# Molecular Packing of High-Density and Low-Density Lipoprotein Surface Lipids and Apolipoprotein A-I Binding†

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ABSTRACT: The surface pressure ($\pi$)–molecular area ($A$) isotherms for monolayers of human high-density lipoprotein ($HDL_3$) and low-density lipoprotein (LDL) phospholipids and of mixed monolayers of these phospholipids with cholesterol spread at the air–water interface were used to deduce the likely molecular packing at the surfaces of $HDL_3$ and LDL particles. LDL phospholipids form more condensed monolayers than $HDL_3$ phospholipids; for example, the molecular areas of LDL and $HDL_3$ phospholipids at $\pi$ = 10 dyn/cm are 88 and 75 Å$^2$/molecule, respectively. The closer packing in the LDL phospholipid monolayer can be attributed to the higher contents of saturated phosphatidylcholines and sphingomyelin relative to $HDL_3$. Cholesterol condenses both $HDL_3$ and LDL phospholipid monolayers but has a greater condensing effect on the LDL phospholipid monolayer. The $\pi$–$A$ isotherms for mixed monolayers of $HDL_3$ phospholipid/cholesterol and LDL phospholipid/cholesterol at stoichiometries similar to those at the surfaces of lipoprotein particles suggest that the monolayer at the surface of the LDL particle is significantly more condensed than that at the surface of the $HDL_3$ particle. The closer lateral packing in LDL is due to at least three factors: (1) the difference in phospholipid composition; (2) the higher unesterified cholesterol content in LDL; and (3) a stronger interaction between cholesterol and LDL phospholipids relative to $HDL_3$ phospholipids. The influence of lipid molecular packing on the affinity of human apolipoprotein A-I (apo A-I) for $HDL_3$ and LDL surface lipids was evaluated by monitoring the adsorption of $^{14}$C-methylated apo A-I to monolayers of these lipids spread at various initial surface pressures ($\pi_i$). At a given $\pi_i$, apo A-I adsorbs more to the $HDL_3$ surface lipid monolayer than to the LDL surface lipid monolayer and fails to adsorb to the $HDL_3$ and LDL surface lipid monolayers at $\pi_i \geq 26$ and 20 dyn/cm, respectively. Cholesterol generally decreases the adsorption of apo A-I to the lipoprotein surface lipid monolayers. These studies suggest that the molecular packing at the surfaces of lipoprotein particles influences the binding of apo A-I and that the surface lipid composition of LDL is, at least partially, the reason for the absence of apo A-I in LDL particles in vivo.

It is well established that native lipoproteins consist of a core of neutral lipids encapsulated by a monolayer of polar lipids and apolipoproteins [for reviews, see Jackson et al. (1976), Tall and Small (1980), Scanu et al. (1982), and Atkinson and Small (1986)]. Although this "oil-drop" model of lipoprotein particles is well characterized, the molecular packing and lipid–protein interactions in the outer shell of various types of lipoprotein particles are not well understood. The surface lipid compositions of various lipoprotein classes are known to differ [for reviews, see Skipski (1972) and Scanu (1979)]. For instance, low-density lipoprotein (LDL) has higher contents of saturated phosphatidylcholine (PC), sphingomyelin, and unesterified cholesterol compared to high-density lipoprotein (HDL). It is likely that these differences in surface lipid composition result in different molecular packing in the surfaces of the lipoprotein particles. Spectroscopic techniques have been utilized to study the molecular packing of the lipids in HDL and LDL particles [for a review, see Keim (1979)]. These studies have suggested that the surface lipids of HDL and LDL particles exist in a fluid state but no systematic differences between these lipoproteins have been reported. Previous nuclear magnetic resonance (NMR) studies from this laboratory have suggested that cholesterol oscillatory motions are slower and/or more restricted in the surface of LDL compared to the surface of HDL (Lund-Katz & Phillips, 1984, 1986). However, the influence of the differences in HDL and LDL phospholipid composition on the packing of lipid molecules at the surfaces of these lipoprotein particles has not been evaluated.

In addition to the differences in surface lipid composition, the apolipoprotein compositions of the various lipoprotein classes also differ [for a review, see Mahley et al. (1984)]. For example, apolipoprotein A-I (apo A-I) is the major protein component of HDL particles, but it is not present in the surface of LDL particles. This occurs although apolipoproteins are known to transfer and exchange between lipoprotein classes in vivo during metabolism (Eisenberg & Levy, 1975; Schaefer et al., 1978; Daerr et al., 1986). The effects of surface lipid composition and packing on apolipoprotein exchange and transfer are not known. It follows that in order to understand the factors that determine the apolipoprotein composition of lipoprotein particles, it is necessary to understand the molecular packing of lipoprotein surface lipids and its influence on apolipoprotein association with the surface lipids.

Here we use the monolayer system to investigate the likely lipid packing in the surfaces of human HDL and LDL particles and to compare the ability of apolipoprotein A-I to bind to monolayers of HDL and LDL surface lipids.

## Experimental Procedures

*Isolation of Human $HDL_3$ and LDL.* Human $HDL_3$ ($1.125 < d < 1.21$ g/mL) and LDL ($1.019 < d < 1.063$ g/mL) were isolated from the fresh serum of normal donors by sequential

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ultracentrifugation in KBr (Havel et al., 1955; Hatch & Lees, 1968). The plasma was treated with 5 mM *N*-ethylmaleimide to inhibit lecithin–cholesterol acyltransferase (Johnson et al., 1986). The lipoprotein fractions were dialyzed extensively against saline (0.15 M NaCl, 0.001 M EDTA, and 0.02% w/v $NaN_3$, pH 7.6) prior to use. The purities of the lipoprotein fractions were assessed by agarose gel electrophoresis (Noble, 1968) using precast agarose slides (Bio-Rad Laboratories, Rockville Center, NY); the LDL and $HDL_3$ gave single bands on staining with Sudan Black. At least three different preparations of $HDL_3$ and LDL from different normal donors were used in the experiments described in this study.

*Extractions of Lipoprotein Phospholipids.* Fresh human LDL and $HDL_3$ were delipidated with 3:2 (v/v) ethanol/diethyl ether at 0 °C (Scanu & Edelstein, 1971). The lipids were concentrated and applied under nitrogen to silica gel G thin-layer chromatography plates (1000-$\mu$m thickness) (Analtech, Newark, DE). The solvent system used for separation was 20:1 (v/v) benzene/ethyl acetate, and the phospholipids were extracted from the silica gel using 50:50:10 (v/v) chloroform/methanol/water. The lipoprotein phospholipid samples were not contaminated by other lipids such as free cholesterol, triglycerides, or cholesterol esters as assessed by thin-layer chromatography on silica gel G plates using two solvent systems: (1) petroleum ether/diethyl ether/acetic acid (75:24:1 v/v); (2) chloroform/methanol/water (65:25:4 v/v).

The purified lipoprotein phospholipids were dried under nitrogen and then dissolved in hexane/ethanol (9:1 v/v) for the monolayer experiments. Because surface chemistry techniques are particularly sensitive to impurities, 99 mol % chromatographically pure hexane was purchased from Fisher Scientific Co. (Malvern, PA) and further purified on an activated alumina column prior to use. Lipid solutions were stored at 4 °C in a desiccator saturated with hexane vapor to minimize evaporation of the solvent. Cholesterol purchased from Sigma Chemical Co. (St. Louis, MO) was added to the extracted lipoprotein phospholipids for experiments in which cholesterol was required. The concentrations of phospholipid and cholesterol in solution were determined by phosphorus assay and gas–liquid chromatography, respectively (Sokoloff & Rothblat, 1974).

*Radiolabeling of Apolipoprotein A-I.* Human apolipoprotein A-I was isolated from HDL ($1.063 < d < 1.21$ g/mL) as described previously (Ibdah & Phillips, 1988). Apo A-I was labeled by reductive methylation of lysine residues using [$^{14}$C]formaldehyde and sodium cyanoborohydride reducing agent as described by Jentoft and Dearborn (1979, 1983). In a typical reaction, 1 mg of apo A-I freshly desalted from 3 M guanidine hydrochloride (Gdn·HCl) in a reaction volume of 0.5 mL of phosphate buffer (5.65 mM $Na_2HPO_4$, 3.05 mM $NaH_2PO_4$, and 0.08 M NaCl, pH 7) was incubated with 2.5 $\mu$Ci of [$^{14}$C]formaldehyde of high specific activity (40–60 Ci/mol; New England Nuclear, Boston, MA) and 25 $\mu$L of 0.1 M sodium cyanoborohydride (Aldrich Chemical Co., Milwaukee, WI). The mixture was held at 4 °C for 18 h, and then the reaction was stopped by dialysis against several changes of 0.1 M NaCl (pH 7). According to this method, the specific activity of $^{14}$C apo A-I was about 2 $\mu$Ci/mg which corresponds to single methylation of about 5% of the lysine residues in the apolipoprotein molecule (i.e., approximately one lysine residue per apo A-I molecule was modified) as determined by amino acid analysis (Krebs et al., 1988). At this degree of labeling, the surface properties of $^{14}$C apo A-I at the lipid–water and air–water interfaces as well as its electrophoretic mobility on SDS–PAGE were identical with those of native apo A-I (Ibdah & Phillips, 1988; Krebs et al., 1988).

Protein determination was carried out by using the SDS–Lowry method of Markwell et al. (1978), and the $^{14}$C radioactivity was assessed by standard liquid scintillation procedures (McLean & Phillips, 1981).

*Surface Pressure–Molecular Area Isotherms.* A Langmuir–Adam surface balance was used to measure the surface pressure–molecular area ($\pi$–$A$) isotherms for spread lipid films as described elsewhere (Phillips & Chapman, 1968; Phillips & Krebs, 1986). The numbers of phospholipid and cholesterol molecules delivered to the air–water interface were computed by using the phosphorus and cholesterol concentrations of the spreading solutions. Ten minutes after the lipid film was spread, the surface area was reduced with a movable Teflon barrier in 1–3 Å$^2$/molecule decrements and 20-s intervals between successive compressions until the collapse $\pi$ was attained. The $\pi$ value was recorded and plotted as a function of the molecular area of the lipid molecules. The estimated error in $\pi$ was ±1 dyn/cm for a given amount of lipid spread onto a constant surface area.

*Adsorption of Apolipoproteins.* The adsorption of $^{14}$C-labeled apo A-I to spread lipid monolayers was investigated as described previously (Ibdah & Phillips, 1988). Briefly, a lipid monolayer was spread to a certain initial surface pressure ($\pi_i$) over the clean air–water interface in a Teflon dish, 10.8 cm in diameter, containing 80 mL of phosphate buffer (5.65 mM $Na_2HPO_4$, 3.05 mM $NaH_2PO_4$, and 0.08 M NaCl, pH 7, 25 ± 1 °C); the $^{14}$C apo A-I was then injected beneath the lipid monolayer to give the desired subphase concentration. Adsorption of $^{14}$C apo A-I to the lipid–water interface was monitored over time by following the increases in surface pressure ($\Delta\pi$) and surface radioactivity using a Wilhelmy plate and a gas flow counter, respectively [for a review, see Phillips and Krebs (1986)]. The surface radioactivity of adsorbed apo A-I was converted to surface concentration ($\Gamma$) using a calibration curve. The errors in $\pi$ and surface radioactivity measurements were approximately ±1 dyn/cm and ±5%, respectively. It should be noted that apo A-I solutions were kept in 2 M Gdn·HCl at concentrations <0.5 mg/mL to avoid aggregation of the apolipoprotein. The final subphase concentration of Gdn·HCl (<1 mM) was too low to cause any denaturation of the protein (Reijngoud & Phillips, 1982) and had no effect on the surface properties of the apolipoprotein at the lipid–water interface (Ibdah & Phillips, 1988). The adsorption experiments were carried out at a subphase protein concentration of $3 \times 10^{-5}$ g/100 mL; at this subphase concentration, adsorption of apo A-I to the lipid–water interface was maximal (Ibdah & Phillips, 1988).

## Results and Discussion

*Molecular Packing of HDL and LDL Surface Lipid Monolayers.* The surface pressure–molecular area isotherms of $HDL_3$ and LDL phospholipids spread as monolayers at the air–water interface are shown in Figure 1A. It is clear that the LDL phospholipid monolayer is condensed relative to the $HDL_3$ phospholipid monolayer; for a given $\pi$, the LDL phospholipids occupy a lower molecular area than the $HDL_3$ phospholipids. For instance, the molecular areas of $HDL_3$ and LDL phospholipids at $\pi$ = 10 dyn/cm are 88 and 75 Å$^2$/molecule, respectively. In addition, the apparent collapse pressures for $HDL_3$ and LDL phospholipid monolayers were attained at molecular areas of 60 and 52 Å$^2$/molecule, respectively. The tighter molecular packing of the LDL phospholipid monolayer relative to that of the $HDL_3$ phospholipid monolayer is a reflection of differences in the phospholipid

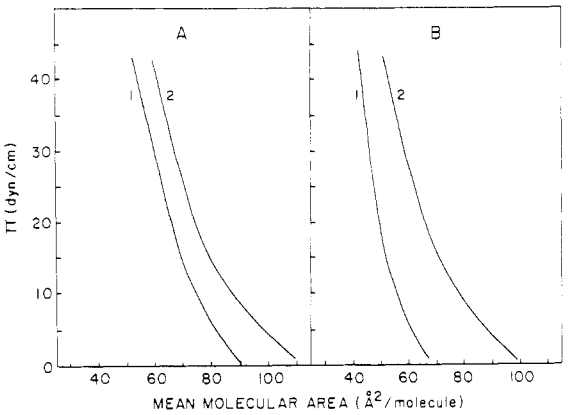


FIGURE 1: Surface pressure ($\pi$)–mean molecular area isotherms for LDL phospholipid (1) and $HDL_3$ phospholipid (2) monolayers (panel A) and for mixed cholesterol/LDL phospholipid (1) and cholesterol/$HDL_3$ phospholipid (2) monolayers at cholesterol:phospholipid molar ratios of 0.55 and 0.15, respectively (panel B). The lipid monolayers were spread on a subphase of phosphate buffer (5.65 mM $Na_2HPO_4$, 3.05 mM $NaH_2PO_4$, and 0.08 M NaCl, pH 7) at 25 ± 1 °C; see Experimental Procedures for details. The depicted $\pi$–$A$ isotherms were obtained by drawing smooth curves through at least 30 data points for each isotherm (the data points are omitted for clarity).

composition in the two lipoproteins. The phospholipid in LDL is more enriched with saturated PC relative to $HDL_3$; the reported ratios of unsaturated PC/saturated PC in $HDL_3$ and LDL are 1.3 and 0.6, respectively (Skipski, 1972). Saturated PC molecules are known to form more condensed monolayers than unsaturated PC molecules (Demel et al., 1967). In addition, LDL phospholipids contain more sphingomyelin compared to $HDL_3$ phospholipids; densitometric scanning of stained phospholipid bands on a thin-layer chromatography plate indicated that the ratios of sphingomyelin/PC in $HDL_3$ and LDL were close to the reported values of 0.12 and 0.38, respectively (Skipski, 1972). Although the sphingomyelin in LDL and $HDL_3$ has the same ratio of unsaturated to saturated fatty acids, the sphingomyelin contains much longer chains than the PC (Skipski, 1972). It should be noted that sphingomyelin has been reported to form more condensed monolayers than the equivalent PC (Ibdah & Phillips, 1988; Lund-Katz et al., 1988); the longer saturated acyl chains in sphingomyelin presumably further enhance the van der Waals cohesion and closeness of molecular packing of sphingomyelin relative to PC.

The influence of cholesterol on the molecular packing of lipoprotein surface lipids was explored by studying mixed monolayers of cholesterol and $HDL_3$ or LDL phospholipids. The $\pi$–$A$ isotherms for $HDL_3$ phospholipid/cholesterol and LDL phospholipid/cholesterol mixed monolayers containing various mole fractions of cholesterol are depicted in Figure 2. The condensation of the phospholipid monolayers by cholesterol is demonstrated by plotting the mean molecular area at $\pi$ = 10 dyn/cm against the mole fraction of cholesterol (Figure 3). Ideally, if there is no interaction between cholesterol and the phospholipid molecules, the mean molecular area can be calculated according to an equation which describes the average area of two immiscible components (Chapman et al., 1969):

$$A_{12} = nA_1 + (1 - n)A_2 \quad (1)$$

$A_{12}$ is the average area for the mixed components at a certain $\pi$, $n$ is the mole fraction of component 1, and $A_1$ and $A_2$ are the molecular areas of components 1 and 2, respectively, obtained from their $\pi$–$A$ isotherms at the same $\pi$. The deviation of the mean molecular area–cholesterol mole fraction plot from

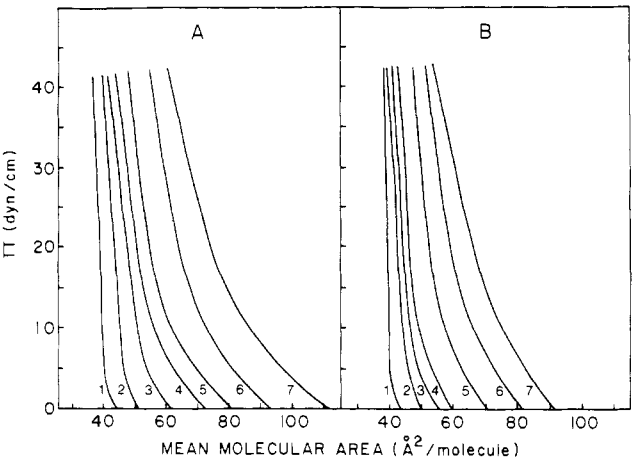


FIGURE 2: Surface pressure ($\pi$)–mean molecular area isotherms for mixed monolayers of cholesterol with $HDL_3$ phospholipids (panel A) and LDL phospholipids (panel B). The isotherms labeled 1–7 correspond, respectively, to cholesterol mole fractions of 1, 0.8, 0.6, 0.5, 0.4, 0.2, and 0. Isotherm 7 is for the pure lipoprotein phospholipid. See the legend to Figure 1 for further details.

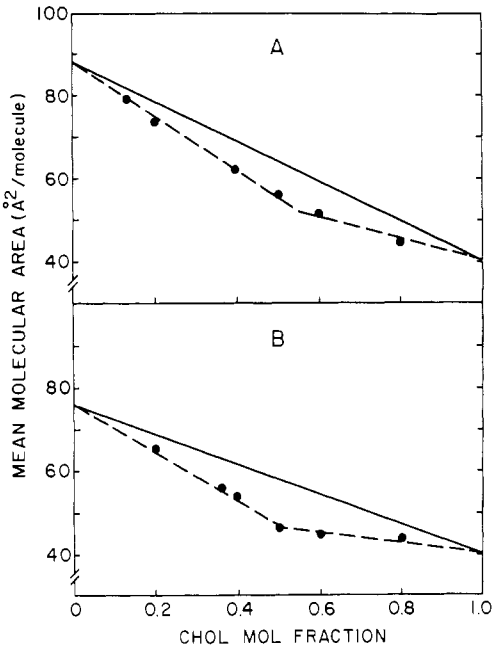


FIGURE 3: Dependence of the mean molecular area of cholesterol/$HDL_3$ phospholipid (panel A) and cholesterol/LDL phospholipid (panel B) mixed monolayers on cholesterol content, at a surface pressure ($\pi$) of 10 dyn/cm. The solid line (—) represents the average areas which would be observed if ideal mixing of cholesterol and phospholipid occurs. The dashed line (●---●) represents the measured mean molecular areas obtained from $\pi$–$A$ isotherms of the mixed cholesterol/phospholipid monolayers shown in Figure 2.

ideality is a measure of the condensation by cholesterol of the phospholipid monolayers [for a review, see Phillips (1972)]. The condensation is due to an interaction between cholesterol and the acyl chains of the phospholipid molecules (Demel et al., 1967; Chapman et al., 1969; Phillips, 1972). The relative condensation of $HDL_3$ and LDL phospholipids by cholesterol can be estimated by computing the molecular areas (at a given $\pi$) of these phospholipids in the mixed cholesterol/phospholipid monolayers. This is possible because pure cholesterol is known to form a solid condensed monolayer with a limiting area of approximately 40 Å²/molecule (Demel et al., 1967; Chapman et al., 1969). If it is assumed that cholesterol occupies this same area in the mixed monolayer, then the area per molecule of the phospholipid can be calculated from the mean molecular

area [for details, see Phillips (1972)]. Calculation of the molecular areas of phospholipid molecules in the pure and mixed films indicates that the same content of cholesterol condenses LDL phospholipids to a greater extent than $HDL_3$ phospholipids. For instance, LDL phospholipids occupy 54 $Å^2$/molecule when mixed in an equimolar ratio with cholesterol at $\pi$ = 10 dyn/cm compared to 75 $Å^2$/molecule when spread alone as a monolayer at the same surface pressure. In comparison, $HDL_3$ phospholipids occupy 72 $Å^2$/molecule in an equimolar film with cholesterol at $\pi$ = 10 dyn/cm and 88 $Å^2$/molecule in the pure film at the same surface pressure. These figures indicate condensations of 18% and 28% of the molecular areas of $HDL_3$ and LDL phospholipids, respectively, due to interaction with cholesterol. Similar calculations show that equimolar concentrations of cholesterol reduce the molecular areas of $HDL_3$ and LDL phospholipids by about 24% and 30%, respectively, at $\pi$ = 20 dyn/cm. The greater condensation of LDL phospholipids compared to $HDL_3$ phospholipids is presumably a reflection of stronger interactions between cholesterol and LDL phospholipids due to increased van der Waals cohesion between the relatively saturated phospholipid hydrocarbon chains and cholesterol. This is consistent with previous studies which showed that cholesterol condenses saturated PC monolayers more than unsaturated PC monolayers (Chapman et al., 1969) and sphingomyelin monolayers more than the equivalent PC monolayers (Lund-Katz et al., 1988).

Figure 1B depicts the $\pi$–$A$ isotherms for $HDL_3$ phospholipid/cholesterol and LDL phospholipid/cholesterol mixed monolayers at cholesterol:phospholipid molar ratios of 0.15 and 0.55, respectively, which correspond to the stoichiometries in the surfaces of $HDL_3$ and LDL particles in vivo (Lund-Katz & Phillips, 1986). It is clear that cholesterol condenses the LDL phospholipids more than the $HDL_3$ phospholipids (cf. panels A and B of Figure 1). For example, the mean molecular areas of $HDL_3$ and LDL surface lipids at $\pi$ = 10 dyn/cm are 78 and 56 $Å^2$/molecule, respectively (Figure 1B); from these figures, $HDL_3$ and LDL phospholipids are calculated to occupy areas of about 85 and 65 $Å^2$/molecule, respectively, in the mixed cholesterol/phospholipid monolayers. This represents reductions in the molecular areas of pure $HDL_3$ and LDL phospholipids of about 4% (from 88 to 85 $Å^2$/molecule) and about 13% (from 75 to 65 $Å^2$/molecule), respectively, due to the interaction with cholesterol. Similar calculations at $\pi$ = 20 dyn/cm show that cholesterol reduces the molecular areas of $HDL_3$ and LDL phospholipids by 6% and 17%, respectively. This greater condensation of LDL phospholipids by cholesterol relative to $HDL_3$ phospholipids is due to the higher content of cholesterol in the LDL surface as well as the stronger interaction between cholesterol and the LDL phospholipids relative to the $HDL_3$ phospholipids. Figure 3 also confirms that cholesterol exerts a greater condensing effect on the lipoprotein phospholipids at a cholesterol mole fraction of 0.36 (mol of chol/mol of phospholipid = 0.55) compared to a mole fraction of 0.13 (mol of chol/mol of phospholipid = 0.15) (cf. Figure 1B). It should be noted that at these molar ratios 60% and 70% of the total unesterified cholesterol in $HDL_3$ and LDL, respectively, are assumed to be present at the surface of the particles as suggested by NMR studies (Lund-Katz & Phillips, 1984, 1986). Similar conclusions apply if it is assumed that all the unesterified cholesterol molecules are present at the surface of the lipoprotein particles. The molar ratios of cholesterol/phospholipid in $HDL_3$ and LDL particles are 0.25 (chol mole fraction = 0.2) and 0.8 (chol mole fraction = 0.44), respectively (Scanu, 1979); at these molar ratios, cholesterol condenses LDL phospholipids to a greater extent than $HDL_3$ phospholipids (cf. panels A and B of Figure 3).

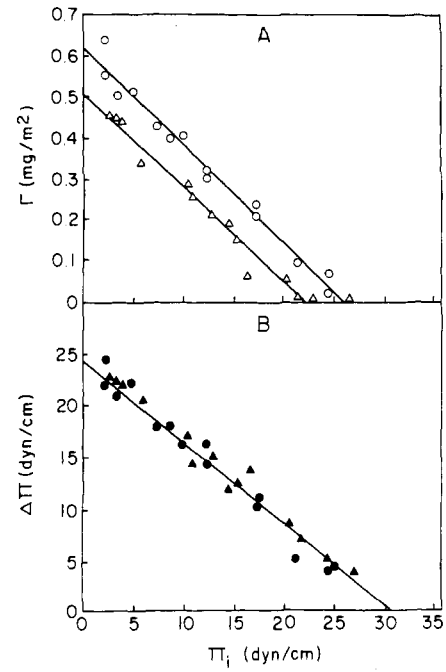


FIGURE 4: Surface concentration ($\Gamma$)–initial surface pressure ($\pi_i$) plot (panel A) and increase in surface pressure ($\Delta\pi$)–$\pi_i$ plot (panel B) for adsorption of $^{14}C$-labeled apo A-I to monolayers of $HDL_3$ phospholipid (○, ●) and LDL phospholipid (△, ▲). The experimental conditions are described under Experimental Procedures. The subphase concentration of apolipoprotein A-I was $3 \times 10^{-5}$ g/100 mL. The steady-state $\Gamma$ and $\Delta\pi$ values are plotted against the $\pi_i$ of the lipid monolayer. Linear regression coefficients for the lines through the $HDL_3$ phospholipid and LDL phospholipid data in panel A are 0.99 and 0.98, respectively. The $\Delta\pi$ values (panel B) are similar and therefore fitted with one regression line (the linear regression coefficient is 0.98). The estimated errors in $\Gamma$ and $\Delta\pi$ measurements are ±0.03 mg/$m^2$ and ±1 dyn/cm, respectively.

In summary, these studies show that LDL surface lipids form a more closepacked monolayer than HDL surface lipids. This can be attributed to the higher contents of saturated PC, sphingomyelin, and cholesterol in LDL as well as to the stronger interaction between cholesterol and phospholipids in LDL compared to HDL.

*Adsorption of Apo A-I to Monolayers of LDL and $HDL_3$ Surface Lipids.* To explore the influence of the molecular packing of lipoprotein surface lipids on the binding of apo A-I, the adsorption of $^{14}C$-labeled apo A-I to monolayers prepared with $HDL_3$ and LDL phospholipids spread at various $\pi_i$ was monitored. It is apparent from the $\Gamma/\pi_i$ plot that there is a linear decrease in the $\Gamma$ of apo A-I with increasing $\pi_i$ of both the $HDL_3$ and LDL phospholipid monolayers (Figure 4A). At any given initial surface pressure, $\Gamma$ for apo A-I with the $HDL_3$ phospholipid monolayer is significantly higher than that with the LDL phospholipid monolayer. The higher affinity of apo A-I for the $HDL_3$ phospholipid monolayer correlates with the more expanded $\pi$–$A$ isotherm of the $HDL_3$ phospholipid monolayer compared to the LDL phospholipid monolayer (cf. Figure 1A and 4A). It follows that the association of apo A-I with the $HDL_3$ and LDL phospholipid monolayers is dependent on the physical state of the lipid monolayers, in agreement with studies of the adsorption of apo A-I and other proteins to synthetic phospholipid monolayers (Camejo & Munos, 1981; Colacicco, 1970; Phillips et al., 1975 a,b; Ibdah & Phillips, 1988). It is evident that the higher contents of sphingomyelin and saturated acyl chains in LDL phospholipid result in a lower adsorption of apo A-I compared

to the $HDL_3$ phospholipid monolayer.

Plots of $\Delta\pi/\pi_i$ show linear decreases in $\Delta\pi$ with increasing $\pi_i$ of the $HDL_3$ and LDL phospholipid monolayers (Figure 4B). It is apparent that adsorption of apo A-I to $HDL_3$ and LDL phospholipid monolayers results in similar changes in surface pressure. This occurs, although apo A-I adsorbs to a higher $\Gamma$ with the $HDL_3$ phospholipid monolayer compared to the LDL phospholipid monolayer for a given $\pi_i$. The lateral compressibility of a lipid monolayer $[=-1/A(\delta A/\delta\pi)_T]$, where $A$ is molecular area and $T$ is temperature, is the parameter which best reflects the effect of the physical state of the lipid monolayer on protein penetration (Phillips et al., 1975 a–c; Ibdah & Phillips, 1988). $\Delta\pi$ is inversely related to the lateral compressibility of the lipid monolayer and can be described by the equation (Phillips et al., 1975a):

$$\Delta\pi = \Gamma A_p/C \quad (2)$$

where $A_p$ is the average area per protein molecule and $C$ is the lateral compressibility of the monolayer. Figure 1A demonstrates that the LDL phospholipid monolayer has a lower lateral compressibility than the $HDL_3$ phospholipid monolayer; this is evident from the difference in the slopes of the $\pi$–$A$ isotherms of LDL and $HDL_3$ phospholipid monolayers especially at $\pi < 25$ dyn/cm where adsorption of apo A-I to these lipid monolayers occurs (Figure 4A). Therefore, the same surface concentration of apo A-I results in a greater increase in the surface pressure of the LDL phospholipid monolayer compared to the $HDL_3$ phospholipid monolayer (cf. panels A and B of Figure 4). For example, 0.5 mg/m$^2$ of adsorbed apo A-I in the LDL and $HDL_3$ phospholipid monolayers results in $\Delta\pi$ values of 24 and 20 dyn/cm, respectively. Extrapolation of $\Delta\pi$ to zero reveals that apo A-I fails to penetrate the $HDL_3$ and LDL phospholipid monolayers at $\pi_i \geq 30$ dyn/cm. Extrapolation of data in Figure 4A suggests that zero surface concentration of apo A-I is expected at $\pi_i \geq 26$ and 22 dyn/cm for the $HDL_3$ and LDL phospholipid monolayers, respectively. The surface concentration of apo A-I at $\pi_i \geq 26$ and 22 dyn/cm in the $HDL_3$ and LDL phospholipid monolayers, respectively, is minimal and cannot be detected by the gas flow counter but still can result in a $\Delta\pi$ value of several dynes per centimeter due to the low compressibility of these lipid monolayers at high $\pi_i$ values.

The effects of cholesterol on the affinity of apo A-I for HDL and LDL surface lipids were also investigated. Plots of $\Gamma$/cholesterol mole fraction and $\Delta\pi$/cholesterol mole fraction for the adsorption of apo A-I to the mixed $HDL_3$ and LDL phospholipid/cholesterol monolayers spread at $\pi_i = 10$ dyn/cm are shown in panels A and B, respectively, of Figure 5. It is interesting to note that the effects of cholesterol on the adsorption of apo A-I to the $HDL_3$ and LDL phospholipid monolayers (Figure 5) and to the egg PC monolayer (Ibdah & Phillips, 1988) are strikingly similar. It is evident that, in general, addition of cholesterol decreases the adsorption of apo A-I to the lipoprotein phospholipid monolayers. Similarly, cholesterol has been demonstrated to decrease binding of apolipoproteins to microemulsions (Erkelens et al., 1981; Maranhao et al., 1986). The effects of cholesterol are dependent on the cholesterol/phospholipid stoichiometry. $\Gamma$ for apo A-I is independent of cholesterol mole fraction in the range 0.2–0.5; this is probably because the lateral compressibilities of the mixed cholesterol/phospholipid monolayers containing these mole fractions of cholesterol are similar (Figure 2). $\Delta\pi$ due to adsorption of apo A-I is not sensitive to the exact mole fraction of cholesterol present in the mixed monolayer (Figure 5). This occurs because $\Delta\pi$ is influenced by the $\Gamma$ of apo A-I as well as by the lateral compressibility of the lipid monolayer

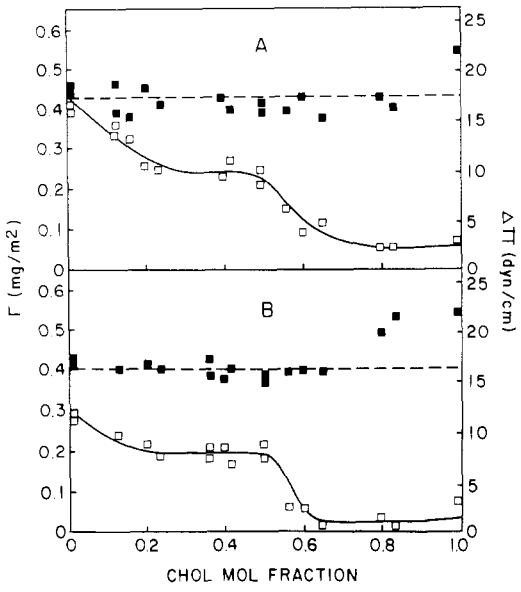


FIGURE 5: Plots of the increase in surface pressure [$\Delta\pi$ (■)] and the surface concentration of apolipoprotein A-I [$\Gamma$ (□)] against cholesterol mole fraction for the adsorption of $^{14}$C apolipoprotein A-I to cholesterol/$HDL_3$ phospholipid mixed monolayers (panel A) and to cholesterol/LDL phospholipid mixed monolayers (panel B) spread at $\pi_i$ = 10 dyn/cm. The experimental conditions are described under Experimental Procedures. The subphase concentration of apolipoprotein A-I was $3 \times 10^{-5}$ g/100 mL. The estimated errors in $\Gamma$ and $\Delta\pi$ measurements are approximately ±0.03 mg/m$^2$ and ±1 dyn/cm, respectively.

(see eq 2) and compensating effects occur.

The effects of cholesterol on the association of apo A-I with HDL and LDL surface lipids were further investigated at cholesterol/phospholipid stoichiometries similar to those existing at the surfaces of these lipoprotein particles in vivo (Lund-Katz & Phillips, 1984, 1986). The adsorption of apo A-I to the monolayers prepared with $HDL_3$ and LDL surface lipid at cholesterol mole fractions of 0.13 and 0.36 (cholesterol:phospholipid molar ratios of 0.15 and 0.55), respectively, is summarized in $\Gamma/\pi_i$ and $\Delta\pi/\pi_i$ plots (Figure 6). The $\Gamma/\pi_i$ plot shows a linear decrease in $\Gamma$ with increasing $\pi_i$ of the $HDL_3$ and LDL surface lipid monolayers. At any given $\pi_i$, the adsorption of apo A-I to the $HDL_3$ surface lipid monolayer is greater than to the LDL surface lipid monolayer (Figure 6A). The greater adsorption of apo A-I to the $HDL_3$ surface lipid monolayer is due to the lower content of cholesterol as well as to the difference in phospholipid composition mentioned earlier. Cholesterol decreases the adsorption of apo A-I to both the $HDL_3$ and LDL phospholipid monolayers, but it has a greater effect on the adsorption to the LDL phospholipid monolayers (cf. Figures 6A and 4A) due to the higher content of cholesterol in the LDL surface lipids as well as the stronger interaction of cholesterol with the LDL phospholipids. The $\Delta\pi/\pi_i$ plot (Figure 6B) shows similar $\Delta\pi$ values for the $HDL_3$ and LDL surface lipid monolayers. Extrapolation of $\Delta\pi$ values to zero reveals that apo A-I fails to penetrate the $HDL_3$ and LDL surface lipid monolayers at $\pi_i \geq 28$ dyn/cm. The dependence of $\Gamma$ on $\pi_i$ shows that the surface concentration of apo A-I at the $HDL_3$ and LDL surface lipid monolayers is zero at $\pi_i \geq 26$ and 20 dyn/cm, respectively. At $\pi_i$ values between 20 and 26 dyn/cm, apo A-I fails to penetrate the LDL surface lipid monolayer but can still adsorb to the $HDL_3$ surface lipid monolayer.

It is clear from the studies described above that the higher contents of saturated PC, sphingomyelin, and cholesterol in the LDL surface relative to the HDL surface result in a greater

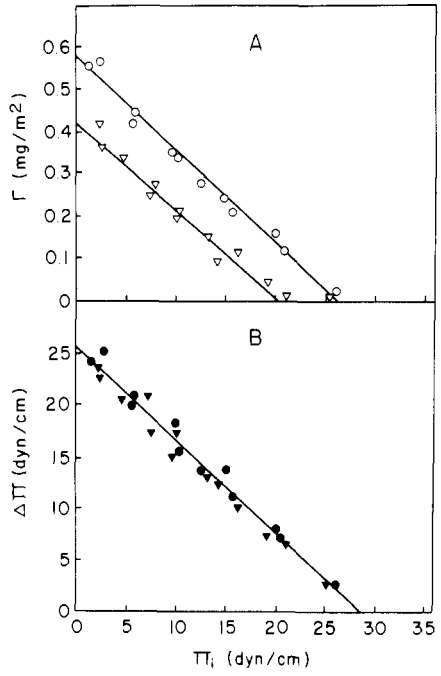


FIGURE 6: Surface concentration ($\Gamma$)–initial surface pressure ($\pi_i$) plot (panel A) and increase in surface pressure ($\Delta\pi$)–$\pi_i$ plot (panel B) for adsorption of $^{14}$C-labeled apo A-I to mixed monolayers of cholesterol/$HDL_3$ phospholipid (○, ●) and cholesterol/LDL phospholipid (▽, ▼) at cholesterol:phospholipid molar ratios of 0.15 and 0.55, respectively. The experimental conditions are described under Experimental Procedures. The subphase concentration of apo A-I was $3 \times 10^{-5}$ g/100 mL. The steady-state $\Gamma$ and $\Delta\pi$ values are plotted against the $\pi_i$ of the lipid monolayer. The linear regression coefficients for the lines through $HDL_3$ and LDL surface lipid data in panel B are similar and therefore fitted with one regression line (the linear regression coefficient is 0.99). The estimated errors in $\Gamma$ and $\Delta\pi$ measurements are ±0.03 mg/m$^2$ and ±1 dyn/cm, respectively.

adsorption of apo A-I to the HDL surface lipids. It also appears that there is a strong correlation between the extent of adsorption of apo A-I and the physical state of the surface lipid monolayers in agreement with previous studies on the adsorption of apo A-I and other proteins to model lipids (Camejo et al., 1968; Phillips et al., 1975c; Ibdah & Phillips, 1988). To determine whether apo A-I simply interacts with the $HDL_3$ and LDL surface lipid monolayers by penetrating and laterally compressing the lipid molecules, it is necessary to relate the mass of adsorbed apo A-I to the space (at the air–water interface) available in the lipid monolayer that can be created by compression of lipid molecules by the adsorbed apo A-I molecules. This area is designated the "available space" and is calculated according to a model which assumes that the lipid molecules are compressed by adsorbing apolipoprotein molecules and ideal mixing of protein and lipid molecules occurs in the resulting monolayer [for details, see Ibdah and Phillips (1988)]. Analysis of adsorption data for apo A-I in terms of this model for various $HDL_3$ and LDL lipid monolayers at various $\pi_i$ values leads to the following findings.

(1) The mass of apo A-I adsorbed to the $HDL_3$ or LDL lipid monolayers is proportional to the available space in the lipid monolayers which indicates that lateral compressibility is a major determinant of the extent of apo A-I adsorption. This is demonstrated by plotting the mass of apo A-I adsorbed to a lipid monolayer against the calculated available space in that lipid monolayer for various $\pi_i$ values [cf. Figure 8 in Ibdah and Phillips (1988)]. The mass/available space plots show constant slopes for $HDL_3$ or LDL lipid monolayers (data not shown); this implies that apo A-I adsorbed to a given lipoprotein lipid monolayer at various $\pi_i$ values adopts the same conformation.

(2) For a given available space, apo A-I adsorbs more to the $HDL_3$ lipid monolayers than to the LDL lipid monolayers. The slopes of apo A-I mass/available space plots for $HDL_3$ and LDL phospholipid monolayers are 1.51 ± 0.1 and 1.28 ± 0.13 mg of apo A-I/m$^2$ of available space, respectively. This suggests that the adsorbed apo A-I molecules interact with the lipid molecules at the interface and that this interaction can influence the degree of adsorption of apo A-I to the surface lipid monolayers. This interaction is probably between the amphipathic helical segments of apo A-I (Segrest et al., 1974) and a boundary layer of lipid molecules (Jost et al., 1973). It is likely that apo A-I condenses lipid molecules which surround it and that this condensing effect of apo A-I is greater in the relatively expanded $HDL_3$ lipid monolayers than in the LDL lipid monolayers. The condensation of the boundary layer of lipid molecules results in an actual available space greater than that calculated from the model which assumes no lipid–protein interaction [for details, see Ibdah and Phillips (1988)]. In agreement with this conclusion, previous calorimetric studies on recombinant HDL particles have demonstrated that the association of apo A-I with HDL phospholipid results in a boundary layer that is perturbed by the apolipoprotein (Tall & Lange, 1978a; Pownall et al., 1979). Alternatively, apo A-I may adopt a more condensed conformation in the mixed apo A-I/$HDL_3$ lipid monolayers compared to the mixed apo A-I/LDL lipid monolayers.

(3) For a given available space, similar masses of apo A-I are adsorbed to the $HDL_3$ phospholipid and the mixed $HDL_3$ phospholipid/cholesterol monolayers. The masses of apo A-I adsorbed to the LDL phospholipid and the mixed LDL phospholipid/cholesterol monolayers are also similar for a given calculated available space. This is consistent with the idea that apo A-I and cholesterol occupy separate regions in the mixed apo A-I/lipid monolayer and do not interact directly.

*Physiological Significance.* The dynamic nature of the association of apolipoproteins with the surface lipids of lipoprotein particles does not lead to indiscriminate mixing because apolipoproteins transfer and exchange preferentially between certain types of lipoprotein particles. Thus, apo A-I is present at the surface of HDL particles but absent from LDL particles. The studies presented here suggest that the affinity of apo A-I for lipoprotein surfaces is sensitive to both the surface pressure at the lipoprotein particle–plasma interface and the surface lipid compositions of the lipoprotein particles. Surface concentration measurements show that apo A-I fails to adsorb to the LDL and $HDL_3$ surface lipid monolayers at $\pi \geq 20$ and $\geq 26$ dyn/cm, respectively (Figure 6). At surface pressures between 20 and 26 dyn/cm, apo A-I adsorbs to the $HDL_3$ surface lipid monolayer but not to the LDL surface lipid monolayer. It would be advantageous to determine the relationship between these surface pressures and the likely surface pressures that exist at the surfaces of lipoprotein particles. It is not possible to measure directly the surface pressure of a lipoprotein particle, but knowledge of the surface area, the number of constituent surface molecules, and the $\pi$–$A$ isotherms of the surface components permits an estimate of the range of pressures likely to be encountered at the lipoprotein surface.

Chemical analysis of lipoproteins has given the number of molecules of the various components per particle for each lipoprotein class (Scanu, 1979). For instance, in $HDL_3$ there are 963 amino acid residues, 51 phospholipid molecules, and 13 unesterified cholesterol molecules; 40% of the free chole-

sterol is believed to reside in the core (Lund-Katz & Phillips, 1984) so that 8 molecules of cholesterol are located in the surface. The diameter of human $HDL_3$ particles is in the range 70–80 Å (Scanu, 1979) so that the average total surface area ($=4\pi r^2$) is about 17700 Å$^2$. It is of interest to compare this number to the likely interfacial area occupied by the surface components of $HDL_3$ particles. Since apolipoprotein A-I adsorbs to the $HDL_3$ surface lipid monolayer at $\pi < 26$ dyn/cm but fails to adsorb at $\pi \geq 26$ dyn/cm, we assume that the steady-state $\pi$ at the surface of $HDL_3$ is approximately 25 dyn/cm. At $\pi = 25$ dyn/cm, the mean molecular area of an $HDL_3$ phospholipid–cholesterol monolayer (cholesterol mole fraction = 0.13) is about 62 Å$^2$/molecule (Figure 3). Given that the total number of surface phospholipid and cholesterol molecules per $HDL_3$ particle is 59, it follows that the surface lipid molecules account for about 3600 Å$^2$ of the surface area. The area of an amino acid in a closed-packed film of $\alpha$-helices is approximately 15 Å$^2$/residue (Malcolm, 1973; Krebs et al., 1988). Assuming that the largely $\alpha$-helical apoproteins have the $\alpha$-helices lying in the plane of the lipid–water interface, it follows that the total area occupied by apolipoprotein molecules at the surface of $HDL_3$ particles amounts to 14400 Å$^2$. The sum of the areas occupied by apolipoproteins and surface lipids in the $HDL_3$ particle at 25 dyn/cm is about 18 000 Å$^2$, which correlates well with the total surface area calculated from the radius of the $HDL_3$ particle. Thus, it is likely that the surface pressure at the $HDL_3$ plasma interface approximates 25 dyn/cm. Moreover, at $\pi = 25$ dyn/cm, the molecular area of $HDL_3$ phospholipids is 65 Å$^2$ (Figure 1A) which agrees with the area of 60–70 Å$^2$/molecule of PC in liquid-crystal bilayers (Phillips, 1972). It is difficult to estimate the surface pressure at the surface of the LDL particle because of uncertainties about the area occupied by the complex apo B-100 molecule. However, our studies suggest that the surface pressure at the LDL–plasma interface should be >20 dyn/cm because apo A-I no longer binds to LDL surface lipids at this point (Figure 6). It is worth noting that the transfer of apolipoproteins among various lipoprotein classes may be a function of changes in surface pressure that occur at the lipoprotein–plasma interface. A decrease in the surface pressure at the surface of a lipoprotein particle may result in adsorption of apolipoprotein molecules whereas an increase in the surface pressure may result in the opposite effect. These changes in surface pressure could be mediated by changes in the size of the lipoprotein particle during metabolism. For instance, hydrolysis of the neutral lipid core of the lipoprotein particle results in a decrease in the surface area, and there is probably a concomitant increase in the surface pressure which favors desorption of apolipoprotein molecules.

Differences in the surface lipid composition of various lipoprotein particles could be an important factor influencing the distribution of apolipoproteins among lipoprotein classes. It has been demonstrated that apo A-I adsorbs more to unsaturated PC monolayers compared to saturated PC or sphingomyelin monolayers spread at the same $\pi_i$ and cholesterol in general decreases the adsorption of apo A-I to phospholipid monolayers (Ibdah & Phillips, 1988). Consistent with this, apo A-I adsorbs more to a spread monolayer of $HDL_3$ surface lipids than to a monolayer of LDL surface lipids at the same $\pi_i$ (Figure 6). These observations suggest that the surface lipid composition of LDL is, at least partially, the reason why apo A-I is absent from LDL particles in vivo. It is also possible that the apo B molecule present at the surface of an LDL particle may have an additional condensing effect on the LDL surface lipids and/or prevent formation of enough available space in one region of the surface for an apo A-I molecule to bind effectively; both effects would further decrease the affinity of apo A-I molecules free in plasma for the LDL surface. It should be noted that NMR studies have demonstrated that about 20% of LDL phospholipid polar groups are immobilized by apo B (Lund-Katz & Phillips, 1986).

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# Laser Flash Photolysis as a Probe of Redox Protein–Membrane Interactions: Effect of Binding of Spinach Plastocyanin and Horse Cytochrome *c* to Lipid Bilayer Vesicles on the Kinetics of Reduction by Flavin Semiquinone†

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ABSTRACT: Spinach plastocyanin binds to both electrically neutral and positively charged lipid bilayer vesicles, whereas cytochrome *c* only binds electrostatically to negatively charged vesicles. Laser flash photolysis using lumiflavin semiquinone as a reductant demonstrates that the reactivity of plastocyanin is increased as much as 6-fold when it is membrane bound whereas the rate constant for cytochrome *c* reduction is decreased by approximately a factor of 3. Membrane-bound plastocyanin reduction occurs via a two-step mechanism, probably involving prior association of lumiflavin semiquinone with the bilayer. In contrast, cytochrome *c* reduction in the membrane-bound state follows simple second-order kinetics, implying that the redox site in the bound state is still accessible to lumiflavin semiquinone in solution, although the rate constant is decreased by approximately 3-fold. These results are interpreted as indicating that the bilayer–protein interaction with plastocyanin leads to a steric blockage of the electron-transfer site from the aqueous phase. Little or no hindrance of the redox site occurs with cytochrome *c*, suggesting a high degree of mobility of this protein on the bilayer surface. Although the increase in plastocyanin reactivity upon binding to the bilayer is quite interesting, its cause remains unclear and requires further study. The results illustrate the utility of laser flash photolysis as a probe of membrane–protein interactions.

It is well-known that many physiological electron-transfer systems are membrane bound and that such membrane binding and organization facilitates compartmentalization and directional electron transfer. Mitochondrial cytochrome *c*, which functions in respiratory electron transport, has been shown to bind electrostatically to vesicles formed from mixtures of negatively charged and neutral phospholipids, and this interaction has been studied quite extensively (Birrel & Griffith, 1976; Brown & Wüthrich, 1977a,b; Chapman & Urbina, 1971; Green & Fleischer, 1963; Kimelberg & Lee, 1969; Kimelberg & Paphadjopoulos, 1971; Mustonen et al., 1987; Nicholls & Malviya, 1973; Nicholls, 1974; Overfield & Wraight, 1980a,b; Quinn & Dawson, 1969; Senthilathipan & Tollin, 1986; Steinemann & Laugher, 1971; Van & Griffith, 1975; Vanderkooi et al., 1973a,b). Recent work (Gupte & Hackenbrock, 1988a,b) has provided evidence that, under physiological conditions, the rate of diffusion of cytochrome *c* along the surface of the inner mitochondrial membrane may be the rate-limiting step in the transport of electrons between the cytochrome $bc_1$ and cytochrome oxidase complexes.

Another electron-transfer protein, plastocyanin, is located on the inside of the photosynthetic thylakoid membrane (Haehnel et al., 1981; Hauska et al., 1971) and also acts as a mobile electron carrier (Olsen, 1982; Takanao et al., 1982) shuttling electrons between the cytochrome $b_6/f$ complex and photosystem I. Unlike cytochrome *c*, there are relatively few reports in the literature on model studies of plastocyanin–

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