44–126 lacks most of the N-terminal half of the molecule. In the  $\Delta$ 123–166 mutant, two putative central helices that have weak lipid affinity (20) and that overlap with a putative lecithin-cholesterol acyltransferase activation domain (18) are deleted. Finally, the  $\Delta$ 190–243 and  $\Delta$ 223–243 mutants, in which either one or two C-terminal putative  $\alpha$ -helical repeats are deleted, lack the last  $\alpha$ -helix (residues 220–241) that has the greatest lipid affinity (20). Among these truncated segments in apoA-I, the N-terminal and central parts are thought to constitute a helix bundle motif, whereas the C-terminal region forms a separate domain (13). The results show that  $\alpha$ -helix formation, especially in the C-terminal domain of apoA-I, provides a major enthalpic contribution to the energetics of lipid binding, indicating a critical role for the C-terminal domain in driving apoA-I to bind to lipid with high affinity.

#### EXPERIMENTAL PROCEDURES

**Materials**—Egg PC was purchased from Sigma. 1-Palmitoyl,2-oleoyl PC (POPC) was from Avanti Polar Lipids (Pelham, AL). 1-Palmitoyl-2-stearoyl-(6-7)-dibromo-phosphatidylcholine (6,7-diBrPC) was from Molecular Probes (Eugene, OR). 1,2- $^3$ H]cholesterol and  $^{14}$ C]formaldehyde were purchased from PerkinElmer Life Sciences. Ultrapure guanidine hydrochloride was from ICN Pharmaceuticals (Costa Mesa, CA). Bacteriological media were obtained from Fisher. The prokaryotic expression vector pET32a was from Novagen (Madison, WI), and the competent *Escherichia coli* strain BL21-DE3 was from Invitrogen. PCR supplies were from Qiagen (Chatsworth, CA). Restriction enzymes were purchased from Promega (Madison, WI). Oligonucleotides were from

IDT (Coraville, IA), and DNA purification kits were from Qiagen. All other salts and reagents were of analytical grade.

Plasma apoA-I was isolated from human HDL as described (21). Human apoA-I and engineered deletion mutants were expressed as a thioredoxin fusion protein in *E. coli* strain BL21-DE3 host and then cleaved and purified as described previously (13). The apoA-I preparations were at least 95% pure as assessed by SDS-PAGE. In all experiments, apoA-I was freshly dialyzed at concentrations  $\leq$  1 mg/ml from 6 M guanidine hydrochloride solution into the appropriate buffer before use.

**Preparation of SUV**—Egg PC dissolved in chloroform:methanol (2:1, v/v) was dried under nitrogen onto the wall of a Corex glass tube and then placed in a vacuum oven to completely remove any remaining solvent. The lipid was then rehydrated in Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.02%  $\text{NaN}_3$ , pH 7.4) and sonicated on ice under nitrogen with a Branson Sonifier 350. A 5-min pulse of sonication was followed by 1 min of cooling. This cycle was repeated up to 10 times until the initially cloudy lipid dispersion became translucent. After spinning in a Beckman GPR tabletop centrifuge for 15 min at 3,000 rpm to remove titanium debris, the samples were centrifuged in a Beckman 50 Ti rotor for 2 h at 4  $^{\circ}$ C at  $145,000 \times g$  to separate any remaining large vesicles. The top layer was collected as SUV with a diameter of  $\sim$ 21 nm (33). For gel filtration experiments requiring incorporation of radioactive lipid,  $^3$ H]cholesterol in trace amounts ( $<$ 1 mol %) was dried along with egg PC in a glass tube and processed as described above for unlabeled SUV. The SUV were stored at 4  $^{\circ}$ C and used within a few days of preparation; the binding of apoA-I to aged SUV is greatly reduced.

**Gel Filtration**—The binding of apoA-I to SUV was assayed by gel filtration as described (34–36). The apoA-I variants were radiolabeled to a specific activity of  $\sim$ 1  $\mu$ Ci/mg of protein by reductive methylation of lysine residues with  $^{14}$ C]formaldehyde as described (37, 38). This trace labeling of apoA-I leads to modification of less than 1 lysine residue in the molecule, and there is no detectable change in the physical properties of the protein. Typically, fresh SUV (1 mg/ml egg PC) containing a trace amount of  $^3$ H]cholesterol was incubated with shaking for 1 h at room temperature with increasing concentrations (10–100  $\mu$ g/ml) of  $^{14}$ C-labeled apoA-I. The mixtures were then applied to a Sepharose

CL-6B column ( $1 \times 28$  cm), and 0.5-ml fractions were collected. The column void and total volumes were determined by eluting blue dextran and cytidine, respectively. Aliquots of each fraction were counted using liquid scintillation procedures to determine the levels of [ $^3$ H]cholesterol (SUV) and [ $^{14}$ C]apoA-I. The radioactivity elution profiles were analyzed in an Excel spreadsheet to determine the ratio of bound to free apoA-I; corrections were made for the small overlap of the elution profiles for bound apoA-I (co-eluting with SUV) and free protein. The elution chromatograms were also fitted with Gaussian distribution functions using Origin software (MicroCal Inc., Northampton, MA), and similar results were obtained. Binding isotherms were obtained by non-linear regression analysis (GraphPad Prism) using a one-binding-site model.

**CD Spectroscopy**—CD spectra were recorded from 184 to 260 nm at room temperature using a Jasco J-600 spectropolarimeter. After dialyzing from 6 M guanidine hydrochloride solution, the apoA-I sample was diluted to 25  $\mu$ g/ml in 10 mM sodium phosphate buffer (pH 7.4), and the CD spectrum was obtained. For the apoA-I-SUV mixture sample, apoA-I was incubated with SUV for 1 h prior to the measurement. The results were corrected by subtracting the buffer baseline or a blank

sample containing an identical concentration of SUV. In addition, spectra of apoA-I-POPC discoidal complexes (PC:protein ratio, 1.7:1 w/w, diameter  $\sim 10$  nm) prepared using the cholera dispersion method (39, 40) were obtained. The  $\alpha$ -helix content was calculated from the molar ellipticity at 222 nm, as described (41). Since there was no significant difference in the CD spectra of buffer and SUV at wavelengths down to 200 nm, the artifact of light scattering coming from SUV particles on the estimation of the  $\alpha$ -helix content was negligible (42).

**Trp Fluorescence Measurements**—Experiments to measure Trp quenching by 6,7-diBrPC were carried out with a Hitachi F-4500 fluorescence spectrophotometer at 25  $^{\circ}$ C. After mixing 25  $\mu$ g/ml apoA-I with 1.5 mg/ml egg PC SUV with or without 33 mol % of 6,7-diBrPC, Trp fluorescence spectra were collected from 300 to 400 nm at an excitation wavelength of 280 nm. To reduce the effect of light scattering caused by vesicles, the sample was excited with vertically polarized light and measured with a horizontal emission polarizer (43). Kinetics data of Trp quenching of apoA-I were obtained by monitoring the emission intensity at 340 nm.

**Isothermal Titration Calorimetry (ITC) Measurements**—Heats of apoA-I binding to SUV were measured with a MicroCal MCS isothermal titration calorimeter at 25  $^{\circ}$ C as described (13, 38). The SUV sample (15 mM) was placed into the sample cell (1.35 ml) and titrated with 8- $\mu$ l aliquots of apoA-I sample (0.8 mg/ml) with continual stirring at 400 rpm. Heats of dilution determined by injecting protein solution into buffer were subtracted from the corresponding heats to give the enthalpy of binding. The decay rate constants of heat were obtained from fitting the titration curves to a one- or two-phase exponential decay model.

**Analytical Procedures**—Protein concentrations were determined by either the Lowry procedure (44) or the absorbance coefficient at 280 nm (13). PC concentrations were determined with an enzymatic assay kit (Wako Chemicals, Richmond, VA).

## RESULTS

**Binding Isotherms of ApoA-I to SUV**—The binding of apoA-I to egg PC SUV was analyzed by gel filtration to compare the lipid binding properties among the apoA-I variants. In our experimental condition, there was no change in the elution profiles of SUV after incubation with apoA-I, indicating that bound apoA-I did not disrupt the structural integrity of the

TABLE I  
Parameters for binding of apoA-I variants to egg PC SUV

ApoA-I variant	$K_d^a$ $\mu$ g/ml	$B_{max}^a$ amino acids/mol PC
Plasma	$1.8 \pm 0.5$	$0.36 \pm 0.03$
WT	$1.7 \pm 0.4$	$0.30 \pm 0.02$
$\Delta 1-43$	$6.3 \pm 0.6$	$0.40 \pm 0.01$
$\Delta 44-65$	$4.2 \pm 1.1$	$0.49 \pm 0.04$
$\Delta 44-126$	$6.5 \pm 1.6$	$0.46 \pm 0.03$
$\Delta 123-166$	$3.3 \pm 0.9$	$0.44 \pm 0.03$
$\Delta 190-243$	$69 \pm 39$	$0.25 \pm 0.08$
$\Delta 223-243^b$	>100	ND <sup>c</sup>

<sup>a</sup> Complete binding isotherms were determined in at least two independent experiments. The data points were pooled and  $K_d$  and  $B_{max}$  values were obtained by nonlinear regression fitting, and expressed as mean  $\pm$  S.D.

<sup>b</sup> One binding site model did not give reasonable values of  $K_d$  and  $B_{max}$  because the binding isotherm did not saturate in the concentration range studied.

<sup>c</sup> ND, not determined.

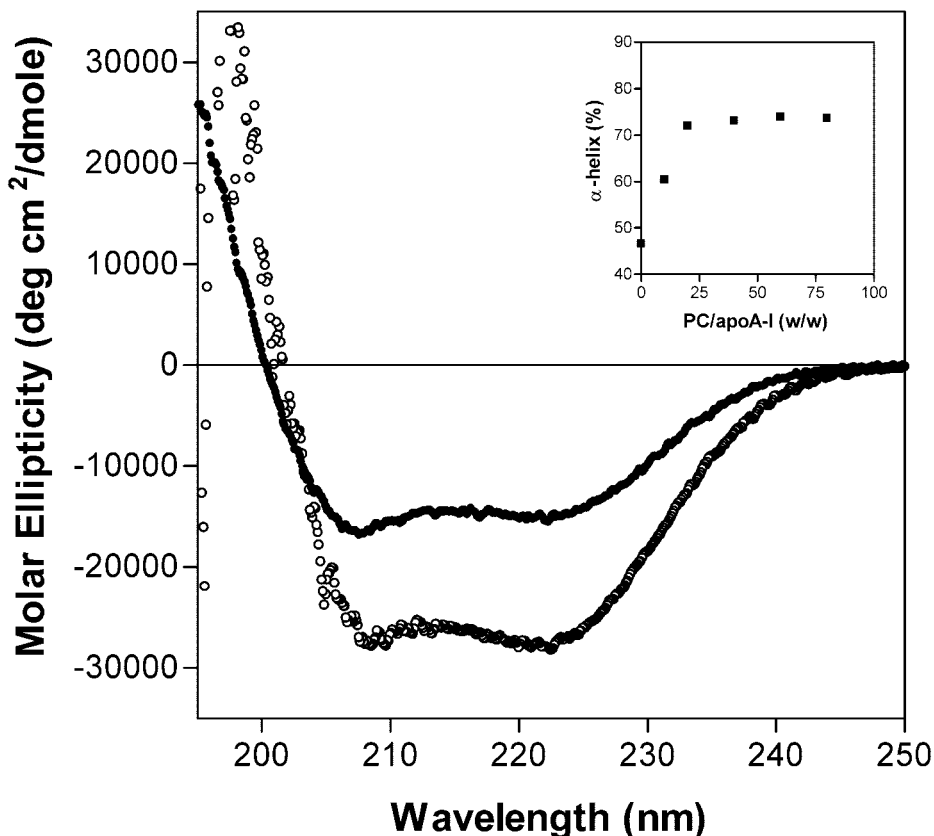


FIG. 2. Far-UV CD spectra of plasma apoA-I in the lipid-free state (●) or bound to egg PC SUV (○). Protein and PC concentrations were 0.025 and 1.5 mg/ml, respectively. The inset shows the increase in  $\alpha$ -helix content of apoA-I as a function of the weight ratio of PC to apoA-I (the apoA-I concentration was 0.025 mg/ml).

TABLE II  
 $\alpha$ -Helix content of apoA-I variants in the lipid-free and lipid-bound states determined by far-UV CD

apoA-I variants	$\alpha$ -Helix content			Increase in $\alpha$ -helix content	
	Lipid-free <sup>a</sup>	SUV <sup>a</sup>	Discs <sup>b</sup>	SUV	Discs
			%	%	
Plasma	46 ± 2	69 ± 3	78	23	32
WT	44 ± 3	69 ± 5	76	25	32
$\Delta$ 1-43	48 ± 2	59 ± 5	73	11	25
$\Delta$ 44-65	43 ± 2	53 ± 4	69	10	26
$\Delta$ 44-126	23 ± 4	49 ± 3	50	26	27
$\Delta$ 123-166	48 ± 4	69 ± 6	76	21	28
$\Delta$ 190-243	51 ± 2	58 ± 2	71	7	20
$\Delta$ 223-243	40 ± 2	45 ± 4	66	5	26

<sup>a</sup> Mean ± S.D. from at least six independent measurements.

<sup>b</sup> Average of duplicate measurements using POPC-apoA-I discoidal complexes.

vesicles (35, 45). Fig. 1A shows typical elution profiles of <sup>14</sup>C-WT and  $\Delta$ 190-243 apoA-I after incubation with SUV containing a trace amount of [<sup>3</sup>H]cholesterol. It is apparent that a significant amount of the WT apoA-I co-eluted with SUV and that this corresponds to the lipid-bound protein. In contrast, most of the  $\Delta$ 190-243 mutant eluted at a position corresponding to free apoA-I. From elution profiles with various concentrations of protein, the binding isotherms of the apoA-I variants to SUV were obtained (Fig. 1B). The binding parameters (the dissociation constant,  $K_d$ , and the maximal binding capacity,  $B_{max}$ ) derived from curve-fitting of these isotherms are listed in Table I. Except for  $\Delta$ 190-223 and  $\Delta$ 223-243 apoA-I, all apoA-I variants displayed high binding affinity to the SUV surface, and the binding capacities (around 0.4 amino acids/PC molecule) are in good agreement with earlier results (34, 35). Deletions in the C-terminal region led to the much lower binding affinity when compared with native apoA-I, indicating a critical role for the C-terminal domain in the binding of apoA-I to lipid (12, 13, 18, 19).

**Effect of Lipid Binding on the Secondary Structure of ApoA-I Variants**—Far-UV CD measurements were employed to determine the  $\alpha$ -helix contents of the apoA-I variants in the lipid-free and lipid-bound states. Fig. 2 shows the far-UV CD spectra of plasma apoA-I in the lipid-free state and bound to SUV. The  $\alpha$ -helix content derived from the molar ellipticity at 222 nm increased with increasing PC to apoA-I ratio and reached a plateau at a PC/apoA-I weight ratio of  $\geq$ 40:1 (Fig. 2, inset), indicating that all apoA-I bound to the SUV surface in that range. Table II summarizes the increases in  $\alpha$ -helix content for the apoA-I variants upon binding to SUV. The increases in  $\alpha$ -helix content for  $\Delta$ 1-43,  $\Delta$ 44-65,  $\Delta$ 190-243, and  $\Delta$ 223-243 apoA-I upon SUV binding were less than half of those WT apoA-I (23-25% increase), suggesting that not only the C-terminal but also the N-terminal regions of apoA-I contribute to the formation of  $\alpha$ -helix upon lipid binding (19). The increases in  $\alpha$ -helix content in POPC discoidal complexes were, however, greater than those on SUV for all apoA-I variants, and only the  $\Delta$ 190-223 mutant displayed a somewhat lower increase. This indicates that the helical structure of apoA-I bound to SUV is different from that in discs (39, 46).

**ITC Measurements**—To determine thermodynamic parameters for the binding of apoA-I variants to SUV, we measured the heats of the binding of apoA-I to SUV using ITC. As shown in Fig. 3, the injections of apoA-I variants into SUV gave large exothermic heats (35). It should be noted that the heats of consecutive injections were virtually identical for each apoA-I variant, indicating that the injected protein is likely to be completely bound to the SUV surface because of the large PC to protein ratio (38, 47). Using the binding constants given in Table I, the thermodynamic parameters for the binding of apoA-I variants to SUV were obtained (Table III). Deletions of residues 1-43, 190-223, or 223-243 led to significantly less

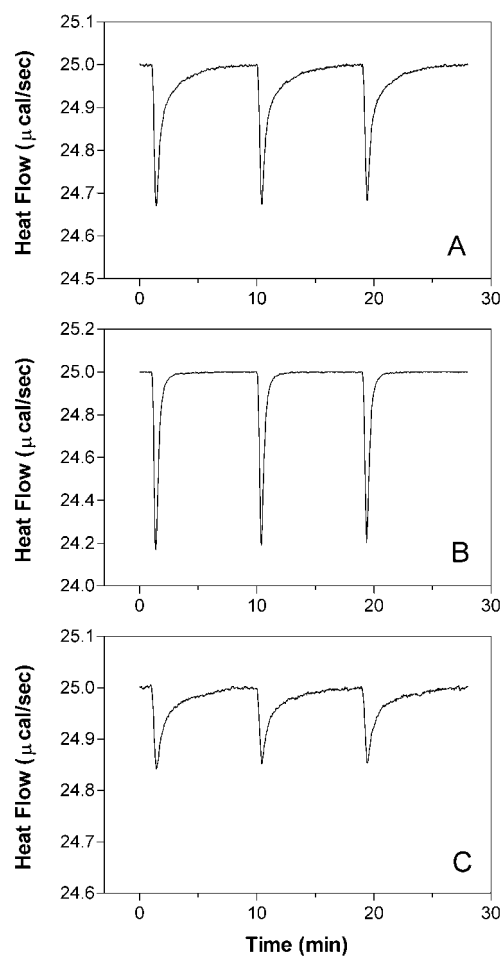


FIG. 3. Isothermal titration curves for WT (A),  $\Delta$ 44-65 (B), and  $\Delta$ 190-243 apoA-I (C) binding to egg PC SUV. 8  $\mu$ l of protein solution (0.8 mg/ml) were injected into SUV (the PC concentration was 15 mM).

exothermic heats of binding to SUV, consistent with the previous observations for the emulsion binding (13). Even in these deletion mutants, the binding of apoA-I to SUV was shown to be an enthalpically driven process. In addition to the thermodynamic parameters, we also analyzed the kinetics of release of heat that appears to reflect the opening of the helix bundle in the N-terminal domain upon the lipid binding of apoA-I (13) (Table III). When compared with WT apoA-I (half-time is 0.45 min), deletions in the N-terminal or central regions that disrupt the helix bundle structure markedly increased the rate of heat decay. This indicates that the conformational rearrangement of the N-terminal domain modulates the process of the binding of apoA-I to the SUV surface.

**Trp Quenching by Dibrominated PC**—To further character-

TABLE III  
Thermodynamic and kinetic parameters of binding of apoA-I deletion mutants to egg PC SUV at 25 °C

apoA-I variants	ITC parameters		$\Delta G^b$	$\Delta S^c$
	$\Delta H^a$	Half-time of decay		
	<i>kcal/mol</i>	<i>min</i>	<i>kcal/mol</i>	<i>cal/mol K</i>
Plasma	$-90.4 \pm 3.7$	0.45	$-12.2 \pm 0.1$	$-263 \pm 13$
WT	$-92.6 \pm 5.3$	0.46	$-12.2 \pm 0.1$	$-270 \pm 18$
$\Delta 1-43$	$-49.4 \pm 1.2$	0.20	$-11.3 \pm 0.1$	$-128 \pm 4$
$\Delta 44-65$	$-90.9 \pm 4.3$	0.19	$-11.6 \pm 0.1$	$-266 \pm 15$
$\Delta 44-126$	$-62.7 \pm 1.6$	0.18	$-11.2 \pm 0.2$	$-173 \pm 6$
$\Delta 123-166$	$-71.6 \pm 3.8$	0.20	$-11.7 \pm 0.1$	$-201 \pm 13$
$\Delta 190-243$	$-39.2 \pm 2.8$	0.70	$-9.9 \pm 0.5$	$-98 \pm 11$
$\Delta 223-243$	$-40.6 \pm 1.9$	0.81	$-9.7^d$	$-104$

<sup>a</sup> Mean  $\pm$  S.D. from at least four measurements.

<sup>b</sup> Free energy was calculated according to  $\Delta G = -RT \ln 55.5(1/K_d)$  using the binding constants given in Table I.

<sup>c</sup> The entropy of binding was calculated from  $\Delta G = \Delta H - T\Delta S$ .

<sup>d</sup> Assuming  $K_d$  value is around 100  $\mu\text{g/ml}$ .

ize the kinetics of the opening of the N-terminal helix bundle upon the binding of apoA-I to SUV, we measured Trp quenching by dibrominated PC incorporated into SUV (48, 49). Because the 4 Trp residues are located in the N-terminal half of apoA-I (positions 8, 50, 72, and 108), monitoring Trp fluorescence is useful for probing the conformational change in the N-terminal domain. Fig. 4A shows fluorescence emission spectra of Trp in plasma apoA-I bound to SUV with or without 33 mol % of 6,7-diBrPC. Although the Trp fluorescence intensity on 6,7-diBrPC-containing SUV decreased to half of that on egg PC SUV, both spectra exhibited a wavelength of maximal fluorescence of 332–333 nm, indicating relatively hydrophobic environments for Trp residues in contact with lipids (12, 14). By monitoring the emission intensity at 340 nm, the time courses of Trp quenching upon SUV binding were obtained (Fig. 4B). The half-time of Trp quenching for WT apoA-I was about 0.7 min, comparable with the rate of ITC decay of this protein (Table III). When compared with WT apoA-I, deletion of residues 123–166 gave much faster Trp quenching (half-time < 0.1 min), whereas  $\Delta 190-223$  exhibited a relatively slow rate (half-time = 2.8 min). This observation is consistent with the notion that the opening of the N-terminal helix bundle is a rate-limiting step in the binding of apoA-I to lipid.

**Energetics of  $\alpha$ -Helix Formation in ApoA-I**—Plotting the increases in  $\alpha$ -helix content (Table II) against the thermodynamic parameters of the binding of apoA-I variants to SUV (Table III) reveals linear correlations. Except for the  $\Delta 44-65$  mutant, the enthalpy of binding is linearly correlated with the number of amino acids in the apoA-I variant molecules forming  $\alpha$ -helix upon lipid binding (Fig. 5). The linear regression analysis yields the following relationship,

$$\Delta H = -1.09 \times (\Delta\alpha\text{-helical residues}) - 24.8 \text{ (kcal/mol)} \quad (\text{Eq. 1})$$

The contribution of  $\alpha$ -helix formation to the lipid binding of apoA-I is thus given as  $-1.1$  kcal/ $\alpha$ -helical residue, in agreement with the previously reported value ( $-1.3$  kcal/ $\alpha$ -helical residue) for human plasma apolipoproteins (30). The non-helix contribution to the energetics of the lipid binding of apoA-I that is likely to come from lipid-protein interaction on the SUV surface (50) can be estimated by extrapolation as  $-25$  kcal/mol of apoA-I. In addition, the relationship of the free energy of binding with the increases in  $\alpha$ -helix content (Fig. 5, inset) is given by the following equation,

$$\Delta G = -0.042 \times (\Delta\alpha\text{-helical residues}) - 9.7 \text{ (kcal/mol)} \quad (\text{Eq. 2})$$

#### DISCUSSION

**Conformational Reorganization of ApoA-I upon Lipid Binding**—Lipid-free apoA-I is likely to be organized into two structural domains in which the N-terminal domain forms a helix

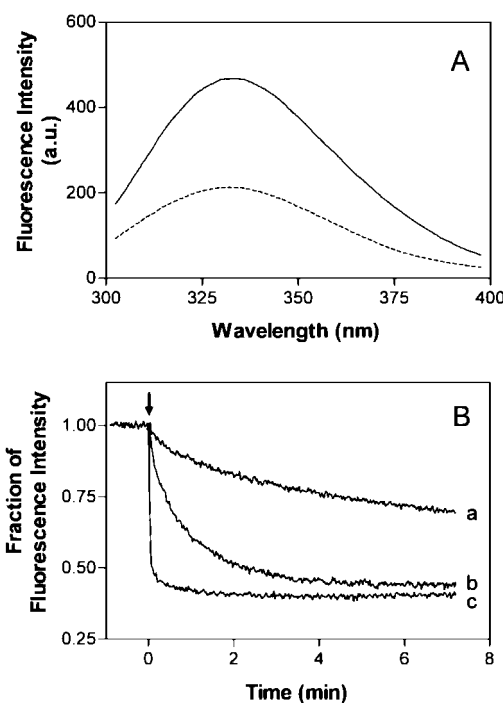
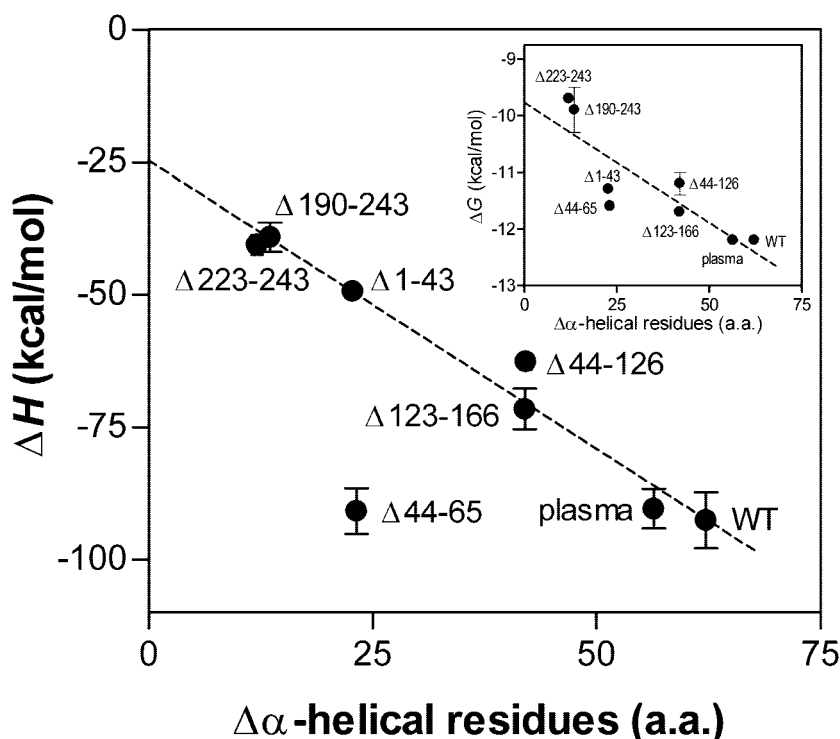


FIG. 4. Binding of apoA-I to egg PC SUV monitored with Trp quenching by 6,7-diBrPC. A, fluorescence emission spectra of Trp in plasma apoA-I bound to egg PC SUV with (dashed line) or without 33% of 6,7-diBrPC (solid line). a.u., arbitrary units. B, time course of Trp quenching by 6,7-diBrPC-containing SUV in  $\Delta 190-243$  (trace a), WT (trace b), and  $\Delta 123-166$  apoA-I (trace c).

bundle, whereas the C-terminal domain exists as a discrete, less organized structure (11–13). Based on spectroscopic and calorimetric measurements, we have recently proposed a two-step mechanism for the lipid binding of apoA-I: apoA-I initially binds to a lipid surface through  $\alpha$ -helices in the C-terminal domain followed by the opening of the helix bundle in the N-terminal domain (13). In this study, we further examined the conformational change of apoA-I upon lipid binding on the SUV surface.

Gel filtration experiments showed that WT apoA-I binds to SUV with high affinity and that deletions in the C-terminal domain lead to a marked reduction in the binding affinity. This finding is consistent with previous reports showing that the C-terminal  $\alpha$ -helix is critical to the lipid binding of apoA-I (12, 13, 18, 19). This C-terminal region is very hydrophobic as revealed by hydrophathy analysis of the amino acid sequence of human apoA-I (51), and a recent electron paramagnetic resonance spectroscopic study demonstrated that it exists as a

FIG. 5. Correlation of the binding enthalpy of apoA-I variants with the increase in  $\alpha$ -helix content upon binding to egg PC SUV. The data point of  $\Delta 44-65$  apoA-I was not included in a best fit regression line ( $r^2 = 0.92$ ). The inset shows the correlation of the free energy of binding with the increase in  $\alpha$ -helix content ( $r^2 = 0.72$ ).



stable  $\alpha$ -helical structure in the lipid-free state (17).

The comparison between the  $\alpha$ -helix contents of apoA-I variants bound to SUV and incorporated into discoidal HDL particles (Table II) suggests that the secondary structure of apoA-I on SUV is less helical when compared with that on the discoidal complex. Two general models have been proposed for the organization of apoA-I molecules in discoidal HDL particles: a “picket fence” model in which the  $\alpha$ -helix repeats of apoA-I are arranged parallel to the phospholipid acyl chains (52) and a “belt” model in which continuous series of  $\alpha$ -helices of apoA-I are aligned perpendicular to the acyl chains (53). Several recent studies support the conclusion that lipid-bound apoA-I adopts a “belt-like” arrangement around the edge of discoidal complexes (54–56), consistent with the crystal structure of the N-terminal truncated  $\Delta 1-43$  apoA-I (57). In contrast, the organization of the  $\alpha$ -helices in apoA-I molecules on spherical HDL particles is not known. On a spherical surface, the amphipathic  $\alpha$ -helices of apoA-I are thought to be embedded between PC molecules with their hydrophobic faces in contact with PC acyl chains (8). In a soluble apolipoprotein such as apoA-I and apoE consisting of a number of different amphipathic helices, the conformational flexibility of the proteins could allow some helices with low lipid affinity to be excluded from the particle surface (58, 59). Indeed, we demonstrated recently that the two-domain structure in apoE leads to two different lipid-bound conformations on lipoprotein-like spherical particles. The N-terminal four-helix bundle can adopt either open or closed conformations, resulting from binding competition with the C-terminal domain that has a high lipid affinity (38). Given the similar domain structure of apoA-I to apoE (13), it is conceivable that the structural organizations of apoA-I on discoidal and spherical particles are also different with some helices in apoA-I being out of contact with lipid on the spherical surface like a “hinged domain” (8, 60). Supporting this idea, a recent fluorescence resonance energy transfer study suggested that apoA-I undergoes a conformational change as it adapts from a discoidal to a spherical surface (46).

Contribution of  $\alpha$ -Helix Formation in ApoA-I to Lipid Binding—ITC measurements demonstrated that the binding of

apoA-I to SUV is accompanied by a large exothermic heat, indicating that the apoA-I binding to SUV is enthalpically driven. This observation is consistent with previous studies of the interaction of plasma apoA-I (35) or apoA-I model peptides (31) with POPC SUV. In addition, the enthalpies of the binding of apoA-I variants to SUV were found to be comparable with those to large emulsion particles (13) (e.g.  $-90$  and  $-84$  kcal/mol of plasma apoA-I for SUV and emulsions, respectively), suggesting that the binding of apoA-I to both types of particle is an energetically similar process.

A significant result in this study is the discovery that the enthalpy of the binding of apoA-I to lipid is linearly correlated with the number of amino acids forming  $\alpha$ -helix. This indicates that the contribution of  $\alpha$ -helix formation to the energetics of the lipid binding of apoA-I is  $-1.1$  kcal/ $\alpha$ -helical residue (Fig. 5). This value is in agreement with an early estimate ( $-1.3$  kcal/ $\alpha$ -helical residue) for plasma apolipoproteins using different membrane systems (30) and within a range of calorimetric values for the enthalpy of  $\alpha$ -helix formation of synthetic peptides (from  $-1.3$  to  $-0.7$  kcal/mol of residue) (50, 61, 62). We have suggested recently that the C-terminal region in the lipid-free apoA-I (especially residues 190–220) is in a predominantly non-helical conformation and that lipid binding induces a random coil to  $\alpha$ -helix transition in this region (13). This concept is consistent with the recent demonstration that the secondary structure of residues 188–220 in apoA-I converts from random coil and  $\beta$ -sheet to  $\alpha$ -helices upon incorporation into a reconstituted HDL particle (17). The large reduction in the enthalpy of binding by deleting residues 190–243 or 223–243 (Table III) is most probably a consequence of the lack of  $\alpha$ -helix-forming ability in the C-terminal domain of these truncated apoA-I molecules.

A linear correlation between the free energy of the binding of apoA-I to lipids and the number of amino acids forming  $\alpha$ -helix (Fig. 5, inset) reveals that the contribution of  $\alpha$ -helix formation to the free energy of binding is  $-42$  cal/ $\alpha$ -helical residue, demonstrating that  $\alpha$ -helix formation in apoA-I leads to an increase in the favorable free energy of binding to lipid. The non-helical contribution (a hypothetical free energy of the binding of

apoA-I that does not undergo a conformational transition upon lipid binding) has a large negative value of  $-9.7$  kcal/mol that corresponds to a  $K_d$  value of  $120 \mu\text{g/ml}$  ( $\sim 4.3 \mu\text{M}$ ). The increase in  $\alpha$ -helix content of 62 residues when WT apoA-I binds to lipid (Table II) contributes  $-2.5$  kcal/mol to the free energy of binding. This additional free energy leads to a reduction in  $K_d$  to  $\sim 2 \mu\text{g/ml}$  ( $\sim 0.06 \mu\text{M}$ ). This approximately 2 orders of magnitude increase in the affinity of binding is demonstrated by the binding isotherms for WT apoA-I and  $\Delta 190$ – $243$  apoA-I (Fig. 1B). This analysis of free energy changes indicates that  $\alpha$ -helix formation plays a critical role in facilitating the binding of apoA-I to a lipid surface with high affinity.

Deletions of some segments in the N-terminal domain also led to reductions in the enthalpy of the binding of apoA-I to lipid. Residues 123–142 are suggested to be in the disordered structure and become helical upon lipid binding (63), consistent with our observations on the  $\Delta 123$ – $166$  mutant. The deletion of the extreme N-terminal region (residues 1–43) also induces a large decrease in the enthalpy of binding. Since an interaction between the N- and C-terminal regions in apoA-I has been suggested (64), it is possible that the deletion of the N-terminal region leads to unfolding of the C-terminal helix (65), resulting in lower lipid binding ability, as is seen with the C-terminal deletion mutants. However, this seems improbable because the  $\Delta 1$ – $43$  mutant exhibited similar lipid binding ability to WT apoA-I (Table I). Rather, it is more likely that residues 1–43 are in a predominantly non-helical conformation in the lipid-free state and form  $\alpha$ -helices upon lipid binding. This idea is supported by studies of synthetic peptides (66) and deletion mutants (19), suggesting a significant role for the first 43 residues of apoA-I in lipid binding. It is not clear why the  $\Delta 44$ – $65$  mutant lacks the correlation between the increase in  $\alpha$ -helix content and the enthalpy of binding (Fig. 5). A peptide corresponding to residues 44–65 has strong lipid binding properties, suggesting a significant role for this domain in the binding of apoA-I to lipids (20). It is conceivable that deletion of residues 44–65 induces a different conformation of apoA-I bound to the SUV surface, although this mutant exhibits an enthalpy of binding similar to that of native apoA-I. This notion may be related to the finding that the  $\Delta 44$ – $126$  mutant, which also lacks residues 44–65, exhibited a much lower exothermic heat of binding when compared with native apoA-I, although this region is thought to form predominantly  $\alpha$ -helical structure in the lipid-free state (13).

In summary, we have provided the first complete set of thermodynamic parameters characterizing the binding of WT apoA-I and some helix-deletion mutants to lipid particles. The correlation of the number of amino acids forming  $\alpha$ -helix with the thermodynamic parameters of the binding of apoA-I to lipid reveals the contribution of  $\alpha$ -helix formation to the energetics of the lipid binding of apoA-I. Because the non-helical contribution to the free energy of binding is not enough for apoA-I to bind to lipid with high affinity, the transition of random coil to  $\alpha$ -helix plays a critical role in driving apoA-I to interact with the lipid membrane. This may be related to the finding that deletion of the C-terminal domain containing predominantly random coil structure greatly reduces the affinity of lipid efflux by apoA-I from cells (25).

**Acknowledgments**—We thank Dr. Kenichi Akaji (Institute for Protein Research, Osaka University) for assistance with ITC measurements and Faye Baldwin for expert technical assistance.

## REFERENCES

- Fielding, C. J., and Fielding, P. E. (1995) *J. Lipid Res.* **36**, 211–228
- Rothblat, G. H., de la Llera-Moya, M., Atger, V., Kellner-Weibel, G., Williams, D. L., and Phillips, M. C. (1999) *J. Lipid Res.* **40**, 781–796
- Yokoyama, S. (2000) *Biochim. Biophys. Acta* **1529**, 231–244
- Oram, J. F. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23**, 720–727
- Wang, N., and Tall, A. R. (2003) *Arterioscler. Thromb. Vasc. Biol.* **23**, 1178–1184
- Fielding, C. J., and Fielding, P. E. (2001) *Biochim. Biophys. Acta* **1533**, 175–189
- 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
- Segrest, J. P., Jones, M. K., De Loof, H., Brouillette, C. G., Venkatchalapathi, Y. V., and Anantharamaiah, G. M. (1992) *J. Lipid Res.* **33**, 141–166
- Frank, P. G., and Marcel, Y. L. (2000) *J. Lipid Res.* **41**, 853–872
- Brouillette, C. G., Anantharamaiah, G. M., Engler, J. A., and Borhani, D. W. (2001) *Biochim. Biophys. Acta* **1531**, 4–46
- Roberts, L. M., Ray, M. J., Shih, T. W., Hayden, E., Reader, M. M., and Brouillette, C. G. (1997) *Biochemistry* **36**, 7615–7624
- Davidson, W. S., Hazlett, T., Mantulin, W. W., and Jonas, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13605–13610
- Saito, H., Dhanasekaran, P., Nguyen, D., Holvoet, P., Lund-Katz, S., and Phillips, M. C. (2003) *J. Biol. Chem.* **278**, 23227–23232
- Davidson, W. S., Arnvig-McGuire, K., Kennedy, A., Kosman, J., Hazlett, T. L., and Jonas, A. (1999) *Biochemistry* **38**, 14387–14395
- Kiss, R. S., Kay, C. M., and Ryan, R. O. (1999) *Biochemistry* **38**, 4327–4334
- Gorshkova, I. N., Liadaki, K., Gursky, O., Atkinson, D., and Zannis, V. I. (2000) *Biochemistry* **39**, 15910–15919
- Oda, M. N., Forte, T. M., Ryan, R. O., and Voss, J. C. (2003) *Nat. Struct. Biol.* **10**, 455–460
- Holvoet, P., Zhao, Z., Vanloo, B., Vos, R., Deridder, E., Dhoest, A., Taveirne, J., Brouwers, E., Demarsin, E., Engelborghs, Y., Rosseneu, M., Collen, D., and Brasseur, R. (1995) *Biochemistry* **34**, 13334–13342
- Fang, Y., Gursky, O., and Atkinson, D. (2003) *Biochemistry* **42**, 13260–13268
- Palgunachari, M. N., Mishra, V. K., Lund-Katz, S., Phillips, M. C., Adeyeye, S. O., Alluri, S., Anantharamaiah, G. M., and Segrest, J. P. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 328–338
- Gillotte, K. L., Zaiou, M., Lund-Katz, S., Anantharamaiah, G. M., Holvoet, P., Dhoest, A., Palgunachari, M. N., Segrest, J. P., Weisgraber, K. H., Rothblat, G. H., and Phillips, M. C. (1999) *J. Biol. Chem.* **274**, 2021–2028
- Burgess, J. W., Frank, P. G., Franklin, V., Liang, P., McManus, D. C., Desforges, M., Rassart, E., and Marcel, Y. L. (1999) *Biochemistry* **38**, 14524–14533
- Panagotopoulos, S. E., Witting, S. R., Horace, E. M., Hui, D. Y., Maiorano, J. N., and Davidson, W. S. (2002) *J. Biol. Chem.* **277**, 39477–39484
- Favari, E., Bernini, F., Tarugi, P., Franceschini, G., and Calabresi, L. (2002) *Biochem. Biophys. Res. Commun.* **299**, 801–805
- Liu, L., Bortnick, A. E., Nickel, M., Dhanasekaran, P., Subbaiah, P. V., Lund-Katz, S., Rothblat, G. H., and Phillips, M. C. (2003) *J. Biol. Chem.* **278**, 42976–42984
- Wang, N., Silver, D. L., Costet, P., and Tall, A. R. (2000) *J. Biol. Chem.* **275**, 33053–33058
- Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) *J. Biol. Chem.* **275**, 34508–34511
- Fitzgerald, M. L., Morris, A. L., Rhee, J. S., Andersson, L. P., Mendez, A. J., and Freeman, M. W. (2002) *J. Biol. Chem.* **277**, 33178–33187
- Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001) *J. Biol. Chem.* **276**, 9955–9960
- Massey, J. B., Gotto, A. M., Jr., and Pownall, H. J. (1979) *J. Biol. Chem.* **254**, 9559–9561
- Gazzara, J. A., Phillips, M. C., Lund-Katz, S., Palgunachari, M. N., Segrest, J. P., Anantharamaiah, G. M., and Snow, J. W. (1997) *J. Lipid Res.* **38**, 2134–2146
- Rogers, D. P., Brouillette, C. G., Engler, J. A., Tendian, S. W., Roberts, L., Mishra, V. K., Anantharamaiah, G. M., Lund-Katz, S., Phillips, M. C., and Ray, M. J. (1997) *Biochemistry* **36**, 288–300
- McLean, L. R., and Phillips, M. C. (1984) *Biochim. Biophys. Acta* **776**, 21–26
- Yokoyama, S., Fukushima, D., Kupferberg, J. P., Kozdy, F. J., and Kaiser, E. T. (1980) *J. Biol. Chem.* **255**, 7333–7339
- Tricerri, M. A., Sanchez, S. A., Arnulphi, C., Durbin, D. M., Gratton, E., and Jonas, A. (2002) *J. Lipid Res.* **43**, 187–197
- Schulthess, G., Compassi, S., Werder, M., Han, C. H., Phillips, M. C., and Hauser, H. (2000) *Biochemistry* **39**, 12623–12631
- Lund-Katz, S., Weisgraber, K. H., Mahley, R. W., and Phillips, M. C. (1993) *J. Biol. Chem.* **268**, 23008–23015
- Saito, H., Dhanasekaran, P., Baldwin, F., Weisgraber, K. H., Lund-Katz, S., and Phillips, M. C. (2001) *J. Biol. Chem.* **276**, 40949–40954
- Sparks, D. L., Phillips, M. C., and Lund-Katz, S. (1992) *J. Biol. Chem.* **267**, 25830–25833
- Thuahnai, S. T., Lund-Katz, S., Williams, D. L., and Phillips, M. C. (2001) *J. Biol. Chem.* **276**, 43801–43808
- Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1992) *J. Biol. Chem.* **267**, 25839–25847
- Mao, D., and Wallace, B. A. (1984) *Biochemistry* **23**, 2667–2673
- Ladokhin, A. S., Jayasinghe, S., and White, S. H. (2000) *Anal. Biochem.* **285**, 235–245
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Saito, H., Miyako, Y., Handa, T., and Miyajima, K. (1997) *J. Lipid Res.* **38**, 287–294
- Li, H. H., Lyles, D. S., Pan, W., Alexander, E., Thomas, M. J., and Sorci-Thomas, M. G. (2002) *J. Biol. Chem.* **277**, 39093–39101
- Seelig, J. (1997) *Biochim. Biophys. Acta* **1331**, 103–116
- Gonzalez-Manas, J. M., Lakey, J. H., and Pattus, F. (1992) *Biochemistry* **31**, 7294–7300
- Mishra, V. K., and Palgunachari, M. N. (1996) *Biochemistry* **35**, 11210–11220
- Wieprecht, T., Apostolov, O., Beyersmann, M., and Seelig, J. (1999) *J. Mol. Biol.* **294**, 785–794

51. Lund-Katz, S., Liu, L., Thuahnai, S. T., and Phillips, M. C. (2003) *Front. Biosci.* **8**, d1044–d1054
52. Phillips, J. C., Wriggers, W., Li, Z., Jonas, A., and Schulten, K. (1997) *Biophys. J.* **73**, 2337–2346
53. Klön, A. E., Segrest, J. P., and Harvey, S. C. (2002) *Biochemistry* **41**, 10895–10905
54. Koppaka, V., Silvestro, L., Engler, J. A., Brouillette, C. G., and Axelsen, P. H. (1999) *J. Biol. Chem.* **274**, 14541–14544
55. Li, H., Lyles, D. S., Thomas, M. J., Pan, W., and Sorci-Thomas, M. G. (2000) *J. Biol. Chem.* **275**, 37048–37054
56. Panagotopoulos, S. E., Horace, E. M., Maiorano, J. N., and Davidson, W. S. (2001) *J. Biol. Chem.* **276**, 42965–42970
57. Borhani, D. W., Rogers, D. P., Engler, J. A., and Brouillette, C. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12291–12296
58. Narayanaswami, V., and Ryan, R. O. (2000) *Biochim. Biophys. Acta* **1483**, 15–36
59. Wang, L., Atkinson, D., and Small, D. M. (2003) *J. Biol. Chem.* **278**, 37480–37491
60. Corsico, B., Toledo, J. D., and Garda, H. A. (2001) *J. Biol. Chem.* **276**, 16978–16985
61. Scholtz, J. M., Marqusee, S., Baldwin, R. L., York, E. J., Stewart, J. M., Santoro, M., and Bolen, D. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2854–2858
62. Lopez, M. M., Chin, D. H., Baldwin, R. L., and Makhatadze, G. I. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 1298–1302
63. Gorshkova, I. N., Liu, T., Zannis, V. I., and Atkinson, D. (2002) *Biochemistry* **41**, 10529–10539
64. Tricerri, M. A., Behling Agree, A. K., Sanchez, S. A., and Jonas, A. (2000) *Biochemistry* **39**, 14682–14691
65. Fang, Y., Gursky, O., and Atkinson, D. (2003) *Biochemistry* **42**, 6881–6890
66. Mishra, V. K., Palgunachari, M. N., Datta, G., Phillips, M. C., Lund-Katz, S., Adeyeye, S. O., Segrest, J. P., and Anantharamaiah, G. M. (1998) *Biochemistry* **37**, 10313–10324