Calorimetry of Apolipoprotein-A1 Binding to Phosphatidylcholine-Triolein-Cholesterol Emulsions

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ABSTRACT The thermotropic properties of triolein-rich, low-cholesterol dipalmitoyl phosphatidylcholine (DPPC) emulsion particles with well-defined chemical compositions (~88% triolein, 1% cholesterol, 11% diacyl phosphatidylcholine) and particle size distributions (mean diameter, ~1000–1100 Å) were studied in the absence and presence of apolipoprotein-A1 by a combination of differential scanning and titration calorimetry. The results are compared to egg yolk PC emulsions of similar composition and size. Isothermal titration calorimetry at 30°C was used to saturate the emulsion surface with apo-A1 and rapidly quantitate the binding constants (affinity K_a = 11.1 ± 3.5 × 10^6 M^{-1} and capacity N = 1.0 ± 0.09 apo-A1 per 1000 DPPC) and heats of binding (enthalpy H = −940 ± 35 kcal mol^{-1} apo-A1 or −0.92 ± 0.12 kcal mol^{-1} DPPC). The entropy of association is −3070 cal deg^{-1} mol^{-1} protein or −3 cal deg^{-1} mol^{-1} DPPC. Without protein on the surface, the differential scanning calorimetry heating curve of the emulsion showed three endothermic transitions at 24.3°C, 33.0°C, and 40.0°C with a combined enthalpy of 1.53 ± 0.2 kcal mol^{-1} DPPC. With apo-A1 on the surface, the heating curve showed the three transitions more clearly; in particular, the second transition became more prominent by significant increases in both the calorimetric and Van't Hoff enthalpies. The combined enthalpy was 2.70 ± 0.12 kcal mol^{-1} DPPC and remained constant upon repeated heating and cooling, indicating that the newly formed DPPC emulsion-Apo-A1 complex is thermally reversible during calorimetry. Thus there is an increase in ΔH of 1.17 kcal mol^{-1} DPPC after apo-A1 is bound, which is roughly balanced by the heat released during binding (−0.92 kcal) of apo-A1. The melting entropy increase, +3.8 cal deg^{-1} mol^{-1} DPPC of the three transitions after apo-A1 binds, also roughly balances the entropy (−3 cal deg^{-1} mol^{-1} DPPC) of association of apo-A1. These changes indicate that apo-A1 increases the amount of ordered gel-like phase on the surface of DPPC emulsions when added at 30°C. From the stoichiometry of the emulsions we calculate that the mean area of DPPC at the triolein/DPPC interface is 54.5 $Å^2$ at 41°C and 54.2 $Å^2$ at 30°C. The binding of apo-A1 at 30°C to the emulsion reduces the surface area per DPPC molecule from 54.2 $Å^2$ to 50.8 $Å^2$. At 30° apo-A1 binds with high affinity and low capacity to the surface of DPPC emulsions and increases the packing density of the lipid domain to which it binds. Apo-A1 was also titrated onto DPPC emulsions at 45°C. This temperature is above the gel liquid crystal transition. No heat was released or adsorbed. Furthermore, egg yolk phosphatidylcholine emulsions of nearly identical composition were also titrated at 30°C with apo-A1 and were euthermic. Association constants were previously measured using a classical centrifugation assay and were used to calculate the entropy of apo-A1 binding (+28 cal deg^{-1} mol^{-1} apo-A1). This value indicates that apo-A1 binding to a fluid surface like egg yolk phosphatidylcholine or probably DPPC at 45°C is hydrophobic and is consistent with hydrocarbon lipid or protein moities coming together and excluding water. Thus the binding of apo-A1 to partly crystalline surfaces is entropically negative and increases the order of the already partly ordered phases, whereas binding to liquid surfaces is mainly an entropically driven hydrophobic process.

INTRODUCTION

Apolipoproteins (Apo), like apo-A1, have loosely folded structures in aqueous solution (Atkinson and Small, 1986; Tall et al., 1976) and are surrounded by a hydration shell. Soluble apolipoproteins bind to phospholipids and convert liposomes made from saturated lecithins such as dimyristoyl (DM) and dipalmitoyl (DP) phosphatidylcholine (PC) into discs (Tall et al., 1975, 1977). In plasma these apolipoproteins are found on triglyceride-rich lipoproteins as well as high-density lipoproteins (Scanu et al., 1969), whereas on the lipoprotein surface they stabilize particle structure, act as cofactors for enzymes, and are ligands for membrane receptor-mediated cell uptake. Because these proteins also transfer between lipoprotein particles in circulation, they belong to a group of proteins widely known as exchangeable apolipoproteins (Jonas, 1992; Small, 1993).

Because lipoproteins can be thought of as emulsion particles (Miller and Small, 1987) to which apolipoproteins are adsorbed, we have generated reproducible emulsion particles made with triolein cores and phospholipid surfaces, e.g., egg yolk PC (Miller and Small, 1982), distearoyl PC, DPPC, and DMPC (Small et al., 1988). Physical properties of these emulsions have been studied by EM (Gantz et al., 1990) and calorimetry (Small et al., 1988). The binding of iodinated apo-A1 and apoE-3 to egg PC/triolein emulsions containing different amounts of cholesterol (Derksen and Small, 1989) or fatty acid (Derksen et al., 1989) have been determined by means of a centrifugation assay. The obtained binding isotherms yield a dissociation constant (K_d)

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and the maximum number of protein molecules bound per 1000 PC (N). However, the heat used or released during the binding process is not measured by this assay. To measure the heat change involved in the binding process of exchangeable apolipoproteins to the lipid surfaces of emulsion particles, we have employed a titration calorimeter. This instrument has been recently developed, is computerized, and is capable of measuring heats of ligand binding with high sensitivity and of calculating all binding parameters (Wiseman et al., 1989). Importantly, there is no need to radiolabel the protein or to use a chemically modified product. In this study we measure the heat of apo-A1 binding to triolein particles emulsified with DPPC at 30°C and 45°C and to egg yolk phosphatidylcholine (EYPC) emulsions at 30°C. Both the phospholipid (Small, 1986a) and the protein (Tall et al., 1976) have been extensively studied individually and as complexes (Gwynne et al., 1975; Tall et al., 1975, 1977; Massey et al., 1979; Wald et al., 1990) in a number of laboratories (for reviews, see Atkinson and Small, 1986; Jonas, 1992). In addition to isothermal titration we have obtained thermograms by differential scanning calorimetry on DPPC emulsion before and after titrating apo-A1 onto the emulsion at 30°C. We have found that DPPC emulsion particles exhibit three transitions, indicative of three lipid domains, presumably of different composition. When the emulsion is titrated with apo-A1 at 30°C until the surface is saturated with protein, a large amount of heat is released and a striking increase occurs in the calorimetric and Van't Hoff enthalpy of the middle transition. The entropy of this process is negative and suggests that apo-A1 causes an increased order, perhaps related to a decrease in surface area of the DPPC. When the DPPC emulsions are titrated at 45°C above the gel-to-liquid transition of DPPC, then no heat is adsorbed or given off. Likewise, when EYPC emulsions are titrated at 30°C when the chains are liquid the transition is also euthermic. From the association constant of apo-A1 binding to similar EYPC emulsions determined in a centrifugation assay (Derksen and Small, 1989), we calculated the binding entropy, which was positive, suggesting a hydrophobic interaction.

MATERIALS AND METHODS

Chemicals

Nonradioactive lipids were used without further purification. All lipids were >99% pure, as stated by the supplier and confirmed by thin-layer chromatography for lipid class purity. Cholesterol and DPPC were obtained from Sigma Chemical Co. (St. Louis, MO). EYPC was obtained from Lipid Products (Nutfield Ridge, England), and triolein was from Nu Chek Prep (Elysian, MN). L-3-Phosphatidylcholine, 1,2-[1-^{14}C]palmitoyl, and [^{3}H]phosphatidylcholine were from Research Products, Amersham Corp. (Arlington Heights, IL) were shown to be >99% pure by thin-layer chromatography of lipid classes and liquid scintillation spectrometry. Phosphate-buffered saline (PBS), pH 7.4, was purchased from Sigma Co. as prepackaged dry powder blends, which are freshly diluted with H_2O as needed and to which 0.02% NaN_3 was added.

Emulsions

Aliquots of chloroform solutions of triolein (40 mg), cholesterol (0.75 mg), and phospholipid (9.25 mg), [^{14}C]DPPC (final specific activity, 300–500 dpm/μg PC) or ^{3}H-EYPC (final specific activity, 800 cpm/μg PC) were pipetted into 15 × 45 mm glass vials. Solvent was blown off with a stream of N_2, and the vials were placed into a vacuum desiccator at 4°C for at least 18 h. Ten milliliters of PBS, 0.02% NaN_3 was added to the dry lipid film, and the vials were placed in a waterbath with melting ice. Emulsions were prepared by sonication above the PC chain melting temperatures (Small et al., 1988). Temperature was monitored throughout sonication by a copper/constantin thermocouple (Omega Engineering, Stamford, CT) inserted directly into the vial. The mixtures were sonicated continuously at temperatures ranging between 39°C and 44°C for 12 min at approximately 30% of maximum power output (Branson Sonifier Model 350, 1 cm probe tip). The sonicated mixtures were then transferred, at room temperature, to ultracentrifuge tubes (SW41). The tubes were filled through a syringe with 1.5 ml of warm PBS, 0.02% NaN_3 to facilitate overlayering and centrifuged for 15 min at 30,000 rpm at 25°C. The floated emulsion particles were harvested by tube slicing and aliquots were taken for lipid analysis and EM. The large particles harvested from four emulsions were combined to do one experiment using the two calorimeters.

Apolipoprotein A-I purification and refolding

Human plasma HDL was isolated by ultracentrifugation (Havel et al., 1955) in a 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA) at a density between 1.080 and 1.121 g/ml and treated with 6 M guanidine HCl to produce free apo-A1 and A-I poor HDL (Cheung and Albers, 1977).

The purity of apo-A1 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) using a Phast System (Pharmacia). The pure apo-A1 was dialyzed against 0.01% EDTA (pH 8), lyophilized, and stored at −80°C as a dry solid. Three milligrams of apo-A1 was dissolved in 3 ml 8 M urea, 50 mM Tris (pH 8), refolded by six sequential overnight dialysis; against 6 M guanidine HCl, twice diluted with H_2O to 4 M and 2 M salt; and finally changed three times against 1 liter of PBS, 0.02% NaN_3. This protein solution was stable when stored at 4°C for at least 24 days, as measured by circular dichroism spectroscopy. No changes occurred in the molar ellipticity [$\ominus$] 222 nm (Dr. M. T. Walsh, personal communication), which showed that the secondary structure of refolded apo-A1 contained 60% α-helix.

Analytical

Emulsion phospholipid content was determined by radioactivity counting as described (Derksen and Small, 1989; Derksen et al., 1989). Emulsion triolein and cholesterol were measured by standard enzymatic methods (Sigma Kit no. 320 UV) (Bennett Clark et al., 1987) and cholesterol oxidase (Tercyak, 1991), respectively. The composition of the DPPC emulsions is shown in Table 1. The composition of the EYPC emulsions was virtually the same, except that EYPC replaces DPPC. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951) or absorption reading at 280 nm.

High-sensitivity titration calorimetry

Heats of reaction were measured in a Omega Reaction Cell with a Microcal high-sensitivity titration calorimeter (Microcal, Northampton, MA). The calorimeter was calibrated electrically and reproduced binding curves of 2′ CMP to ribonuclease A as described previously (Wiseman et al., 1989). Solutions were degassed under vacuum before use. The protein solution was placed in a 250-μl syringe at a concentration of 0.028 mM, and the emulsion was placed in the 1.3684-ml cell at a concentration of 4.2 mM. Injections of 8 μl each, at a rate of 1 μl/2 s, were made every 3 min with continual stirring at 300 rpm and were terminated at saturation. The interaction of protein and lipid was analyzed with a one-site binding model

TABLE 1 Lipid composition and particle size of large triolein-DPPC emulsion particles with low cholesterol content*

Lipid class	Weight %	Surface lipid composition§	Particle size‡ Method	Particle size‡ Mean diameter (Å)
Triolein	87.9 ± 0.5	2.2¶		
			Lipid composition	1080 ± 40
Cholesterol	1.1 ± 0.1	9.5	Negative stain EM	1040 ± 10
DPPC	11.0 ± 0.4	88.3		

Lipid analysis was performed as described in Materials and Methods.
*Values are the mean ± SE of three experiments.
‡The mean particle diameter of each emulsion was calculated from the lipid composition data with the aid of a computer program (Miller and Small, 1987) or measured by negative stain EM (see Fig. 1). Values are the mean ± SE of four separate emulsions.
§The surface lipid composition was calculated from the lipid composition data with the aid of a computer program (Miller and Small, 1987).
¶Maximal solubility of triolein in DPPC is about 3 mol% (Hamilton, 1989), but cholesterol decreases the solubility (Spooner and Small, 1987). Small amounts of other neutral lipids, such as diolein, oleic acid, and lyso compounds, are also present and come from impurities in the starting materials or are generated during hydration and sonication of the lipid mixtures (see Derksen et al., 1989).

in which the protein binds to a cluster of 1000 phospholipid molecules. This procedure was repeated with four emulsions.

Differential scanning calorimetry

All experiments were performed on a Microcal MC-2 scanning calorimeter (Microcal, Northampton, MA) at a scanning rate of 90°C/h. All samples were degassed before use. First, a buffer heating run from 3°C to 50°C was recorded by loading both sample and reference cells at room temperature with 1.3 ml buffer. Next the sample cell was loaded with 1.3 ml DPPC emulsion at a concentration of 3.0 mM. Samples were cooled to approximately 1°C, and data were collected on heating runs from 3°C to 50°C. Samples were heated five times and were cooled to 1°C for 70 min between runs. After filling the cell, the first heating run always showed an upward-sloping baseline and an apparent change in heat capacity roughly estimated at ~0.8 kcal deg^{-1} mol^{-1}; the second and subsequent runs were reproducible with nearly flat baselines and very small changes in heat capacity. Because the first runs were variable and had sloping baselines, making them very difficult to quantitate, these first runs on emulsions were not used. Data presented on emulsions alone represent the results of the last four heating runs. While data were collected on an emulsion in the MC-2, apo-A1 was titrated onto another sample of the same emulsion preparation at 30° using the Omega titration calorimeter. After the titration was finished 1.3 ml of this sample containing emulsion with apo-A1 was transferred to the MC-2 and scanned as described above. Data presented on emulsions with apo-A1 represent the results of the last four heating runs only. The data were collected and processed by computer using Origin software developed by Microcal. The procedure was repeated with four emulsions. One emulsion in the absence of apo-A1 coalesced and creamed up during repeated scanning and accumulated inside the injection cavity. The data collected in this experiment were not used in the results. EYPC-triolein-cholesterol emulsions with composition and particle size distribution virtually identical to those of the DPPC emulsions were also run in the MC-2. They gave a flat, featureless curve with no transitions (not shown). These emulsions were titrated with apo-A1 at 30°C using the same protocol as with DPPC. DPPC emulsions were also titrated above the gel-to-liquid-to-crystal transition at 45°C in the Omega titration calorimeter.

Statistical analysis of the data was performed with the RS1 laboratory software package (BBN Software, Cambridge, MA).

Transmission electron microscopy

Each isolated emulsion fraction was diluted with deionized water and fixed for at least 1 h with OsO_4 in cacodylate buffer (pH 7.4). Final lipid and osmium concentrations were 1–2 mg/ml and 0.6–0.8%, respectively. A 5-μl droplet of fixed emulsion was applied for 10 s to a Formvar carbon-coated 300 mesh copper grid. The carbon film surface had been made hydrophilic by glow discharging in a Balzers Union CTA 010 glow discharge apparatus just before use. Excess emulsion was removed by blotting with filter paper and immediately replaced with a 5-μl droplet of 1% sodium phosphotungstate (pH 7.4). After a few seconds, excess stain was removed and the grid was air-dried. Five random fields, each containing 100–200 particles, were photographed with a Philips CM12 electron microscope at a magnification of 13,000. Particles were measured on the negatives with a Peak 7× magnifier with a graticule 1–20 mm in 0.1-mm divisions. Only discrete spherical particles greater than 155 Å were tabulated, and a minimum of 300 particles were measured for each sample.

RESULTS

Characterization of size and composition of emulsion particles

Emulsion composition was characterized chemically by lipid analysis, and size was determined by negative stain EM (Table 1 and Fig. 1 *A*). The estimated surface composition (Miller and Small, 1987) is also given (Table 1). The average particle diameter calculated from the lipid composition was 1080 ± 40 Å and was very similar to the average size found by negative stain EM. Particles were spherical, and sizes ranged from 220 to 3600 Å, with a mean size around 1040 Å; less than 1% of the particles were vesicles, 80% of the particles were between 400 and 2000 Å, and 5% were above 2000 Å. The particle size distribution did not change from one emulsion to the next (Fig. 1 *A*). Titration of emulsion with apo-A1 did not change the morphology, number of vesicles, or particle size distribution ($p > 0.4$) (Fig. 1 *B*). That apo-A1 was bound to the emulsion particles after titration was confirmed by floating an aliquot in the centrifuge and measuring the protein concentration in the infranatant, which showed that less than 25% of the protein remained here.

Reaction of Apo-A1 with emulsion particles

The apo-A1 was titrated with the DPPC emulsion particles at 30°C (Fig. 2 *A*) and 45°C (Fig. 2 *C*) and with EYPC emulsion (Fig. 2 *D*). At 30°C an automated sequence of 37 injections of 6.1 μg apo-A1 each, spaced at 3-min intervals, was required to saturate the surface of emulsion particles containing 4.17 mg DPPC. Initial injections released about 35 μcal/μg protein bound (Fig. 2 *A*). The total heat produced exothermally ranged from 3900 to 6600 μcal per titration curve (0.68 to 1.09 kcal released/mol DPPC) in three experiments. The data were not corrected for ligand heats of dilution because they were not significant, as revealed by the heat generated on the final injection. After

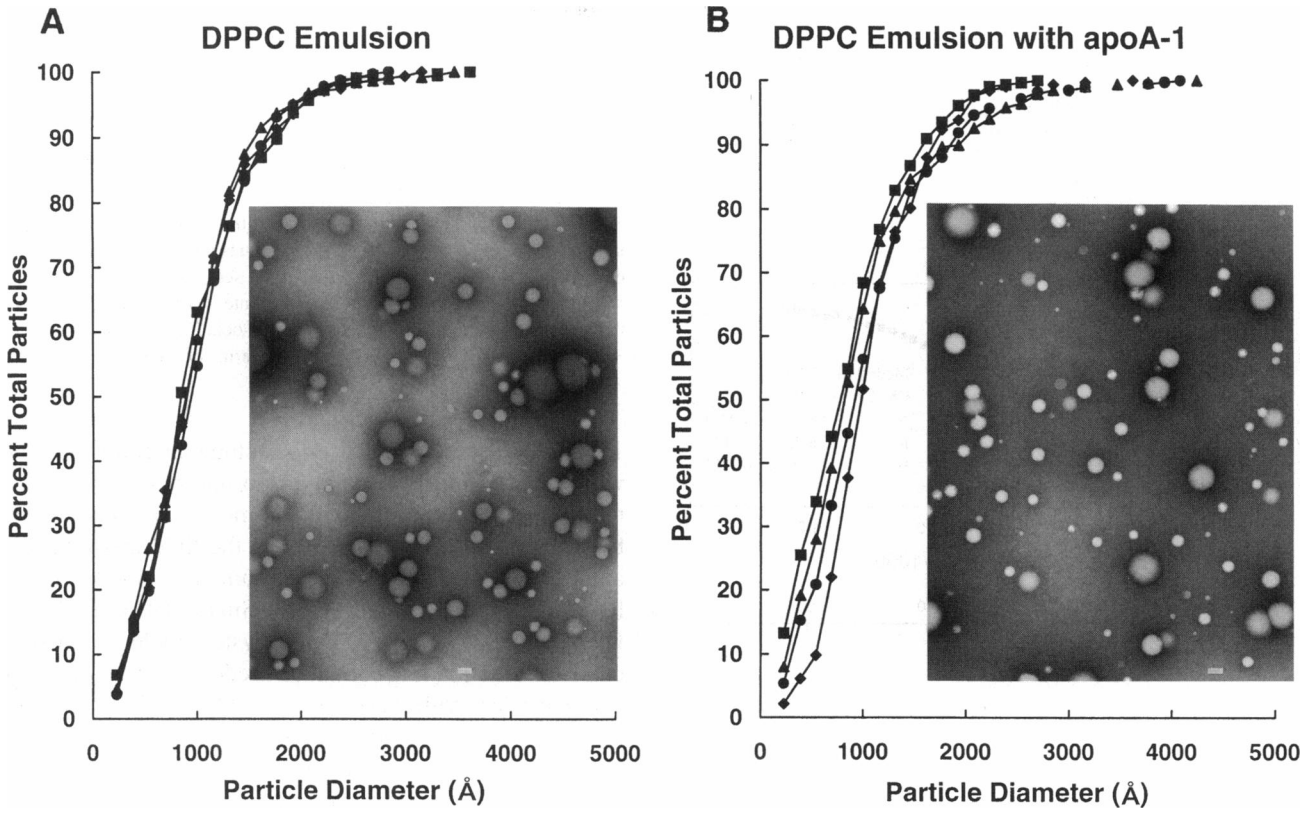

FIGURE 1 Particle size distributions of four (●, ■, ▲, ♦) DPPC emulsions in the absence (*A*) or presence (*B*) of apo-A1, and representative electron micrographs (*insets*) of one experiment (▲). The molar ratio of apo-A1 to DPPC varied from 0.0012 to 0.0015. The bar equals 1100 Å, and the magnification is 22,750.

normalization by computer for concentrations, the data were deconvoluted using an algorithm based on the Marguardt method (Wiseman et al., 1989). Results from the deconvolution of the data in Fig. 2 *A* are shown in Fig. 2 *B*, where the points are experimental and the solid line is the calculated best-fit curve using parameter values indicated in the figure legend.

For all experiments performed at 30.0°C, the average best values for N, K, and H of the apo-A1 binding to a lipid surface of specific composition are compiled in Table 2. In addition, the free energy of association, F_a, and the entropy of association, S, are calculated.

For titration of apo-A1 onto DPPC emulsions at 45°C (Fig. 2 *C*) and onto EYPC emulsions (Fig. 2 *D*), no heat was evolved or adsorbed.

Differential scanning calorimetry of emulsion particles in the absence or presence of apo-A1

A set of thermograms of a DPPC emulsion in the absence and presence of saturating apo-A1 is shown in Fig. 3, and the deconvoluted curves after subtraction of buffer baseline and normalization for concentration are shown in Fig. 4. Without apo-A1 proteins (Fig. 3, *top scans*), the heating runs of emulsions exhibit three distinct and broad endothermic transitions. With apo-A1 on the surface there was a gain in enthalpy of the middle transition that persisted on subsequent heating (Fig. 3, *bottom scans*). Fig. 4 displays the deconvoluted curves of two thermograms shown in Fig. 3 of emulsion without (*top plot*) and with (*bottom plot*) apo-A1. After curve fitting, the location, size, and shape of three transitions are evident as peaks 1, 2, and 3. Note that upon apo-A1 binding the second peak becomes much larger and sharper (compare Fig. 4 top with bottom). A summary of the processed calorimetric data of the three experiments is listed in Table 3, which reveals that upon apo-A1 binding to the emulsion the midpoint of the second transition temperature is unchanged (32.8 ± 0.1°C) but both the calorimetric (H_c) and Van't Hoff (H_v) enthalpy increase in a statistically significant way ($p < 0.001$). The changes in the peaks 1 and 3 are insignificant (Table 3). The total enthalpy under the curve (the sum of all three transitions) was 1.53 ± 0.20 kcal/mol DPPC and increased significantly with apo-A1 added to 2.70 ± 0.12 kcal/mol DPPC. Microcalorimetry of emulsion with apo-A1 did not change the morphology, number of vesicles, or particle size distribution as determined by negative stain EM (results not shown). Microcalorimetry of EYPC-triolein-cholesterol emulsions yielded flat, featureless curves between 5°C and 50°C, and no transitions or changes in specific heat were seen (data not shown).

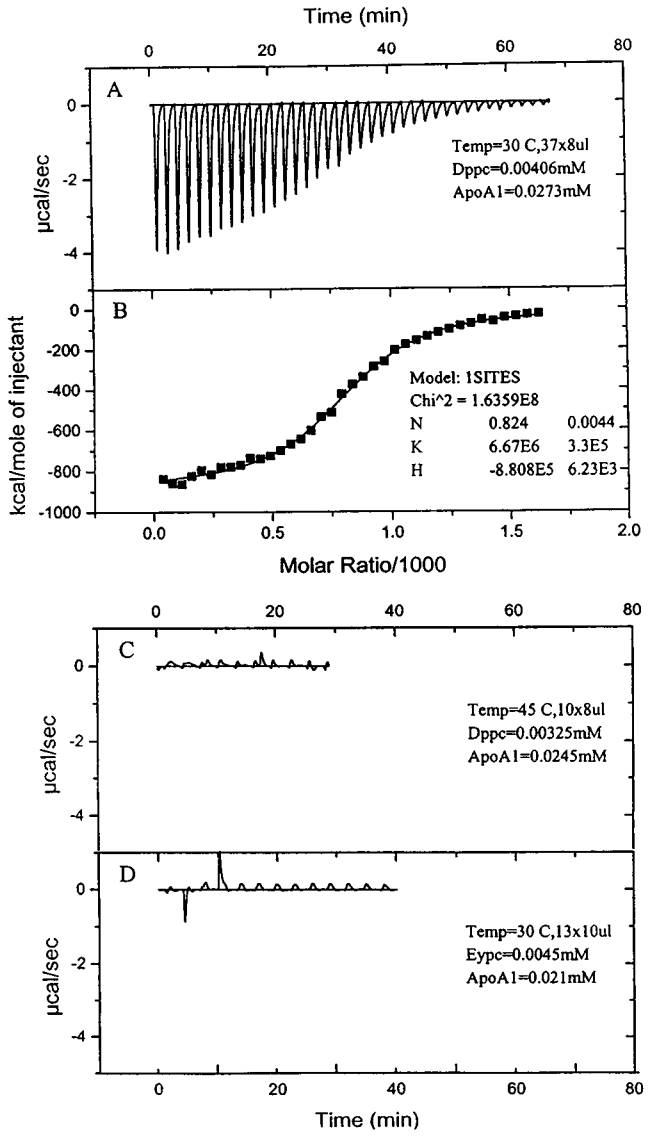

FIGURE 2 (*A*) Isothermal titration curve for apo-A1 (0.0273 mM) injected into a DPPC/triolein/cholesterol emulsion (DPPC = 4.06 mM). Injection schedule was 40 × 8 µl into 1.4 ml emulsion at 30.0°C. Injections were 3 min apart and titration was stopped after 37 injections. (*B*) Graph derived from Fig. 1 *A* of kcal/mol of injected apo-A1 versus the molar ratio of apo-A1 per 1000 DPPC (DPPC = 0.00406 mM). The solid line corresponds to the best-fit curve obtained by least-squares deconvolution and was achieved without fixing any of the parameters. The best values of the fitting parameters using a one-binding site model are 0.824 for N, 6.67 × 10^6 M^{-1} for K, and −880.8 kcal/mol for ΔH. (*C*) Isothermal titration of apo-A1 (0.0245 mM) injected into a DPPC-triolein-cholesterol emulsion at 45.0°C (DPPC = 3.25 mM). Injections were 3 min apart and titration was stopped after 10 injections of 8 µl. (*D*) Isothermal titration of apo-A1 (0.021 mM) injected into a EYPC-triolein-cholesterol emulsion at 30.0°C (EYPC = 4.5 mM). Injections were 3 min apart and titration was stopped after 13 injections of 10 µl.

DISCUSSION

The studies presented here extend previous studies on apo-A1 binding to emulsion models with fluid or viscous surfaces using a centrifugation assay (Derksen and Small, 1989; Derksen et al., 1989), to include an emulsion model with a partly solid surface and calorimetry as the analytical tool. The change in the emulsion model was accomplished by replacing EYPC with DPPC in the lipid mixture. Small amounts of cholesterol were retained to model nascent lipoprotein particles (Miller and Small, 1983, 1987). The DPPC/triolein/cholesterol model system yielded an isolated emulsion fraction that contained predominantly large spherical particles with very reproducible lipid compositions (Table 1) and size distributions (Fig. 1). The lipid composition and particle size distribution of the low-cholesterol DPPC emulsion were nearly identical to that of the low-cholesterol egg PC emulsion in a previous study (Derksen and Small, 1989). Because the purity of the starting lipids

TABLE 2 Binding parameters of Apo-A1 to DPPC emulsion surface at 30°C*

Capacity (N)	1.0 ± 0.09 protein/1000 DPPC
Association (K)	11.1 ± 3.5 × 10^6 M^{-1}
Enthalpy (H)	−940 ± 35 kcal/mol protein
Free energy of association (F_a)	−9760 cal/mol protein‡
Entropy of association (S)	−3070 cal/°/mol protein‡

*Determined by ultrasensitive isothermal titration calorimetry. For experimental conditions see legend of Fig. 2. Values are the mean ± SE of three experiments. For surface lipid composition see Table 1.

‡H enthalpy expressed in terms of DPPC content is H enthalpy = −0.92 ± 0.12 kcal/mol DPPC. F_a free energy of association in terms of DPPC = −9.76 cal/mol. S entropy of association in terms of DPPC = −3 cal/°/mol.

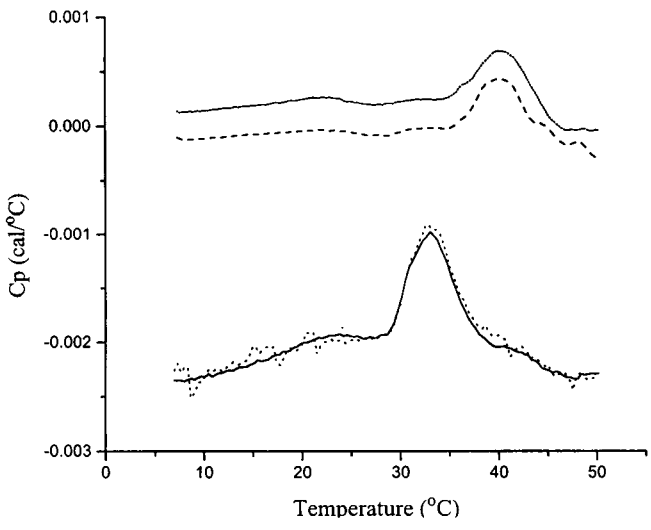

FIGURE 3 Four differential scanning calorimetry thermograms performed on two samples of one DPPC/triolein/cholesterol emulsion either in the absence (*top two scans*) or presence of apo-A1 (*bottom two scans*) containing 2.889 and 3.077 mM DPPC, respectively. The molar ratio of apo-A1 to DPPC was 0.0015. For lipid composition of emulsion see Table 1. The change in heat capacity (C_p) in cal/°C is plotted versus temperatures. Each sample was scanned five times at a heating rate of 90°C/h from 3°C to 50°C, and cooled down for 70 min between runs. Shown are the second and third heating runs for emulsion in the absence of apo-A1 (*top scans*) and the third and fourth heating runs of the same emulsion with apo-A1 (*bottom scans*). Examples of curve fitting and deconvolution of two of these thermograms are shown in Fig. 4.

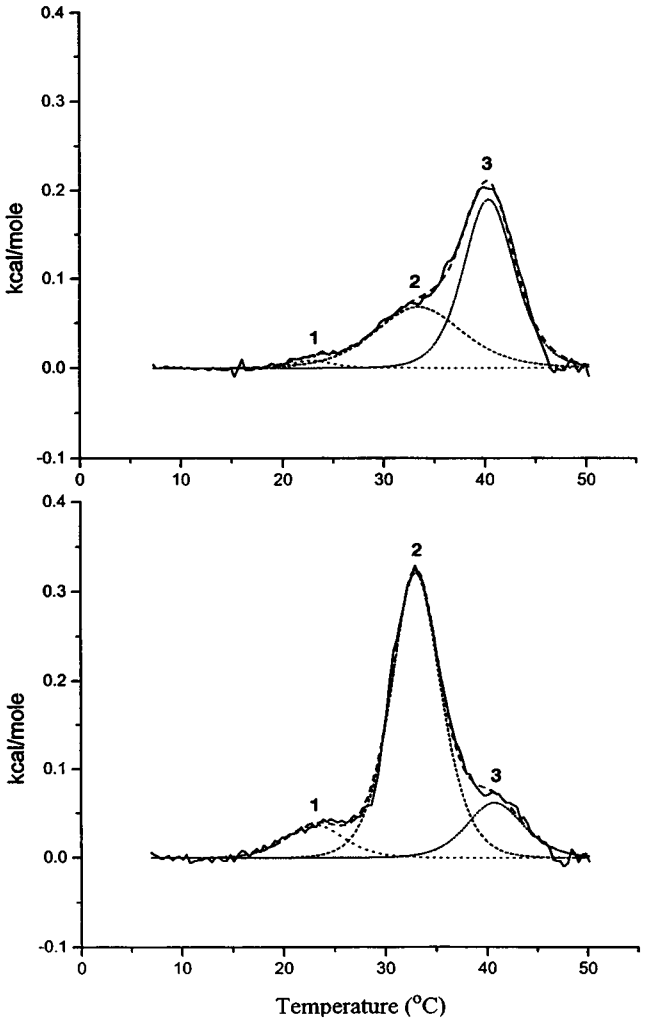

FIGURE 4 Plots of processed data corresponding to raw data of two thermograms in Fig. 3. The top plot is derived from the third heating scan of emulsion in the absence of apo-A1. The bottom plot is derived from the fourth heating run of the same emulsion with apo-A1. The solid line represents the normalized data after subtraction of the buffer (PBS) run, normalization for concentration and subtraction of a baseline constructed with "Progress Baseline" (pb) from the baseline menu. The dashed lines represent the best fit (χ^2) to the normalized data using the model 2 curve fitting program from the DSC menu. This program, also known as Non-2-state:Cursor-Init, further calculates the parameters T_m, the midpoint of the transition temperature; H_c, calorimetric enthalpy; and H_v, Van't Hoff enthalpy for each transition initialized by the cursor. The best fit to the normalized data and the best values for the parameters T_m, H_c, H_v were always obtained when deconvolution was initiated for three consecutive transitions. The dotted, short dotted, and short dashed lines represent the three consecutive meltings (peaks 1–3). All data, files, and programs used in processing the raw data, and the best values for T_m, H_c, and H_v are summarized in Table 3.

TABLE 3 Thermodynamic parameters of DPPC emulsion transitions in the absence and presence of apo-A1*

Transition	Parameter‡	Emulsion§	Emulsion + Apo-A1¶	p‖
1	T_m	24.3 ± 0.6	23.2 ± 0.2	—
	H_c	0.1 ± 0.05	0.23 ± 0.03	0.03
	H_v	10.4 ± 2.2	9.5 ± 0.6	—
2	T_m	33.0 ± 0.3	32.8 ± 0.1	—
	H_c	0.64 ± 0.11	1.77 ± 0.09	<0.001
	H_v	7.1 ± 0.5	12.1 ± 0.2	<0.001
3	T_m	40.0 ± 0.1	39.5 ± 0.4	—
	H_c	0.78 ± 0.1	0.69 ± 0.42	—
	H_v	14.5 ± 0.9	12.0 ± 1.8	—
	TH	1.53 ± 0.20	2.70 ± 0.12	<0.001

*Derived from differential scanning calorimetry as described in Figures 3 and 4.

‡T_m = midpoint of transition temperature in °C; H = enthalpy in kcal mol^{-1} DPPC; H_c = calorimetric enthalpy; H_v = Van't Hoff enthalpy × 10^{-4}; TH = combined calorimetric enthalpies of all three transitions.

§Mean ± SEM from six of the last four heating runs of two independent experiments.

¶Mean ± SEM from nine of the last four heating runs of three independent experiments.

‖Statistical probability from unpaired t-test performed on data sets (see § and ¶) using the RS1 laboratory software system; not significant.

and the procurement procedure are also practically the same, small amounts of diolein and oleic acid may also be present in the surface (Derksen et al., 1989), but they were not quantitated in this study. Thus, the estimate that 2.2 mol% of the emulsion surface lipids are neutral lipids other than cholesterol (Table 1) is conservative.

Performing differential scanning calorimetry on polydisperse emulsion particles raises several concerns about surface changes during heating and cooling that may affect the binding of proteins. For instance, how much does the surface area of a small particle change with temperature compared to larger ones? To approach this problem we have chosen three triolein droplet sizes of 500, 1000, and 1500 Å in diameter and calculated volume and surface area. At 25°C the volume of triolein is 1.10156 ml/g, whereas at 50°C and 3°C these volumes are 1.12440 and 1.08147 ml/g, respectively, adjusted using an average coefficient of expansion of 0.000913 ml/g per degree Celsius (Small, 1986b). Using these values, one can calculate the volume change of these particles at these temperatures and derive the diameter and surface area from these volumes. This calculation shows that when these particles are heated from 3°C to 50°C the surface area of each particle size increases by +2.63%. Thus, the change in surface area of a spherical triolein core droplet upon heating (or cooling) is independent of size.

Minor changes in particle dimensions and surface area of DPPC are calculated to occur with change in temperature, as shown in Table 4. An average emulsion particle of about 1065 Å diameter contains 347,049 triolein and 1,932 cholesterol molecules in the core and 1,347 triolein, 5,847 cholesterol, and 54,381 DPPC molecules in the surface, as calculated with the aid of a computer program (Miller and Small, 1987) from the lipid composition data. The total core volume of this particle at 25°C is derived from the sum of the molecular triolein and cholesterol volumes multiplied by the number of these molecules in the core (= 559,989,234 $Å^3$). Assuming a spherical particle morphology diameter,

TABLE 4 Effect of temperature on neutral lipid core dimensions, the DPPC area per molecule, and calculated angle of tilt in the surface of an emulsion particle*

Temperature (°C)	Emulsion lipid volumes (Å^3)‡			Neutral lipid		DPPC surface parameters		
	Triolein	Cholesterol	DPPC	d(Å)§	SA (Å^2)¶	SA (Å^2)‖	*h***	Θ‡‡
3	1580.63	642	1123	1016.41	3,245,528	53.26	21.09	44.3
25	1610.00	642	1153	1022.65	3,285,518	53.99	21.35	43.5
30	1616.67	642	1153	1024.06	3,294,566	54.16	21.29	43.7
41	1631.35	642	1228	1027.14	3,314,436	54.53	22.52	NA
50	1643.37	642	1228	1029.70	3,330,662	54.82	22.40	NA

*The emulsion used in Figs. 3 and 4 had a weight percent composition of 87.9% triolein, 0.9% cholesterol, and 11.2% DPPC and is used for these calculations. The average emulsion particle has a diameter of 1065 Å at 25°C and contains 347,049 triolein and 1932 cholesterol molecules in the core, and 1347 triolein, 5847 cholesterol, and 54,381 DPPC molecules in the surface as calculated with the aid of a computer program from the lipid composition data (Miller and Small, 1987).

‡Molecular lipid volumes for triolein, cholesterol, and DPPC were taken from tables 10–3, 11–9, and 12–4 in Small (1986a). The molecular triolein volume at 25°C was set at 1610 Å (Small, 1986b), and other volumes were temperature adjusted using an average coefficient of expansion of 0.000913 ml/g per degree Celsius.

§The diameter (*d*) of the core lipid droplet was calculated from the volume $\pi d^3/6$, assuming spherical particle morphology and using appropriate numbers of molecules (see *) and lipid volumes (see ‡) for the various temperatures.

¶The core surface area (SA) = πd^2.

‖Phospholipid SA = Core SA − [Cholesterol SA (5847 × 39Å^2) + Triolein SA (1347 × 90Å^2)]. SA per molecule = phospholipid SA/54381.

**Phospholipid height (*h*) = phospholipid volume/phospholipid SA. Phospholipid SA, see ‖. Phospholipid volume = 54,381 × appropriate volume for temperature (see b).

‡‡Angle of tilt (Θ) = $\cos^{-1}$ of phospholipid *h*/phospholipid length (*l*). The length of DPPC in the gel state was calculated as the sum of the thickness of the polar region (10.4 Å) plus 15 carbon lengths of 1.27 Å each (19.05 Å) for a total of 29.45 Å (Small, 1986a). NA, not applicable, because hydrocarbon chains are melted.

d = 1,022.65 Å, then *S* the surface area = 3,285,518 Å^2. This surface area contains 1,347 triolein, 5,847 cholesterol, and 54,381 DPPC molecules. With an estimated surface area of 90 Å^2 per triolein and 39 Å^2 per cholesterol molecule, it follows that 54,381 DPPC molecules occupy 2,936,255 Å^2 at 25°C (53.99 Å^2 per DPPC molecule). At 41°C the DPPC surface area would be 54.5 Å^2. This is consistent with the formation temperature of the emulsion being 41 ± 2°C, i.e., a temperature at which the DPPC chains are likely to be melted. Apparently 54.5 Å^2 is the area each DPPC occupies at the triolein interface when the emulsion is formed. To calculate the surface area per DPPC molecule distributed equally over the core lipid droplet interface of this particle at 30°C, this exercise is repeated using a molecular triolein volume that is temperature adjusted with an average coefficient of expansion of 0.000913 ml/g per degree Celsius. The surface area at 30°C is 54.16 Å^2 (Table 4), an imperceptible change. In fact, over the 3–50°C range, the surface area is nearly constant (2.2% increase). The molecular volume divided by the surface area of a DPPC molecule yields the height of this lipid in the surface, and the length (Small, 1986b) of the DPPC molecule permits an estimate of the angle of tilt. Calculated height (~22 Å) and tilt (~44°) change little with temperature (Table 4).

When the newly formed emulsions are cooled, parts of DPPC chains may solidify. If all the DPPC solidified and stood perpendicular to the triolein interface they would occupy only ~40 Å^2/DPPC. This would leave a fraction (54.16 − 40)/54.16 × 100 = 26% of the triolein surface naked and would favor rapid fusion. At 30°C the potential maximal lipid free surface area on the same particle is 770,035 Å^2. This can be obtained by subtracting the surface area per DPPC at 30°C (54.16 Å^2) from the minimal surface area of DPPC in the gel state (40 Å^2) (Small, 1986a) and multiplying this by the number of phospholipids in the surface, 54,381 × 14.16 = 770,035 Å^2. We attempted to fuse these particles by ultracentrifugation using the technique of Miller and Small (1982), which readily fuses, aggregates, and coalesces EYPC-triolein-cholesterol emulsions, but the emulsions would not coalesce. Because fusion does not easily occur, we suggest that the DPPC molecules are partly solidified and tilted to cover the whole surface.

The surface area occupied by the protein in the surface of the average particle like that described in Table 4 is 206,148 Å^2 and may be obtained from the number of proteins per particle (*N* × no. of phospholipids/1000) (Table 2) multiplied by the surface area of apo-A1 on the DPPC monolayer = 243 × 15.6 Å^2 (number of amino acids × surface area per residue) (Ibdah and Phillips, 1988). With protein on the surface the surface area available to phospholipids is reduced by the area the protein occupies. At 30°C this reduction decreases the overall area per DPPC molecule from 54.16 Å^2 to 50.75 Å^2 (6.3%). The putative angle of tilt decreases from 43.7° to 39.5° (9.6%). These changes are probably underestimated by about 50% because the molecules melting in peak 3 (high melting) probably are not influenced by protein binding. The change in surface area and possible decreased tilt of the DPPC when apo-A1 binds to the emulsion surface may be related to the observed increase in the calorimetric and Van't Hoff enthalpies of the second transition (Table 3). With apo-A1 on the surface of the DPPC emulsion the calorimetric enthalpy of the middle transition (transition 2) is increased by about 1 kcal mol^{-1}

DPPC, which is very close to the amount of heat released during titration if expressed per mole of DPPC (0.92 ± 0.12 kcal mol^{-1} DPPC). Thus, apo-A1 may increase the lipid packing density of the domain to which it binds, converting a fraction of the DPPC into a gel-like phase.

The third binding parameter listed in Table 2 is the enthalpy of binding. There was 940 kcal released per mol of apo-A1 bound to lipid or, if expressed as lipid, 0.92 kcal mol^{-1} DPPC. The amount of heat involved in protein conformation during lipid binding may be deduced from the difference in the heat of denaturation of free (unbound) apo-A1, and lipid bound apo-A1 in aqueous solutions, which is about 90 kcal (Tall et al., 1975, 1976, 1977) and less than 10% of the total heat released in the binding of apo-A1 to a DPPC emulsion surface. About half of this small enthalpy (50 kcal) may come from the induction of α-helical content from 60% to 75% of the secondary structure (Lux et al., 1972; Massey et al., 1979; Privalov, 1982). However, the great fraction of heat released (~90%) must come from lipid-lipid or lipid-protein interactions resulting from protein binding. This appears to be the direct result of carrying out the binding experiments at 30°C, where part of the lipid is solid. When binding titrations were carried out on these emulsions at 45°C, where the DPPC is melted, no heat is evolved or adsorbed (Fig. 2 *C*). Furthermore, in 18 separate experiments titrating apo-A1 onto EYPC-cholesterol-triolein emulsions of similar size and composition, virtually no heat was evolved or absorbed (for example, see Fig. 2 *D*). These surfaces were fluid. Thus, the vast majority of the heat evolved must result from protein realigning and/or reordering the partly solid lipid domain. This is supported by the negative entropy of the binding process (Table 2), because crystallization of lipid is accompanied by a negative entropy. When entropy of binding is expressed in terms of DPPC it is about -3 cal mol^{-1} deg^{-1}. If the process simply moved $-CH_2-$ from an aqueous to hydrocarbon environment, the process ought to have a positive entropy (Tanford, 1973). After binding, an increase in H of the lipid transitions (transition 2 is doubled; Table 3) or an increase of about 1.17 kcal/mol DPPC is seen. Assuming ΔG to be 0 at the lipid transitions one can calculate the entropy change at about -3.8 cal mol^{-1} deg^{-1}. Because H for the L_a gel transitions is about -9 kcal/mol DPPC, S is -28.7 cal mol^{-1} deg^{-1}. Thus 3–4 cal mol^{-1} deg^{-1} could represent the conversion of about 10–15% of the DPPC from the liquid state to the ordered gel state.

It is of interest that the titration of apo-A1 onto EYPC emulsions at 30°C was euthermic—no heat was evolved or taken up. Because we had measured the association constant in a prior publication (Derksen and Small, 1989), we calculated the free energy of association (~8400 cal/mol apo-A1) and used it to calculate the entropy. The entropy of apo-A1 binding to EYPC is positive at about 28 cal/mol^{-1} deg^{-1}, and this may represent the apo-A1 pushing out some water (i.e., condensing) the monolayer on the EYPC emulsion. Thus it would appear that the binding mechanism to DPPC emulsions at 30° differs from that to egg lecithin at 30° and probably to DPPC at 45°.

Pure multilamellar DPPC liposomes in aqueous suspension melt in two transitions at 35.3°C and 41.4°C with a combined enthalpy of 10.3 kcal mol^{-1} and cooperative units ranging between 260 and 290 (Mabrey and Sturtevant, 1976). In this study the DPPC on the surface of the emulsion is about 88.3% pure (Table 1) and melts in three transitions at 24.3°C, 33.0°C, and 40.0°C with a combined enthalpy of 1.53 kcal mol^{-1} DPPC. The observed low enthalpy, the shift downward in transition temperatures (Mabrey and Sturtevant, 1976; Singer and Finegold, 1990), and the broad transitions (small cooperative units) are consistent with this level of purity. None of the lipid compositions of the three domains undergoing the three transitions are currently known, but it seems reasonable that at 30°C, the lower melting domain is fluid, the middle is viscous and partly melted, and the higher is solid. Apoproteins most probably bind to the fluid and viscous domains and not to solid domains.

In summary, we have developed an emulsion system in which chemical composition and physical properties are suitable to effectively serve as a model for studying the binding of exchangeable apolipoproteins to triglyceride-rich lipid particles with partially ordered surfaces. The use of titrating calorimetry shows that the binding process is exothermic, and analysis suggests that most of the heat evolved can be attributed to reordering of partly melted DPPC molecules at the surface. On the other hand, the binding of apo-A1 to fluid surfaces is an entropy-driven hydrophobic effect that is euthermic.

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