Kinetics and Mechanism of Transfer of Reduced and Carboxymethylated Apolipoprotein A-II between Phospholipid Vesicles†

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ABSTRACT: The transfer of ^{14}C-labeled, reduced and carboxymethylated human apolipoprotein A-II (RCM-AII) between small unilamellar vesicles (SUV) has been investigated. Ion-exchange chromatography was used for rapid separation of negatively charged egg phosphatidylcholine (PC)/dicetyl phosphate donor SUV containing bound ^{14}C-labeled RCM-AII from neutral egg PC acceptor SUV present in 10-fold molar excess. The kinetics of ^{14}C-labeled RCM-AII transfer in incubations of up to 12 h at 37 °C are consistent with the existence of fast, slow, and apparently "nontransferrable" pools of SUV-associated apolipoprotein; the transfers from these pools occur on the time scales of seconds or less, hours, and days/weeks, respectively. For donor SUV (0.15 mg of phospholipid/mL reaction mixture) containing about 15 RCM-AII molecules per vesicle, the sizes of the fast, slow, and nontransferrable pools are 13, 69, and 18%, respectively. The transfer of RCM-AII from the slow kinetic pool follows first-order kinetics, and the half-time ($t_{1/2}$) is about 3 h. The different kinetic pools of SUV-associated RCM-AII probably reflect apoprotein in different conformations of the SUV surface. Increasing the number of RCM-AII per donor SUV enlarges the size of the fast pool and increases the $t_{1/2}$ of transfer from the slow pool. In contrast, raising the incubation temperature reduces the $t_{1/2}$ of slow transfer. The $t_{1/2}$ of RCM-AII transfer from the slow kinetic pool is inversely proportional to the acceptor/donor SUV ratio which suggests that the transfer of apoprotein molecules in this kinetic pool is mediated by SUV collisions. The transition state probably involves the desorption of some segments of the transferring apolipoprotein molecule from the surface of the donor SUV and the adsorption of these segments to the acceptor SUV to form a transient, ternary complex.

It is well established that serum apolipoproteins exist in a dynamic state at the surface of lipoprotein particles. Thus, during metabolism of plasma lipoproteins, there is transfer and exchange of apolipoproteins between various lipoprotein classes [for a review, see Eisenberg (1984)]. For instance, hydrolysis of very low density lipoprotein (VLDL)[1] triglycerides by lipoprotein lipase results in the transfer of apolipoprotein C (apo C) from VLDL to high-density lipoprotein (HDL), while during the action of lecithin–cholesterol acyltransferase (LCAT) on HDL, apo E is transferred from HDL to VLDL (Eisenberg et al., 1972; Glangeaud et al., 1977). There is also evidence that apolipoproteins A-I and A-II can exchange in vivo between HDL_2 and HDL_3 subclasses (Shepherd et al., 1978). A detailed understanding of the kinetics and molecular mechanisms involved in apolipoprotein transfer will be valuable because alterations in the apolipoprotein content of a lipoprotein particle can have profound effects on the metabolism of the lipoprotein. Besides being important structural elements of lipoproteins, apolipoproteins can function as cofactors for various enzymes of lipid and cholesterol metabolism as well as being the ligands by which cell surface receptors recognize lipoprotein particles [for reviews, see Eisenberg (1986) and Innerarity et al. (1986)].

The exchange of ^{125}I-labeled apoproteins between HDL subclasses in vitro has been studied by using centrifugation and gradient gel electrophoresis (Grow & Fried, 1977, 1978; Grow, 1983). The rate of apolipoprotein exchange between HDL_2 and HDL_3 subfractions was found to be influenced by both the temperature and the concentrations of donor and acceptor particles. It has been suggested that collision complexes may be involved in the exchange of apoproteins between HDL subfractions (Grow & Fried, 1978; Grow 1983). However, other studies have suggested that ^{125}I-labeled apo A-I exists in the aqueous phase in equilibrium with HDL particles (Pownall et al., 1978). Fluorescence quenching studies of apo A-I in solution and in lipid–protein complexes have suggested that rates of protein folding may significantly affect apoprotein transfer rates (Mantulin et al., 1986). The mechanisms involved in the dissociation of apoproteins from lipid–protein complexes due to denaturation have also been studied (Reijngoud & Phillips, 1982, 1984). In a recent study, the kinetics of apolipoprotein transfer between phospholipid vesicles have been investigated by using fluorescence quenching methods (McKeone et al., 1988). The transfer of apo C molecules is rapid and follows biexponential kinetics, suggesting the presence of more than one kinetic pool of apoprotein at the surfaces of lipid–protein complexes.

To date, the likely mechanisms involved in the transfer of apoprotein molecules have not been addressed in detail. In addition, there has been no quantitative data on the size of different kinetic pools of apolipoproteins that may exist at the surfaces of lipid–protein complexes. Moreover, in most cases, ^{125}I-apoproteins have been utilized to monitor transfer, and it is now apparent that iodination can modify the physical properties of apo A-I (Osborne et al., 1984; Patterson & Lee,

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[1] Abbreviations: apo, apolipoprotein; Gdn-HCl, guanidine hydrochloride; HDL, high-density lipoprotein; PC, phosphatidylcholine; RCM-AII, reduced and carboxymethylated apolipoprotein A-II; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle(s); VLDL, very low density lipoprotein(s).

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1986). This perturbation reinforces the need for reliable measurements of the rate constants for transfer of apoproteins with properties the same as those of the native molecules. Here we use a ^{14}C-labeled reduced and carboxymethylated apo A-II (RCM-AII) as a model apoprotein to study the kinetics and mechanism of apolipoprotein transfer between small unilamellar vesicles (SUV). The thermodynamics of RCM-AII association with lipids have been investigated previously (Pownall et al., 1981). The present study indicates that the kinetics of ^{14}C-labeled RCM-AII transfer are consistent with the existence of fast, slow, and apparently nontransferrable pools of SUV-associated apoprotein; the transfers occur on the time scales of seconds or less, hours, and days/weeks, respectively. About 70% of the apoprotein molecules are in the slow pool, and their transfer on the time scale of hours seems to be mediated by contact between donor and acceptor SUV.

Experimental Procedures

Materials

Lipids. Egg yolk phosphatidylcholine (PC) was obtained from Calbiochem (La Jolla, CA). Dicetyl phosphate was purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol tri[9,10(N)-^{3}H]oleate (1 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). The purities of lipid samples were assayed by thin-layer chromatography on silica gel G plates (Analtech, Newark, DE) using two solvent systems: (1) chloroform/methanol/water (65/25/4 v/v) for phospholipids and (2) petroleum ether/diethyl ether/acetic acid (75/24/1 v/v) for triacylglycerol. All samples were >99% pure.

Preparation of Human Reduced and Carboxymethylated Apolipoprotein A-II. The human reduced apo A-II (monomer) was isolated from the total high-density lipoprotein (HDL) fraction. Human HDL was isolated by sequential ultracentrifugation in the density range 1.063–1.21 g/mL (Havel et al., 1955; Hatch & Lees, 1968). The total HDL fraction was delipidated with 3/2 (v/v) ethanol/ether at 4 °C (Scanu & Edelstein, 1971), and the protein was chromatographed on a Sephacryl S-200 column (200 × 2.5 cm) using 6 M urea, 1 M NaCl, 10 mM Tris-HCl (pH 8.6), 1 mM EDTA, and 0.02% NaN_3 as the elution buffer (Scanu et al., 1969). The column fractions which corresponded to the elution positions of apolipoproteins A-I and A-II were pooled, dialyzed against 50 mM ammonium bicarbonate (pH 8), and lyophilized. The lyophilized sample was dissolved in a small volume of the above elution buffer to a protein concentration of 60 mg/mL. Dithiothreitol (DTT) and 2-mercaptoethanol were added at final concentrations of 5 mM and 10% (v/v), respectively, to reduce all disulfide bonds in apo A-II. The sample was then chromatographed on a Sephacryl S-200 column (140 × 1.6 cm) maintained under reducing conditions to separate apo A-I (M_r 28 000) from reduced apo A-II monomer (M_r 8700) using the above elution buffer combined with 10 mM DTT. The combined reduced apo A-II fractions were dialyzed against 0.1 M Tris-HCl buffer (pH 8.6) containing 10 mM DTT. The sample was concentrated to approximately 3 mg/mL, and guanidine hydrochloride (Gdn-HCl) was added to a final concentration of 3 M. The cysteine residue of reduced apo A-II monomer was then carboxymethylated using iodoacetic acid to prevent oxidation and formation of apo A-II dimer, using procedures described previously (Jackson et al., 1973; Pownall et al., 1981). An approximately 3-mL sample containing 10 mg of reduced apo A-II was incubated at 30 °C for 2 h, and then 30 mg of iodoacetic acid (Sigma Chemical Co., St. Louis, MO) was added to carboxymethylate cysteine residues. The pH of the reaction mixture was maintained at 8.6 by addition of 0.1 M NaOH, and then the sample was incubated for another 2 h at 30 °C. With this method, only the single cysteine residue was alkylated per molecule as determined by amino acid analysis (Pownall et al., 1981). The reduced and carboxylated apo A-II sample was then dialyzed extensively against 5.65 mM Na_2HPO_4, 3.05 mM NaH_2PO_4, and 80 mM NaCl, pH 7. The freshly dialyzed sample was then used for radiolabeling of RCM-AII as described below. The RCM-AII preparation gave a single band identical with that of reduced apo A-II (apo A-II + DTT) on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentrations of solutions were determined by the SDS procedure of Markwell et al. (1978) adapted from that of Lowry et al. (1951).

Radiolabeling of Human Reduced and Carboxymethylated Apo A-II. RCM-AII was radiolabeled by reductive methylation of lysine residues using [^{14}C]formaldehyde as described by Jentoft and Dearborn (1979, 1983). In a typical reaction, 1 mg of RCM-AII freshly desalted from 3 M 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 μCi of [^{14}C]formaldehyde (40–60 Ci/mol; New England Nuclear, Boston, MA) and 25 μL 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 buffer solution (20 mM phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 6). The resulting specific activity of the labeled apoprotein was about 2 μCi/mg. At this specific activity, about half of the RCM-AII molecules are ^{14}C labeled assuming single methylation of lysine residues whereas less than half of the molecules are labeled if monomethyl and dimethyl derivatives are formed [cf. Jentoft and Dearborn (1983)]. However, it should be stressed that the ^{14}C-methylated and native apoproteins have identical structural and physical properties, whether monomethyl or dimethyl derivatives are formed. Surface pressure measurements demonstrated that the surface activities of the ^{14}C-methylated apoproteins at the air–water interface and their affinities for various lipid monolayers are identical with those of native apoproteins (Ibdah & Phillips, 1988; Krebs et al., 1988; Ibdah et al., 1989). In addition, the ^{14}C-methylated and native apoproteins have identical electrophoretic mobilities on SDS–PAGE.

Methods

Preparation of Small Unilamellar Vesicles (SUV). Phospholipid vesicles were prepared by the method of Barenholz et al. (1977). Appropriate aliquots of lipid stocks were added to a 15-mL Corex tube, mixed, and dried under nitrogen. Traces of remaining solvent were removed by drying under vacuum overnight at room temperature (25 ± 2 °C). Ten milliliters of buffer (20 mM sodium phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 6) was added to give a final lipid concentration of approximately 5 mg/mL. The lipids were dispersed by sonication under nitrogen at 4 °C with a Branson Model 350 sonifier at setting 4; the tapered microtip was immersed 2 cm into the solution. Lipid solutions were sonicated for a total of 30–40 min in 5-min intervals separated by 2-min cooling periods. The lipid solutions were then centrifuged at 40 000 rpm in a Beckman 50 Ti rotor for 2 h at 4 °C. The clear supernatant was removed and stored under nitrogen at 4 °C and used within 1 week of preparation. For determination of phospholipid concentrations, the lipids were extracted by the method of Bligh and Dyer (1951), and the inorganic phosphorus was analyzed by the method of Goodwin

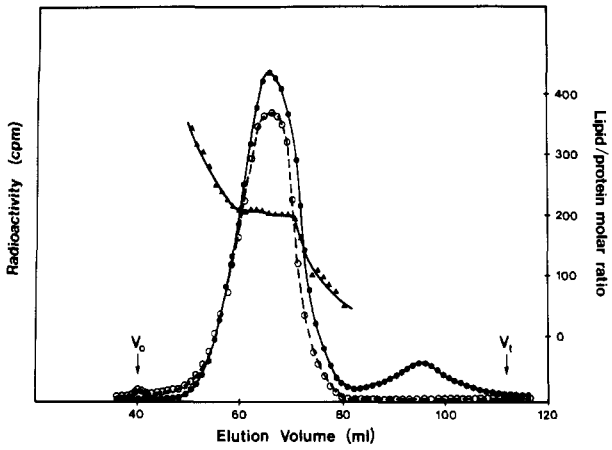

FIGURE 1: Gel filtration profile of an incubation mixture containing ^{14}C-labeled reduced and carboxymethylated apo A-II (RCM-AII) and egg phosphatidylcholine (PC) small unilamellar vesicles (SUV) containing [^{3}H]triolein as a lipid marker. [^{14}C]RCM-AII (specific activity ~2 μCi/mg of protein) was incubated with negatively charged egg PC/dicetyl phosphate (85/15 mol/mol) SUV containing [^{3}H]-triolein (specific activity 0.1 μCi/mg of phospholipid) at an initial lipid/protein molar ratio of ~170/1 in buffer (20 mM phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 6) overnight at room temperature (25 ± 2 °C). The reaction mixture was chromatographed on a Sepharose 4B-CL column (56 × 1.6 cm) and eluted with the above buffer at a rate of 15 mL/h in 1.2-mL fractions. The [^{14}C]protein (●) and [^{3}H]lipid (○) contents of various fractions were determined by using liquid scintillation counting (Beckman LS 6800 scintillation counter) and plotted versus elution volume. The ^{3}H and ^{14}C radioactivity in the peak fraction was 154800 and 176400 cpm, respectively. The final lipid/protein molar ratios in various fractions (▲) are also shown.

et al. (1958) as modified by Sokoloff and Rothblat (1974).

In a typical experiment, neutral and negatively charged vesicles were prepared. The neutral SUV were used as acceptor particles and consisted of 100 mol % egg PC to which trace [^{3}H]triolein was incorporated (0.1 μCi/mg of PC) as a nonexchangeable marker. The negatively charged SUV consisted of 85 mol % egg PC and 15 mol % dicetyl phosphate.

Preparation of Lipid/Protein Donor SUV. ^{14}C-Labeled RCM-AII was incorporated into the negatively charged vesicles which were used as donor particles. ^{14}C-Labeled RCM-AII, freshly desalted from 3 M Gdn-HCl, was incubated with SUV at the desired lipid/protein molar ratio in the range 100/1 to 700/1 in buffer (20 mM phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 6) overnight at room temperature (25 ± 2 °C). Under these conditions, the apoprotein adsorbed spontaneously onto the SUV without formation of discoidal complexes [for a review, see Jonas, 1984). The reaction mixture was chromatographed on a Sepharose 4B-CL column (56 × 1.6 cm) and eluted with the above buffer at a rate of 15 mL/h in 1.2-mL fractions. The protein contents of various fractions were determined by using liquid scintillation counting. Chromatography of protein/SUV mixtures was necessary to separate unbound protein in solution and to obtain donor particles of well-characterized sizes and lipid/protein stoichiometries.

Figure 1 shows a characteristic gel filtration profile for an egg PC/RCM-AII SUV in which both lipid and protein were labeled. It is clear that the protein has two separate elution peaks; the first coincides with that of the lipid elution peak and represents SUV-associated protein while the second represents protein free in solution. The peak fractions of donor SUV were pooled; the protein and lipid contents were determined and used to compute the final lipid/protein molar ratio. The donor SUV preparation was stored at 4 °C and used within 1 week.

Characterization of SUV. (1) Particle Size. Gel filtration chromatography was used to determine the particle sizes of the various vesicles. A 4B-CL Sepharose column (56 × 1.6 cm) was calibrated with bovine erythrocyte carbonic anhydrase, bovine serum albumin, yeast alcohol dehydrogenase, horse spleen apoferritin, and bovine thyroglobulin (Stokes radii of 20, 37, 45, 65, and 85 Å, respectively; Sigma Chemical Co., St. Louis, MO). Blue dextran and potassium ferricyanide were used to determine the void and total volumes of the column (40 and 112 mL, respectively). A linear calibration plot of the Stokes radius versus the inverse error function of the partition coefficient (K_{av}) of the standard proteins (not shown) was used to determine the sizes of various particles (Ackers, 1967). The elution volumes of the donor particles (Figure 1) and acceptor particles (not shown) were both 64 ± 1 mL, corresponding to a Stokes radius of 110 ± 10 Å. It should be noted that addition of RCM-AII to SUV does not change their Stokes radius at various lipid/protein ratios. The molar ratio of lipid/protein was constant across the elution peak (Figure 1). Negative-stain electron microscopy was also used as described previously (Collins & Phillips, 1982) to assess the particle sizes of the various vesicles. The results of electron microscopy (not shown) were consistent with the above findings from gel filtration chromatography. No discoidal complexes were apparent by electron microscopy.

(2) Molecular Weight of Vesicles. The mean molecular weight of egg PC vesicles can be calculated from the measured Stokes radius and their partial specific volume by using the expression:

$$M = (N/\bar{V})(4/3\pi r_o^3 - 4/3\pi r_i^3) \quad (1)$$

where M is the molecular weight, N is Avogadro's number, r_o is the outer radius of the vesicle determined by gel filtration and electron microscopy, r_i is the inner radius of the bilayer of the vesicle determined by subtracting the bilayer thickness from the outer vesicle radius, and $\bar{V}$ is the partial specific volume of egg PC. The bilayer thickness is considered to be 29 Å based on the X-ray diffraction data of Lecuyer and Dervichian (1969). When the measured Stokes radius of 110 Å and an egg PC $\bar{V}$ value of 0.98 are used (Huang, 1969; Watts et al., 1978), the calculated molecular weight of egg PC vesicles is 2.1 × 10^6, corresponding to 2800 phospholipid molecules per vesicle. Newman and Huang (1975) reported values of 105 Å, 1.88 × 10^6, and 2330 for the Stokes radius, molecular weight, and number of egg PC molecules per vesicle, respectively, using the Svedberg equation for calculation of molecular weight.

Assay of Apolipoprotein Transfer between SUV. Donor SUV containing ^{14}C-labeled apoprotein were incubated with acceptor SUV containing [^{3}H]triolein as a nonexchangeable marker. Typically, the incubations were carried out in a buffer containing 20 mM sodium phosphate, 1 mM EDTA, and 0.02% NaN_3, pH 6 and 37 °C, at a donor concentration of 0.15 mg/mL and 10-fold molar excess of acceptor SUV. In some experiments, the acceptor/donor ratio and temperature were varied. Rapid separation of negatively charged donor vesicles and neutral acceptor vesicles was achieved by anion-exchange chromatography as adapted from McLean and Phillips (1981) using short (1-mL) columns of DEAE-Sepharose 6B-CL (Pharmacia Fine Chemicals). One milliliter of prewashed gel was poured into a Pasteur pipet plugged with glass wool. The gel was washed with 1 mL of cold (4 °C) buffer and then equilibrated with 0.1 mL of cold buffer containing 0.2 mg of (neutral) egg PC vesicles to bind to any hydrophobic sites. Following a wash with 1 mL of cold buffer, 0.25 mL of the incubation mixture was added to the column

and eluted with another 1 mL of cold buffer. The eluate, containing neutral acceptor vesicles, was collected directly into 7-mL scintillation counting vials. Four milliliters of Scintiverse II (Fisher Scientific) was added, and the sample was counted in a Beckman LS 6800 scintillation counter. A 0.25-mL aliquot of the incubation mixture was also counted in 1 mL of buffer. Measurements were taken in duplicate unless stated otherwise.

The fractional recovery of acceptor vesicles (R) in the eluate was calculated from the relation $R = {}^3H_t/{}^3H_{mix}$ were ${}^3H_t$ and ${}^3H_{mix}$ are ${}^3H$ counts in the eluate and incubation mixture, respectively. The recovery was >90% at all incubation time points. It should be noted that the transfer of negatively charged RCM-AII molecules to acceptor SUV had no significant effect on their recovery; because a large excess of acceptor SUV was used, a small amount of protein was incorporated per acceptor SUV so that the surface charge density never became sufficient to cause binding to the DEAE-Sepharose. The fractional leak (L) of donor SUV in the eluate was also studied by using unlabeled acceptor SUV and donor SUV containing ${}^{14}C$-labeled apoprotein and [${}^3H$]triolein as a nonexchangeable marker. The fractional leak of donor SUV was calculated from the relation $L = {}^3H_e/{}^3H_{mix}$ where ${}^3H_e$ and ${}^3H_{mix}$ are ${}^3H$ counts in the eluate and incubation mixture, respectively. The leak of negatively charged donor vesicles through the column when applied alone was <1%. The leak of donor vesicles was increased in the presence of a large excess of acceptor particles; at a 10-fold excess of acceptor vesicles, the leak at the zero time point was about 1–2% which increased to approximately 5% in the presence of a 40-fold excess of acceptor SUV. The effect of the time of incubation of donor and acceptor SUV on the leak of donor SUV was also studied; the leak of donor SUV after 12 h was in the range 2–7% for incubation mixtures containing 10–40-fold excess acceptor SUV. Fusion of vesicles during 12-h incubations was not significant as determined by electron microscopy and gel filtration chromatography.

Analysis of the Kinetics of Apoprotein Transfer. The ${}^{14}C$ counts in the eluate at time t (${}^{14}C_t$) can be described by the relation ${}^{14}C_t = N_tR + L({}^{14}C_{mix} - N_t)$ here N_t is the number of [${}^{14}C$]RCM-A-II counts transferred from donor to acceptor SUV at time t. R and L are the fractional recovery and leak, respectively (see above), and ${}^{14}C_{mix}$ is the ${}^{14}C$ counts in the incubation mixture so that ${}^{14}C_{mix} - N_t$ represents the ${}^{14}C$ counts remaining in the donor SUV at time t. It follows that N_t can be calculated from the equation:

$$N_t = \frac{{}^{14}C_t - {}^{14}C_{mix}L}{{}^3H_t/{}^3H_{mix} - L} \quad (2)$$

Preliminary experiments indicated that the transfer of [${}^{14}C$]RCM-AII between SUV is consistent with there being at least two pools of RCM-AII which transfer at different rates; the kinetics were on the time scales of seconds or less and hours, respectively. A rapid transfer of a significant proportion of the ${}^{14}C$ radioactivity to the acceptor SUV was detected at the zero time point (i.e., within 1 minute of mixing donor and acceptor SUV). A transfer of more ${}^{14}C$ radioactivity then ensued. It follows that the amount of [${}^{14}C$]RCM-AII transferred at time t to the acceptor vesicles has fast and slow components and can be described by the equation:

$$N_t = N_0 + N_t^s \quad (3)$$

where N_0 is the ${}^{14}C$ counts transferred at the zero time point and N_t^s is the ${}^{14}C$ counts transferred more slowly to the acceptor vesicles in time t. For the purpose of the kinetic analysis of the data, it is assumed that the rapid and slow transfers of RCM-AII occur from independent kinetic pools. Therefore, the slow transfer of [${}^{14}C$]RCM-AII at various incubation times (N_t^s) was analyzed by using eq 2 and fitted by nonlinear regression to a first-order rate equation using the Marquart algorithm [the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.) was used on an IBM XT-compatible computer]. The amount of [${}^{14}C$]RCM-AII transferred slowly from donor to acceptor SUV at equilibrium (N_∞^s) and the associated rate constant (k) were computed; the half-time ($t_{1/2}$) was calculated from the relationship $t_{1/2} = \ln 2/k$. The best fit was determined after evaluating the data by using either one- or two-exponential functions as described under Results. The sizes of the fast and slow pools of SUV-associated RCM-AII were also calculated assuming that each kinetic pool has an independent equilibrium value which varies according to the acceptor/donor SUV ratio (A/D). Thus, when $A/D = 10/1$, values of N_0 and N_∞^s represent 90.9% of the total size of the corresponding kinetic pool associated with donor SUV. It follows that the sizes of the fast and slow pools can be calculated as the fraction of the total [${}^{14}C$]RCM-AII in donor SUV from the following relationships: fast pool = $N_0(D + A)/{}^{14}C_{mix}A$ and slow pool = $N_\infty^s(D + A)/{}^{14}C_{mix}A$. It should also be noted that back-transfer of [${}^{14}C$]RCM-AII molecules from acceptor vesicles to the donor vesicles was not significant because at least a 10-fold excess of acceptor SUV was present in all assays.

Results

Kinetics of RCM-AII Transfer between SUV. The kinetics of [${}^{14}C$]RCM-AII transfer from donor SUV (lipid/protein molar ratio of 175) incubated at 37 °C and a phospholipid concentration of 0.15 mg/mL with 10-fold molar excess of acceptor vesicles were investigated. The various kinetic pools are considered in turn below.

(*A*) *Fast Transfer.* The pool that transfers rapidly was determined by measuring N_0 (at "zero" time) using eq 2. About 11% of the total [${}^{14}C$]RCM-AII in the donor SUV was transferred to acceptor vesicles within 1 min of mixing donor and acceptor particles. This corresponds to a fast pool size of 12%. Rechromatography of the donor SUV conducted at the same time as the transfer experiment (<1 week of storage of the SUV preparation at 4 °C) showed that all ${}^{14}C$ counts were associated with the donor SUV and <2% of the protein was free in solution. Similar results were obtained by rechromatography of other donor SUV preparations conducted 2 weeks after the first chromatography. This excludes the possibility that rapid association of apoprotein free in solution with acceptor vesicles could account for the measured N_0 fraction. The intervesicular transfer occurring on a time scale of seconds or less suggests the presence of a "fast pool" of RCM-AII associated with donor SUV. The rate constant of the fast transfer can not be measured by using our assay of transfer because of the short time scale (seconds). Using fluorescence quenching methods, McKeone and colleagues (McKeone et al., 1988) reported rapid kinetics of apo C transfer between SUV with $t_{1/2}$ values in the range 60–1180 ms.

(*B*) *Slow Transfer.* The pool (N_t^s) of [${}^{14}C$]RCM-AII that transfers slowly from donor to acceptor SUV was analyzed by using eq 2 and 3. Figure 2 shows a fitted curve that represents a single-exponential nonlinear fit of N_t^s as a function of time. The computed rate constant (k) and $t_{1/2}$ values (±SE) for the data in Figure 2 are 3.6×10^{-3} ($\pm 2.5 \times 10^{-4}$) min^{-1} and 192 (±12) min, respectively. The computed RCM-AII transfer equilibrium value (N_∞^s) for this preparation was 4300

Table I: Effects of the Number of RCM-AII Molecules per Vesicle on the Kinetics of Transfer between SUV[a]

no. of RCM-AII molecules per vesicle[b]	phospholipid/protein mole ratio[c]	% fast component[c,d]	slow component		
			%[c,d]	k (min^{-1})[e]	$t_{1/2}$ (min)[e]
15	190 (±15)	13 (±3)	69 (±10)	4×10^{-3} ($\pm 3 \times 10^{-4}$)	173 (±13)
4	680 (±55)	8 (±2)	73 (±14)	8.8×10^{-3} ($\pm 7 \times 10^{-4}$)	79 (±6)

[a] At 37 °C, with a 10-fold molar excess of acceptor vesicles and a donor phospholipid concentration of 0.15 mg/mL of incubation mixture. [b] Calculated from the lipid/protein mole ratios and a molecular weight of egg PC vesicles of 2.1×10^6 (see Methods). [c] Mean ±SD, calculated by using data from three different SUV preparations. [d] Percent of the total protein calculated as described under Methods. The remaining percent protein was not transferrable on the time scale of these experiments. The fast component values are significantly different ($p < 0.05$) while the slow component values are not significantly different ($p < 0.05$) when compared by the paired Student's t test. [e] ±SE, k values were computed by using nonlinear regression analysis (see Methods).

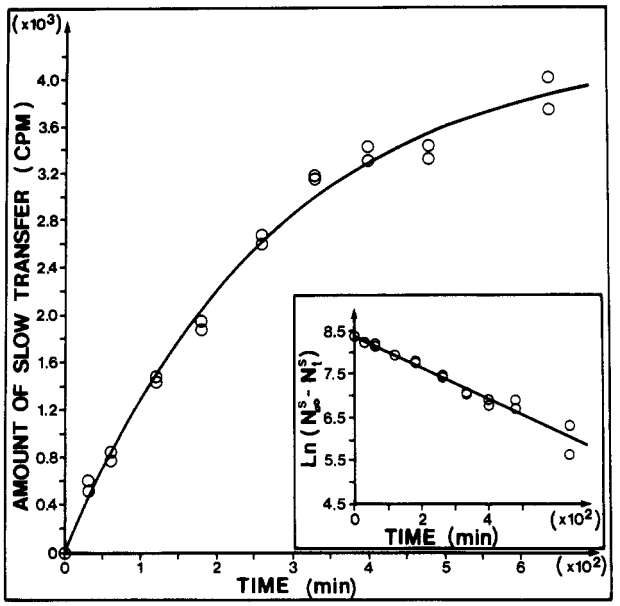

FIGURE 2: Kinetics of transfer of [^{14}C]RCM-AII from donor SUV (lipid/protein molar ratio of 175) incubated at 37 °C with a 10-fold excess of acceptor vesicles at a donor phospholipid concentration of 0.15 mg/mL of incubation mixture. The data points (N_t^s, see eq 3) were taken from duplicate experiments and fitted to a first-order rate equation using nonlinear regression analysis as described under Methods. The inset shows a plot of the natural logarithm of the ^{14}C counts remaining in the slow pool of the RCM-AII associated with donor particles ($N_\infty^s - N_t^s$) as a function of time.

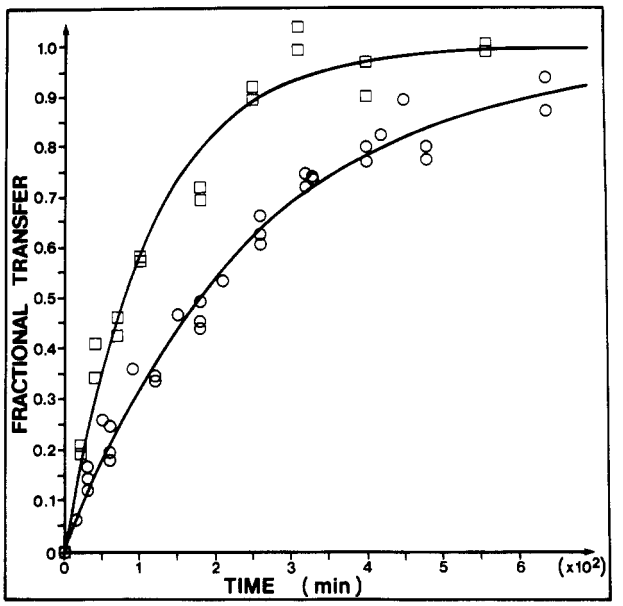

FIGURE 3: Fractional transfer of [^{14}C]RCM-AII from donor SUV with lipid/protein molar ratios of 190 ± 15 (○) and 680 ● 55 (□) (mean ± SD, $n = 3$) as a function of time. The donor vesicles were incubated at a phospholipid concentration of 0.15 mg/mL of incubation mixture with a 10-fold molar excess of acceptor vesicles at 37 °C. The solid lines represent the nonlinear regression of the data using a first-order rate equation as described under Methods.

± 150 cpm (±SE); this corresponds to a "slow pool" size of 61%. The transfer kinetics using different preparations of SUV under the above conditions were similar. The sizes of the fast and slow pools of RCM-AII in donor SUV at lipid/protein molar ratios of 190 ± 15 (±SD, $n = 3$) were 13 ± 3% and 69 ± 10%, respectively, with k and $t_{1/2}$ values for the slow pool of 4×10^{-3} ($\pm 3 \times 10^{-4}$) min^{-1} and 173 (±13) min, respectively (Table I). The slow transfer data were best fitted to a single-exponential decay function as evident from the plot of the natural logarithm of the ^{14}C counts remaining in the slow pool ($N_\infty^s - N_t^s$) as a function of time (inset, Figure 2). However, it should be noted that the [^{14}C]RCM-AII counts transferred at equilibrium from fast and slow pools ($N_0 + N_\infty^s$) account for about 66% of the total [^{14}C]RCM-AII counts in the donor SUV; this is less than the theoretical equilibrium value of 90.9% assuming that all the [^{14}C]RCM-AII in donor SUV is exchangeable.

The total remaining ^{14}C counts in the donor particles [$^{14}C_{mix} - N_t$; where N_t includes the fast pool and the ^{14}C counts transferred slowly at time t (see eq 2)] as a function of time were better fitted to a double-exponential decay function with two rate constants; the first rate constant (k_1) was similar to that computed by using the first-order rate equation for N_t^s data as a function of time (Figure 2), whereas the second rate constant (k_2) was very small compared to k_1 (about 2% of k_1) with a large standard error of estimate. Thus, k_2 would correspond to $t_{1/2}$ values of days/weeks which cannot be measured with accuracy using the time scale of our experiments. This pool is considered as apparently "nontransferrable". A detailed study of the kinetics of this pool would be difficult given the tendency of SUV to aggregate and fuse on storage.

Effects of Number of RCM-AII Molecules per Particle on the Kinetics of Transfer. Figure 3 shows the time course for the fractional transfer of [^{14}C]RCM-AII between SUV using donor particles with high and low lipid/protein molar ratios (190/1 and 680/1) under the conditions described in the legend. The number of RCM-AII molecules per donor particle and the corresponding sizes of the fast and slow pools as well as the k and $t_{1/2}$ values of slow transfer are listed in Table I. It is evident that a higher number of RCM-AII molecules per donor particle enlarges the fast pool without any significant effect on the size of the slow pool. It is also clear from Figure 3 and Table I that the rate constant (k) of RCM-AII slow transfer between SUV is decreased by increasing the number of RCM-AII molecules per particle.

The transfer data from a single experiment using donor particles with a lipid/protein molar ratio of about 100/1 (approximately 28 RCM-AII molecules per SUV) were consistent with the above results. The rate constant of slow

Table II: Effect of Acceptor/Donor Ratio on the Kinetics of RCM-AII Transfer between SUV[a]

acceptor/donor particle ratio	slow component[b]		
	%[c]	k (min^{-1})	$t_{1/2}$ (min)
10	69 (±6)	4.3×10^{-3} ($\pm 5 \times 10^{-4}$)	161 (±19)
20	69 (±5)	7.9×10^{-3} ($\pm 1 \times 10^{-3}$)	88 (±11)
30	67 (±3)	1.4×10^{-2} ($\pm 1.3 \times 10^{-3}$)	50 (±5)
40	73 (±3)	1.7×10^{-2} ($\pm 1.5 \times 10^{-3}$)	40 (±4)

[a] Data from duplicate experiments using donor vesicles containing about 14 RCM-AII molecules per vesicle. The transfer of RCM-AII was monitored under the conditions described in the legend to Figure 4. [b] The values (±SE) were computed by using nonlinear regression analysis (see Methods). [c] Percent of the total RCM-AII in donor particles (see Methods).

transfer was significantly decreased [$k = (2.1 \times 10^{-3}) \pm (9 \times 10^{-4})$ min^{-1}] compared to those of higher lipid/protein molar ratios (cf. Table I). The sizes of the fast and slow pools of transferrable RCM-AII (about 14% and 71%, respectively) were generally similar to those of donor SUV with a lipid/protein molar ratio of 190/1.

Effects of Acceptor/Donor SUV Ratio on the Kinetics of RCM-AII Transfer. The effects of acceptor/donor ratio on the kinetics of [^{14}C]RCM-AII transfer from donor particles containing about 14 RCM-AII molecules/particle (lipid/protein molar ratio ~200/1) were studied by using acceptor/donor SUV phospholipid ratios in the range from 10/1 to 40/1 and a donor phospholipid concentration of 0.15 mg/mL incubation mixture at 37°C. Figure 4 shows the fractional transfer of [^{14}C]RCM-AII as a function of time at the various acceptor/donor particle ratios. The sizes of the slow pool, and $t_{1/2}$ values at different acceptor/donor SUV ratios, are listed in Table II. It is evident that increasing the acceptor/donor SUV ratio significantly reduces the half-time of RCM-AII transfer from donor to acceptor vesicles but there is no change in the size of the RCM-AII pool which is transferred slowly. It should be noted that the sizes of the "slow pools" which are transferred to acceptor SUV at equilibrium are dependent on the acceptor/donor SUV ratio (see Methods); the values in Table II are corrected for the variations in acceptor/donor SUV ratios. It is also evident from Table II that in the range of acceptor/donor SUV ratios from 10/1 to 40/1, $t_{1/2}$ is inversely proportional to the acceptor/donor ratio. In addition, reducing the concentration of phospholipid in the incubation mixture increases the half-time of RCM-AII transfer between SUV. Thus, incubation of donor SUV at 37 °C with 10-fold molar excess of acceptor SUV at a donor phospholipid concentration of 0.075 mg/mL results in the transfer of RCM-AII with a k value of 1.7×10^{-3} min^{-1} ($\pm 6 \times 10^{-4}$); this corresponds to a $t_{1/2}$ of ~410 min compared to a value of 161 min when the donor phospholipid concentration is 0.15 mg/mL.

The effect of the donor/acceptor SUV ratio on the sizes of fast pools was not significant. The computed fast pool sizes at various acceptor/donor molar ratios were in the range 13–15%.

Effects of Temperature on the Kinetics of RCM-AII Transfer. The kinetics of [^{14}C]RCM-AII slow transfer at 37 and 50 °C were measured by using a donor SUV preparation of lipid/protein molar ratio = 175/1 (averages of three kinetic measurements at various incubation times were utilized). The computed k values at 37 and 50 °C were 3.6×10^{-3} ($\pm 2.5 \times 10^{-4}$) and 2.1×10^{-2} ($\pm 2 \times 10^{-4}$) min^{-1}, respectively; these correspond to $t_{1/2}$ values of 192 and 33 min, respectively. The sizes of the slow pools of RCM-AII associated with donor SUV were 61% and 79% at 37 and 50 °C, respectively. It is apparent that increasing the temperature increases the size of the slow pool which is transferred to acceptor SUV and decreases the $t_{1/2}$ of transfer. Similar effects of temperature on the transfer of [^{14}C]RCM-AII from donor SUV containing different levels of protein were observed. For instance, at a lipid/protein molar ratio of about 700/1, the sizes of the slow pools were approximately 71% and 82% at 37 and 55 °C, respectively; the $t_{1/2}$ values were 76 and 18 min, respectively. From these data, the activation energy of RCM-AII transfer derived from the Arrhenius equation is about 25 kcal mol^{-1}.

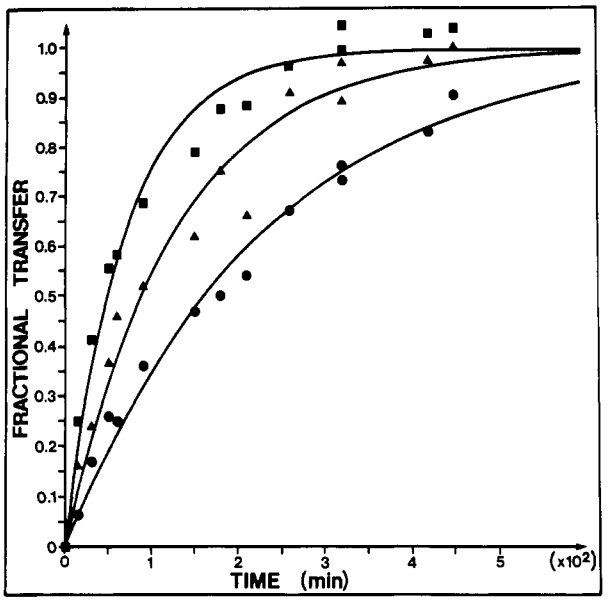

FIGURE 4: Fractional transfer of [^{14}C]RCM-AII at acceptor/donor SUV ratios of 10/1 (●), 20/1 (▲), and 30/1 (■). Data at an acceptor/donor SUV ratio of 40/1 are not shown for clarity. Donor particles containing about 14 RCM-AII molecules/vesicle (lipid/protein molar ratio of 200) were incubated at 37 °C with acceptor vesicles at the desired acceptor/donor particle ratio and at a donor phospholipid concentration of 0.15 mg/mL of incubation mixture. The solid lines represent nonlinear regression of the data using a first-order rate equation as described under Methods.

Discussion

Kinetic Pools of RCM-AII in Donor SUV. The present results suggest that there are fast, slow, and apparently "nontransferrable" kinetic pools of RCM-AII associated with donor SUV; transfers to acceptor SUV are on the time scales of seconds or less, minutes/hours, and days/weeks, respectively. For donor SUV containing about 15 RCM-AII molecules, the sizes of the fast, slow, and "nontransferrable" pools expressed as the percent of the total protein are 13, 69, and 18, respectively. A possible explanation for the existence of these pools is that the apoprotein adopts more than one conformation when bound to donor SUV. Apoprotein molecules in different conformations probably interact with lipids to different extents which in turn may affect the rate of their transfer from donor to acceptor SUV. It is likely that the fast kinetic pool represents apoprotein molecules which are relatively loosely bound to donor SUV. This protein is not removable from donor SUV upon dilution and gel filtration chromatography of the donor SUV preparation. The apoprotein in this pool may adopt a conformation in which most of the amino acid residues form loops or tails outside the plane of the lipid–water interface which facilitates the rapid transfer of this kinetic pool to acceptor SUV. It is apparent that the size of this kinetic pool of RCM-AII is dependent on the number of protein molecules per particle; at a low apoprotein content per donor SUV, the size of the fast kinetic pool is decreased (Table I). Crowding

of lipid and apoprotein molecules at the surface of donor SUV probably gives rise to more apoprotein molecules which are weakly adsorbed to the surface. The rate constant of apoprotein transfer from this kinetic pool can not be assessed by using our transfer assay because the rapid transfer occurs on a time scale of seconds or less. These findings are consistent with those reported recently by McKeone et al. (1988), who studied the transfer of apo C molecules between SUV using fluorescence quenching methods. Apo C was found to undergo spontaneous transfer between SUV; two kinetic components with $t_{1/2}$ values of about 70 and 700 ms were detected. The two pools were attributed to apo C molecules interacting differently with lipid, and both rates increased when the apo C content of the donor SUV was raised. This transfer presumably corresponds to that of the fast pool of loosely bound RCM-AII reported here. However, the sizes of the above kinetic pools of apo C as a percent of the total apo C in donor SUV were not reported.

The slow kinetic pool of RCM-AII associated with donor SUV probably represents protein molecules in a conformation that allows stronger interactions with lipid molecules. About 70% of the total RCM-AII exists in the slow kinetic pool which transfers to acceptor SUV with $t_{1/2}$ values of approximately 170 and 80 min for donor SUV containing 15 and 4 protein molecules, respectively (Table I). Molecular area considerations suggest that the faster rate of transfer at a lower protein content can be attributed to a lower packing density of the lipid molecules at the lipid–water interface compared to that at a higher protein content. This explanation is based on the observation that binding of RCM-AII to the donor SUV at various lipid/protein molar ratios does not change the size of SUV (r = 110 Å). The total surface area of the SUV is about 152 000 Å^2, and the number of lipid molecules in the outer monolayer of SUV is about 1900 molecules (calculated molecular weight = 2.1 × 10^6; two-thirds of the lipid molecules are assumed to be in the outer monolayer). The areas occupied by apoprotein molecules when 4 and 15 RCM-AII molecules are bound per donor SUV are about 4500 and 17 500 Å^2, respectively, assuming that the apoprotein exists in a conformation with an average area of 15 Å^2/residue (Ibdah & Phillips, 1988). It follows that the mean molecular areas of lipid molecules are about 71 and 77 Å^2/molecule at the surface of donor SUV containing 15 and 4 RCM-AII molecules, respectively. Thus, increasing the apoprotein content per SUV results in a concomitant compression of the lipid monolayer at the interface which in turn increases the lipid–protein interaction and causes a slower rate of apoprotein transfer from donor SUV to acceptor SUV. Studies of the adsorption of apoproteins to lipid monolayers show that apoproteins exert similar effects on the packing of lipid monolayers (Phillips & Krebs, 1986; Ibdah & Phillips, 1988).

The remaining apoprotein (18%) exists in a residual pool which is apparently nontransferrable on the time scale of a 12-h incubation of donor and acceptor SUV. Apoprotein molecules in this residual pool probably adopt a conformation that allows maximum interaction with lipid molecules. It appears that increasing the temperature of incubation to 50 °C results in a nearly complete transfer of this residual pool to acceptor SUV with kinetics similar to the slow pool. This can be explained at least partially by heat denaturation causing partial unfolding of apoprotein molecules so that apoprotein transfer to acceptor SUV is facilitated [cf. Reijngoud and Phillips (1984)].

Mechanism of Apoprotein Transfer between SUV. It is evident from the data listed in Table II that the $t_{1/2}$ of slow

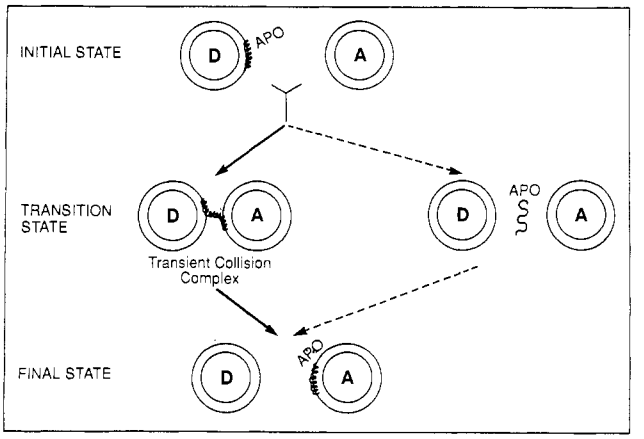

FIGURE 5: Illustration of the likely mechanism of apoprotein (APO) transfer from the slow kinetic pool between small unilamellar vesicles (SUV). Donor (D) and acceptor (A) SUV present in the aqueous medium collide (solid line) to form a transient ternary collision complex (donor SUV–APO–acceptor SUV) followed by dissociation of the complex with complete transfer of APO to acceptor SUV. The dashed lines indicate that a minor mechanism for the transfer of APO to acceptor SUV may involve desorption of APO from the donor SUV, diffusion through the aqueous phase, and adsorption to an acceptor SUV.

transfer of RCM-AII between SUV is inversely proportional to the acceptor/donor SUV ratio. This is consistent with the rate of apoprotein transfer being proportional to the frequency of collisions between the donor and acceptor SUV which suggests that the transfer of apoprotein molecules is mediated by SUV collisions. Figure 5 depicts a possible mechanism of apoprotein transfer between SUV. Partial desorption of apoprotein segments into the aqueous phase may facilitate the formation of ternary complexes (donor SUV–apoprotein–acceptor SUV) during collision of acceptor and donor SUV in which apoprotein is bound to both donor and acceptor SUV. Dissociation of this complex can be accompanied by a complete transfer of apoprotein molecules from donor SUV to acceptor SUV. However, a minor mechanism of apoprotein transfer between SUV may involve diffusion of protein molecules through the aqueous phase; this involves complete dissociation of apoprotein molecules from donor SUV before adsorption to acceptor SUV as illustrated in Figure 5. In this regard, McKeone et al. (1988) have suggested that transfer of apo C between SUV with $t_{1/2}$ < 1 s may occur by diffusion through the aqueous phase. The activation energy of 25 kcal/mol for the transfer of RCM-AII is comparable to values reported previously (Reijngoud & Phillips, 1984) for apoprotein dissociation from lipid bilayers. In terms of the transition state depicted in Figure 5, this activation energy is presumably associated with dissociation of sufficient segments of RCM-AII from the donor SUV so that the ternary complex can form.

It can be inferred from the present study that apoproteins may exist in multiple conformations at the surfaces of lipoprotein particles. This is in agreement with previous immunological studies which suggested that apo A-I adopts heterogeneous conformations at the surface of HDL particles (Ayrault-Jarrier et al., 1988). Apoprotein transfer is a complex process which is influenced by the conformation of the apoprotein, lipid–protein, and perhaps protein–protein interactions. The rates of apoprotein transfer are faster than the turnover times of lipoproteins which underlines the crucial role of apolipoprotein transfer in modulating the metabolism of lipoprotein particles in vivo. Further studies are needed to characterize the transfer of other apolipoproteins between SUV as well as between the various classes of serum lipoproteins.

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