Kinetics and Mechanism of the Spontaneous Transfer of Fluorescent Phosphatidylcholines between Apolipoprotein–Phospholipid Recombinants†

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ABSTRACT: The kinetics and mechanism of the spontaneous transfer of fluorescent phosphatidylcholines (PCs) between model lipoproteins were studied with PCs that contained 9-(1-pyrenyl)nonanoic acid in the *sn*-2 position and myristic, palmitic, stearic, oleic, or linoleic acid in the *sn*-1 position. The model lipoproteins were recombinants of apolipoproteins A-I (apoA-I) and A-II (apoA-II) from human high-density lipoproteins and the synthetic phospholipids dimyristoylphosphatidylcholine (DMPC) and 1-palmitoyl-2-palmitoleoylphosphatidylcholine (PPOPC). The kinetics of transfer of the fluorescent PCs were first order and independent of acceptor concentration and structure when recombinants of different apolipoprotein–phospholipid stoichiometries were used. The addition of neutral salts changed the rate of transfer by almost 3 orders of magnitude. The rates of transfer were also influenced by properties of the lipid–protein surface of the recombinant donor; transfer half-times were 5 times slower from a 240/1 than from a 45/1 DMPC/apoA-II recombinant. The rate of transfer of a pyrene-labeled PC was dramatically affected by the fatty acyl chain composition in the *sn*-1 position. From a 45/1 DMPC/apoA-II recombinant, the half-times of transfer were 9.6, 114, 630, 6.9, and 74.5 min for the myristoyl-, palmitoyl-, stearoyl-, linoleoyl-, and oleoylphosphatidylcholine derivatives, respectively. Thus, the addition of two methylene units to the acyl chains decreased the rate by a factor of 8–10 whereas the addition of a double bond increased the rate by a factor of 10. When a PPOPC matrix was used, the change was a factor of 5–7 per two methylene units and about 3–4 per double bond. Increases in the transfer rate with fatty acyl composition correlated with changes in the aqueous solubility of monomeric phospholipids that could be predicted from the critical micelle concentration of homologous lipids. The dramatic effects of salts, acyl chain length, and unsaturation on the rates of transfer are substantial proof that these phospholipids transfer as monomers via the aqueous phase. The transfer of the pyrenyl-PCs was highly temperature dependent. Activation energies for PC transfer with the host matrix in the liquid-crystalline state was 23–26 kcal/mol. By contrast, the activation energy decreased when the lipid matrix was in the gel state, and the magnitude of change in the enthalpy of transition from the gel to liquid-crystalline state correlated with the differences in the activation energy. Thus, the physical state of the lipid in the donor complex affects the magnitude of the activation energy. Because the spontaneous transfer of phospholipids can produce a net flux that is a function of the structure of both the lipid and the lipid–protein surface from which the lipid dissociates, this mechanism of equilibration can be quantitatively important for unsaturated or short-chain PCs.

The transfer and exchange of phospholipids, apolipoproteins, and free cholesterol are important aspects of human plasma lipoprotein metabolism (Smith et al., 1978). The exchange of phospholipids (PLs) between individual lipoprotein fractions has been demonstrated in vivo and in vitro, and that between lipoproteins, platelets, erythrocyte membranes, and a variety of other cell types and tissues has been demonstrated in vitro [for review, see Bell (1976) and Bruckdorfer & Graham (1976)]. Few studies have addressed the mechanism, kinetics, or specificity of phospholipid exchange; thus, its physiological role in lipoprotein metabolism remains undefined.

Both spontaneous and protein-mediated (Ihm et al., 1980) transfers are possible mechanisms of lipoprotein–phospholipid exchange. Two kinetically distinguishable mechanisms have been proposed for spontaneous transfer: (1) lipid transfers as monomers through the aqueous phase or (2) lipid transfers at the point of collision of two surfaces. A collision between two lipoproteins would have second-order kinetics and thus would be dependent on acceptor concentration and independent of the aqueous solubility of the exchanging lipid. Monomeric phospholipid exchanged through the aqueous phase would have first-order kinetics that are independent of acceptor concentration but dependent on the aqueous solubility of the lipid.

We have used the fluorescence properties of pyrene and pyrene-labeled lipids to measure the kinetics of phospholipid transfer. The excimer fluorescence intensity of pyrene is linearly related to its microscopic concentration so that fluorescence changes with net transfer of the lipid. Thus, the physical separation of donor and acceptor particles is not necessary. This procedure has been used to study the transfer between lipoproteins or phospholipid vesicles of pyrene (Charlton et al., 1976; Almgren, 1980) and several lipids containing a pyrenyl moiety including cholesterol (Kao et al., 1977), a diglyceride (Charlton et al., 1978), a fatty acid (Doody et al., 1980), and phospholipids (Roseman & Thompson, 1980; Massey et al., 1980b). All of these studies support the mechanism in which dissociation from the lipid surface is the rate-limiting step and transfer occurs through the aqueous phase. Studies on phospholipid transfer using other techniques support this mechanism (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Nichols & Pagano, 1981; McLean & Phillips, 1981; Papahadjopoulos et al., 1976). Factors that could regulate the rate of transfer, such as differing polar head groups, fatty acyl chain compositions, or properties of the lipid–protein surface containing a transferring species, have not been studied.

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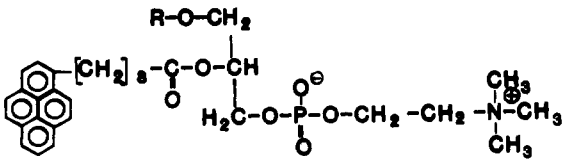

R=14 1-MYRISTOYL-2[9'(3'-PYRENYL)NONANOYL] PHOSPHATIDYLCHOLINE (MPNPC)
R=16 1-PALMITOYL- (PPNPC)
R=18 1-STEAROYL- (SPNPC)
R=18:1 1-OLEYL- (OPNPC)
R=18:2 1-LINOLEYL- (LPNPC)

FIGURE 1: Structures of the pyrene-labeled phosphatidylcholines used in this study.

With a well-defined model system, the mechanism, kinetics, and specificity of phospholipid transfer can be identified unambiguously. To this end, we have prepared apolipoprotein–phospholipid recombinants with well-defined compositions and structures (Massey et al., 1981a; Pownall et al., 1977, 1981). These model lipoproproteins have two advantages for phospholipid exchange. First, they are stable (Massey et al., 1981a,b); their structures do not change during the time course of the experiment. Second, there is no compartmentalization of lipids into an inner and outer monolayer as seen in single bilayer vesicles (Roseman & Thompson, 1980).

To study PC[1] transfer, we have synthesized phosphatidylcholines that vary in number of methylene units and degree of unsaturation (Figure 1). All contain 9-(1-pyrenyl)nonanoic acid in the *sn*-2 position and myristic, palmitic, stearic, oleic, or linoleic acid in the *sn*-1 position. The purpose of this report is to establish the mechanism of phospholipid transfer between model lipoproteins and to identify the factors that regulate the kinetics of this process.

Experimental Procedures

Materials. Apolipoproteins A-I and A-II were purified from human high-density lipoproteins (HDL) as previously described (Pownall et al., 1977; Massey et al., 1980a,b). 9-(1-Pyrenyl)nonanoic acid was either prepared by the method of Doody et al. (1980) or purchased from Molecular Probes (Plano, TX). PPOPC was prepared by acylation of the lysolecithin (Cubero-Robles & van den Berg, 1969). Dioleoyl-PC and dilinoleoyl-PC were prepared by the method of Patel et al. (1979). DMPC and 1-monopalmitoyl-, 1-monomyristoyl-, and 1-monostearoyl-PC were purchased from Calbiochem-Behring Corp. (La Jolla, CA). 1-Monooleoyl- and 1-monolinoleoyl-PC were prepared by digestion of the corresponding lecithin with phospholipase A_2 (*Crotalus adamanteus*) (Miami Serpenterium, Miami, FL).

The pyrene-labeled phosphatidylcholines were prepared by a modification of the procedure of Patel et al. (1979). Lysolecithin (50 mg) was heated in a Abderhalden drying apparatus (Fisher Scientific Co., Houston, TX) at 45 °C for 4 h and then dissolved in 1 mL of methylene chloride. To this, a 4-fold molar excess of the anhydride of 9-(1-pyrenyl)nonanoic acid was added in 1 mL of methylene chloride. After addition of 1 mol of 4-pyrrolidinopyridine, the mixture was heated overnight at 40–45 °C. The mixture was purified by silica gel chromatography eluted with a gradient of methanol in chloroform. The purity of the product was verified by migration of fluorescence and phosphorus (Dittmer & Lester, 1964) on thin-layer plates of silica gel eluted with chloroform–methanol–ammonia (65:34:4).

A molar extinction coefficient of 50 000 M^{-1} cm^{-1} at 347 nm was used to determine the concentration of the pyrenyl-PC (Pownall & Smith, 1973).

Methods. For the preparation of the apolipoprotein–phospholipid recombinants with fluorescent phospholipids, the desired mole fraction of the pyrenyl-PC in DMPC or PPOPC was dissolved in benzene. The benzene was evaporated and the lipid dispersed in buffer. ApoA-I and apoA-II spontaneously associate with DMPC to form well-characterized lipid–protein recombinants (Pownall et al., 1977; Massey et al., 1980a,b, 1981a; Jonas et al., 1977). The PPOPC/apoA-I recombinant[2,3] was prepared by cosolubilization of the protein and lipid in cholate after which the detergent was removed on a desalting column of Bio-Rad P-4. Unless specified, a standard buffer consisting of 10 mM Tris, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, and 1 mM NaN_3 was used.

The fluorescence of pyrene and many of its derivatives is a function of their microscopic concentrations (Birks, 1970). Pyrene has a highly structured fluorescence spectrum with wavelength maxima at less than 400 nm; this is defined as monomer emission (M). However, at higher concentrations of pyrene, a pyrene molecule in its first excited singlet state can, by a diffusion-controlled process, associate with a ground-state pyrene molecule to form an excited dimer or excimer (E). The excimer fluoresces with a wavelength maxima at 470 nm. The ratio of these intensities, E/M, is directly proportional to the pyrene concentration (Pownall & Smith, 1973), as defined by

$$\frac{E}{M} = \frac{\text{excimer fluorescence}}{\text{monomer fluorescence}} = \frac{[\text{pyrene}]Tk}{\eta} \quad (1)$$

where T is the temperature, k is a constant, and η is the viscosity. This property of pyrene and its derivatives can be used to monitor their transfer if a net concentration change occurs. In our assay, a pyrene-labeled PC is incorporated into a model lipoprotein. When unlabeled recombinants are added, the excimer fluorescence decreases as the PC transfers from the donor complex to the acceptor complex. In lipid transfer studies, the change in the E/M ratio is represented by a complex function (Roseman & Thompson, 1980) because the excimer fluorescence represents the concentration of lipid in

[1] Abbreviations: HDL, high-density lipoprotein; PC, phosphatidylcholine; SM, sphingomyelin; DMPC, dimyristoylphosphatidylcholine; T_c, gel–liquid-crystalline transition temperature; apoA-I, apolipoprotein A-I, the most abundant protein of human HDL; apoA-II, apolipoprotein A-II, the second most abundant protein of human HDL; PPOPC, 1-palmitoyl-2-palmitoleoylphosphatidylcholine; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] Throughout this paper the proportion of phospholipid to apolipoprotein is given as a molar ratio of lipid to protein.

[3] H. J. Pownall, Q. Pao, M. Rohde, and A. M. Gotto, Jr., unpublished results.

Table I: Physical Properties of Apolipoprotein-Phospholipid Recombinants

	recombinant[a]				
	DMPC/apoA-II	DMPC/apoA-II	DMPC/apoA-II	DMPC/apoA-I	PPOPC/apoA-I
stoichiometry[b]					
(mol of lipid/mol of protein) ± 5%	45/1	75/1	240/1	100/1	100/1
(g of lipid/g of protein) ± 5%	0.63	0.73	0.90	0.71	0.73
molecular weight	2.3×10^5	3.4×10^5	1.6×10^6	2.3×10^5 [e]	
Stokes' radius[d] (Å)	57 ± 5	65 ± 5	121 ± 5	62 ± 3	62 ± 3
transition temperature (T_c) (°C)	28.5	26.3	24.4		
transition enthalpy (kcal/mol)	0.95	2.1	3.9		

[a] Data for the DMPC/apoA-II recombinants are from Massey et al. (1981a,b). [b] Lipid/protein weight and molar ratios were determined by chemical analysis of complexes isolated by gel filtration. [c] Molecular weight taken from Jonas et al. (1977). [d] Stokes' radius determined by gel filtration chromatography as described by Massey et al. (1981a).

the donor recombinant, whereas the monomer fluorescence is in both the donor and acceptor recombinants. For our fluorescence measurements, a SLM Instruments Model 8000 photon-counting spectrofluorometer was used. Because of the extreme stability of this fluorometer, the direct measurement of only the excimer fluorescence is possible. Because excimer fluorescence represents only the concentration of the pyrenyl-PC in the donor complex, no other data manipulation is necessary. The temperature of the samples in the thermostated sample chamber was monitored by a Bailey Instruments digital thermometer (Model Bat 8) with thermocouple.

Results

The physical and chemical characteristics of the apolipoprotein–phospholipid recombinants of apoA-I and apoA-II with DMPC and of apoA-I with PPOPC used in this study are tabulated in Table I. The DMPC/apoA-II complexes varied in molecular weight from 230 000 for a 45/1 complex to 1 600 000 for a 240/1 complex. The apoA-I/phospholipid complexes were similar in size to the smaller apoA-II complexes. When MPNPC was incorporated into these complexes, a linear increase of the E/M ratio was found with increasing mole percent of the probe in the complex (eq 1) (Mantulin et al., 1981) up to 10 mol %. For the kinetic experiments, a concentration of the pyrenyl-PC of 5 mol % or less was used.

The results of a typical kinetic experiment are shown in Figure 2. The rate of transfer of MPNPC between DMPC/apoA-II recombinants was measured by the decrease in the excimer fluorescence at 470 nm and analyzed as a first-order process by

$$\ln\left(\frac{E_t - E_\infty}{E_0 - E_\infty}\right) = -kt \qquad (2)$$

where E_0 is the initial excimer fluorescence, E_∞ the fluorescence at equilibrium, and E_t the fluorescence at different times. As shown in Figure 2B, the analysis of the decrease in the excimer fluorescence was linear for over 99% of the reaction and gave a first-order rate constant (k) of 0.071 min and a half-time ($t_{1/2}$) of 9.6 min. Similar traces were found when other pyrene-labeled PCs and recombinants were used.

Concentration Dependence of MPNPC Transfer. As seen in Figure 3, the half-time of transfer of MPNPC between DMPC/apoA-II recombinants was independent of acceptor concentration over a 50-fold range. Also, there was no change in the half-time when complexes of different stoichiometries were used as acceptors. However, the half-time was dependent on the structure of the donor complex. The half-time of transfer changed from 9.4 min for transfer from a 45/1 complex to 55 min from a 240/1 complex.

Effect of Neutral Salts on MPNPC Transfer. Neutral salts changed the rate of transfer of MPNPC between DMPC/apoA-II (45/1 M/M) complexes by almost 3 orders of magnitude (Figure 4). The half-times varied from 1.2 min in 3 M NaSCN to 800 min in 3 M $MgCl_2$.

Dependence of Phospholipid Transfer on Fatty Acid Composition. For a 45/1 DMPC/apoA-II complex (Figure 5 and Table II) the half-times of transfer of MPNPC, PPNPC, and SPNPC were 9.6, 114, and 630 min, respectively. The addition

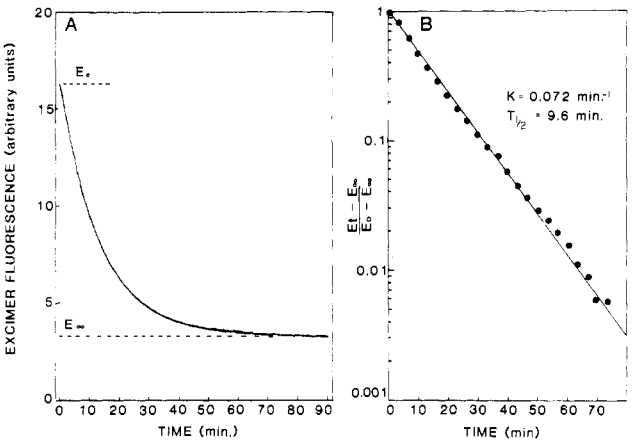

FIGURE 2: Kinetic traces of the exchange of MPNPC between DMPC/apoA-II recombinants (45/1 M/M). The exchange of MPNPC was measured by the decrease in excimer fluorescence at 470 nm. Panel A shows a kinetic trace obtained when a DMPC/apoA-II (45/1) recombinant (0.01 mg of lipid) containing MPNPC (5 mol %) was added to an excess of unlabeled recombinants (0.25 mg of lipid) in a final volume of 1 mL at 37.4 °C. Panel B shows a first-order plot (eq 2) of the data in panel A.

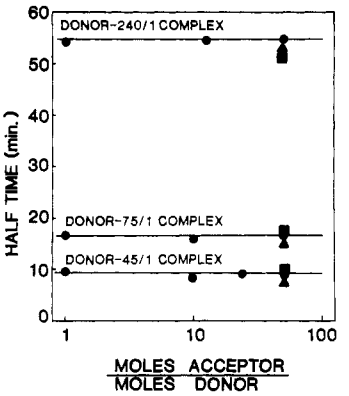

FIGURE 3: Effect of acceptor to donor ratio on the half-times of transfer of MPNPC. The rate of transfer of 5 mol % MPNPC out of 45/1, 75/1, and 240/1 DMPC/apoA-II recombinants to either a 45/1 (●), 75/1 (▲), or 240/1 (■) recombinant was measured at 37.0 °C as a function of the moles of phospholipid molecules in the acceptor complexes to moles of phospholipids in the donor complex. The concentration of acceptor recombinants was varied as indicated. The lines indicate the average value for the half-times.

Table II: Summary of Kinetic Data for the Transfer of Pyrenyl PCs between Model Lipoproteins

lipid	donor complex	transition temp (°C)	rate constant[a] (min^{-1})	half-time[a] (min)	temp range	activation energy[b] (kcal/mol)
MPNPC	DMPC/apoA-II (45/1)	30	0.071	9.8	$>T_c$	23.3
					$<T_c$	20.4
	DMPC/apoA-II (75/1)	30	0.042	16.5	$>T_c$	22.7
					$<T_c$	14.8
	DMPC/apoA-II (240/1)	29	0.0127	54.6	$>T_c$	23.0
					$<T_c$	10.3
PPNPC	DMPC/apoA-II (45/1)		0.0061	114	$>T_c$	23.3
MPNPC	DMPC/apoA-I (100/1)		0.0646	10.7	$>T_c$	23.2
MPNPC	PPOPC/apoA-I (100/1)		0.0895	7.8	$>T_c$	23.8
PPNPC	PPOPC/apoA-I (100/1)		0.0126	54.9	$>T_c$	26.2
SPNPC	PPOPC/apoA-I (100/1)		0.00257	269	$>T_c$	24.3
OPNPC	PPOPC/apoA-I (100/1)		0.00960	72	$>T_c$	22.3
LPNPC	PPOPC/apoA-I (100/1)		0.0373	18.6	$>T_c$	23.7

[a] The rate constants and half-times were measured at 37 °C. [b] The activation energies have an experimental error of ±5%.

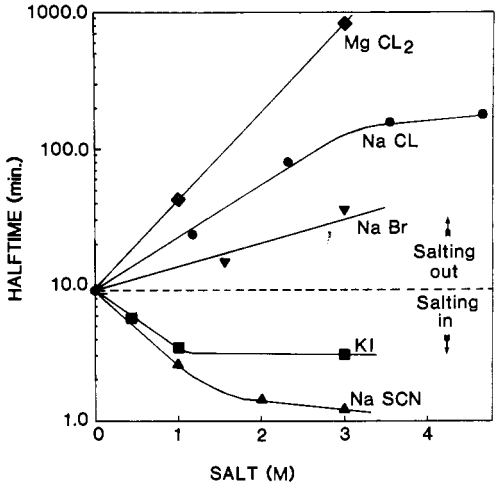

FIGURE 4: Effect of neutral salts on the half-time of transfer of MPNPC between 45/1 DMPC/apoA-II recombinants. Donor recombinants (0.01 mg) with 5 mol % MPNPC were mixed with acceptor recombinant (0.25 mg) at 37.0 °C at the indicated salt concentration.

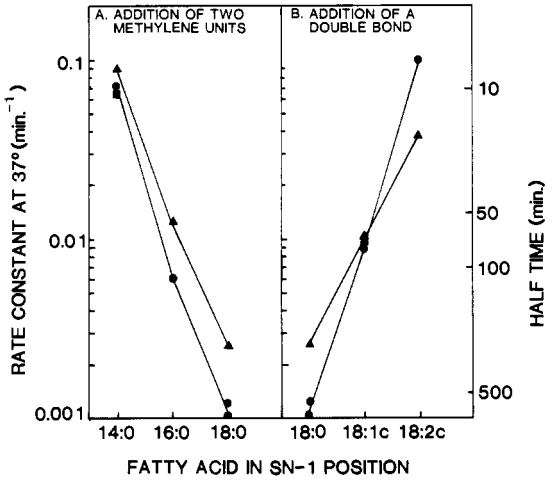

FIGURE 5: Effect of the fatty acid composition of a phospholipid on its rate of transfer between apolipoprotein–phospholipid recombinants. The donor recombinants were DMPC/apoA-II (45/1) (●), DMPC/apoA-I (100/1) (■), and PPOPC/apoA-I (100/1) (▲). Donor recombinants (0.01 mg) with 1 mol % pyrene-labeled PC in STD buffer at 37 °C were mixed with identical acceptor complexes (0.25 mg). In panel A, the fatty acyl composition in the *sn*-1 position of the pyrene-labeled PC contained a different number of methylene units (myristic, palmitic, and stearic acids). In panel B, the fatty acyl composition is varied in the number of double bonds (stearic, oleic, and linoleic acids).

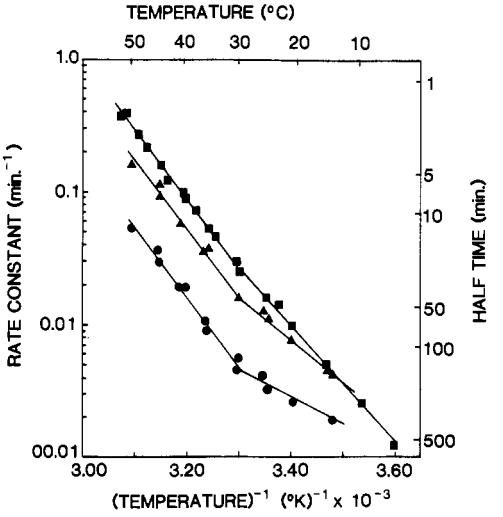

FIGURE 6: Arrhenius plots of the transfer of MPNPC between DMPC/apoA-II recombinants. The donor recombinants (0.01 mg/mL) (5 mol % MPNPC) were DMPC/apoA-II complexes with a stoichiometry of 45 to 1 (■), 75 to 1 (▲), and 240 to 1 (●). The acceptor recombinant was a 45/1 DMPC/apoA-II complex (0.25 mg/mL). A summary of the data is given in Table II.

of two methylene units dramatically decreased the rate of transfer. The change in the rate of the myristoyl derivative transferring between the DMPC/apoA-I recombinant, which has a molecular weight similar to that of the DMPC/apoA-II recombinant, was small (Table II). However, the rate of transfer of the saturated pyrenyl-PCs between apoA-I recombinants containing a lipid with an unsaturated acyl chain, PPOPC, was faster than that observed in those containing DMPC. The addition of a double bond to a PC increased its rate of transfer. As shown in Figure 5B, for the DMPC/apoA-II and the PPOPC/apoA-I recombinants the rate was increased by 10- and 4-fold, respectively. The rate of transfer of the unsaturated pyrenyl-PCs was slow from a PPOPC complex as compared to that of a DMPC complex.

Temperature Dependence of Phospholipid Transfer. Figure 6 contains Arrhenius plots of the transfer of MPNPC between DMPC/apoA-II recombinants. The plots were biphasic and intersected at temperatures which corresponded to the transition of lipid from gel to liquid crystalline in the complex. These data are summarized in Table II. For the three DMPC/apoA-II complexes, the activation energy (ΔE_a) was similar above the transition temperature (T_c). However, below T_c the activation energy decreased dramatically. The differences in ΔE_a below and above T_c for the 45/1, 75/1, and

240/1 complexes were 2.9, 7.9, and 12.7 kcal/mol, and the enthalpies of the thermal transition of the lipid as measured by differential scanning calorimetry were 0.95, 2.1, and 3.9 kcal/mol, respectively (Table I). There was a direct correlation between ΔE_a and the magnitude of change in the enthalpy of the gel to liquid-crystalline transition. Therefore, the physical state of the lipid in the complex affected the magnitude of the activation energies. As shown in Table II, the activation energies above T_c for the transfer of all five fluorescent lipids out of the different model lipoproteins were within experimental error.

Discussion

The physical exchange or transfer of phospholipids has been demonstrated between lipoproteins and between lipoproteins and cells (Bell, 1976; Bruckdorfer & Graham, 1976). To study phospholipid transfer, we have synthesized phosphatidylcholines with a pyrenyl moiety (Figure 1). These PCs exhibit several characteristic properties of more physiological lecithins. For example, they are substrates for human plasma lecithin:cholesterol acyl transferase, phospholipases A_2 (*Crotalus adamanteus*), C (*Bacillus cereus*), and D (cabbage), and the beef liver and plasma phospholipid exchange proteins (J. B. Massey and H. J. Pownall, unpublished results).

Mechanism of Transfer. Several lines of experimental evidence support the hypothesis that the pyrene-labeled phospholipids transfer as monomers through the aqueous phase. First, the kinetics of transfer of the pyrenyl-PCs are a first-order process (Figure 2). Second, the rate of transfer of the PC is independent of the concentration of the acceptor over a 50-fold range (Figure 3) or of the structure of the acceptor recombinant. Third, the rates of transfer are dependent upon the properties of the donor. The rate of transfer of MPNPC from different DMPC/apoA-II complexes can vary by a factor of 5 (Figure 3 and Table II). The fatty acyl composition of the bulk phospholipid of the donor recombinant also affects the rate of transfer (Table II and Figure 5). In all cases, the composition of the donor recombinant affects the rate of transfer; the rate-limiting step is dissociation of the phospholipid from the lipid–protein surface. The PC, once in the aqueous phase, can readily associate with an acceptor lipid structure by an essentially diffusion-controlled process, and net transfer of the lipid occurs at a rate dependent upon the aqueous solubility of the monomeric phospholipid.

Effect of Neutral Salts. The addition of neutral salts changes the rate of transfer of MPNPC almost 3 orders of magnitude (Figure 4). The relative effectiveness of neutral salts in changing the solubility of nonpolar compounds in aqueous solution follows the empirical Setschenow equation

$$\log S = -K_s C_s + \log S_0 \qquad (3)$$

where S_0 and S are the solubility of the solute in water and in the electrolyte solution, K_s is a salting-out coefficient characteristic of the salt, and C_s is the molal concentration of the salt solution. From eq 3, if the half-time is dependent on the aqueous solubility of the exchanging lipid, then the half-time should be a function of the salt concentration. The salting-out coefficient can be written as

$$-K_s = \frac{\log (S/S_0)}{C_s} = \frac{\log [t_{1/2}(\text{salt})/t_{1/2}(0)]}{C_s} \qquad (4)$$

where $t_{1/2}$(salt) and $t_{1/2}(0)$ are the half-time in the presence and absence of salt, respectively. Horvath & Melander (1977) have proposed that the molal surface tension increment (σ) of a salt can be used as a lyotropic series. For our purposes, we use the molal surface tension increment as a numerical Hoffmeister series. The relative effectiveness of the salt (Setschenow constant) which we calculate from eq 4 and the linear portions of the curves in Figure 4 closely follow this lyotropic series. For the salts NaSCN, KI, NaCl, NaBr, and $MgCl_2$, the values of K_s are −1.3, −0.99, 0.36, 0.82, and 1.5 per mol/L and the values of the molal surface tension increment are 0.6×10^3, 0.84×10^3, 1.32×10^3, 1.64×10^3, and 3.16×10^3 [dyn·g/(cm·mol)], respectively. The correlation of the K_s and σ values in both order and magnitude indicate a general salting-in/salting-out phenomenon. We conclude that the changes in the half-times for the transfer of MPNPC between DMPC/apoA-II recombinants are due to a change in the aqueous solubility of the transferring lipid.

Fatty Acyl Chain Dependence. The chemical composition of the fatty acyl chains of a phospholipid can also affect its aqueous solubility (Tanford, 1980). The aqueous concentration of a phospholipid in equilibrium with a lipoprotein is related to that lipid's critical micelle concentration (cmc). As shown in Figure 5, the rate of transfer is dramatically affected by the fatty acyl chain composition in the *sn*-1 position. The addition of two methylene units to a pyrenyl-PC in a 45/1 DMPC/apoA-II recombinant decreases the rate by a factor of 8–10, whereas the addition of one double bond increases the rate by a factor of 10. For the myristoyl derivative transferring between DMPC/apoA-I recombinants, the rate is not significantly different. However, the rate of transfer of the saturated pyrenyl-PC between apoA-I recombinants having a lipid with an unsaturated chain, PPOPC, is much faster. The rate of transfer of the unsaturated pyrenyl-PC has an increase per double bond of approximately four. This change per double bond is what would be expected from the cmc measurements of unsaturated lipids. The dramatic effect of salts, acyl chain length, and unsaturation on the rate of transfer is substantive proof that phospholipids transfer through the aqueous phase. Furthermore, the transfer of PCs by mixing during the formation of a transient collision complex would require identical transfer rates for PCs which differ by two methylene units or a double bond because their lateral diffusion into the confluent region would be identical. Also, dramatic changes with PC chemical composition would not be expected to result from the transfer of the fluorescent phospholipids by dissociation and transfer of phospholipid–apoprotein "subunits" (Grow & Fried, 1978). Finally, due to the stability of our recombinants, transfer by a fusion mechanism is negligible (Massey et al., 1980a,b, 1981a,b).

Dependence of Lipid–Protein Surface. The rate of transfer of the fluorescent lipids is dependent upon the structural and chemical properties of the lipid–protein surface from which it dissociates (Figure 3 and Table II). Fluorescence polarization measurements indicate that the increased apoA-II to DMPC ratio correlated with increased order in the lipid array (Mantulin et al., 1981). The more rigid the lipid matrix of a recombinant, the faster the rate of transfer of MPNPC. A possible explanation of the differences in rates of transfer related to donor structure is that the phospholipid may partition more into the aqueous phase and less into the lipid phase because of a loss of configurational freedom in the more rigid lipid matrix. This relative entropic loss causes an increase in the aqueous concentration of the PC and thus an increase in rate of transfer. The differences in rate when a saturated pyrenyl-PC is in a saturated (DMPC) or unsaturated matrix (PPOPC) as compared to the unsaturated pyrenyl-PC could be due to an unfavorable entropic effect such as nonideal mixing of saturated and unsaturated fatty acyl chains. The

saturated pyrenyl-PCs would partition less into an unsaturated matrix as compared to a saturated matrix, and thus would transfer faster (Figure 5). Other factors of the lipid–protein surface, such as radius of curvature, could also affect the rate. A dependence of the rate of transfer of lipophilic compounds and the radius of a vesicle (Almgren, 1980) or lipoprotein (Charlton & Smith, 1981) has also been suggested to modify the rate. The rate is slowest from the larger recombinants. However, if recombinants of quite similar Stokes' radii (Table I) are used, this concept does not predict the difference for the saturated and unsaturated phospholipid matrixes. Because of the interdependence of recombinant size, lipid–protein stoichiometry, and phospholipid fluidity, the precise physical and chemical properties of the lipid–protein surface that influence the rate of transfer are difficult to identify. However, changes in rate due to the structure of the recombinant are still smaller than those seen for the addition of two methylene units to the fatty acyl chains of the transferring lipid.

Temperature Dependence of Transfer. The transfer of the pyrenyl-PCs is highly temperature dependent. Above the gel to liquid-crystalline transition of the bulk phospholipid in the recombinants, the activation energies range from about 23 to 26 kcal/mol (Table II). For MPNPC, ΔE_a does not change significantly when complexes of DMPC or PPOPC are used. For other lipids, ΔE_a values of 16 (Phillips et al., 1980) and 19.3 kcal/mol (Poznansky & Czekanski, 1979) have been measured for cholesterol in phospholipid vesicles, and values of 16.6 ($T < 36$ °C) and 11.6 kcal/mol ($T > 36$ °C) have been measured for a pyrene-labeled diglyceride from HDL (Charlton et al., 1978). In the transfer of monomeric surfactants of sodium alkyl sulfate between micelles (Aniansson et al., 1976) and of alkyl pyrenes between phospholipid vesicles (H. J. Pownall and L. C. Smith, unpublished results), an increase in the acyl chain length of the lipid gives an increase in the activation energy of about 750 cal/mol per methylene unit. Thus, it is reasonable that the more hydrophobic lipids used in this study have higher activation energies. The change in ΔE_a with acyl chain length is not distinguished in this study because the predicted increase in ΔE_a per methylene unit is small relative to the measured ΔE_a.

Below the thermal transition of DMPC in the apoA-II/DMPC complexes, the activation energy decreases dramatically. There is a direct correlation between the magnitude of change in the enthalpy of the gel to liquid-crystalline transition and ΔE_a. The properties of the physical state of the lipid in the complex affect the magnitude of the activation energy. This effect was also found for the transfer of 9-(1-pyrenyl)-nonanoic acid between DMPC vesicles (Doody et al., 1980). For the 240/1 complex, the differences in ΔE_a above and below T_c is 12.7 kcal/mol whereas the ΔH of melting is 3.9 kcal/mol. Because of the absence of a quantitative correlation between these two parameters, factors other than lipid chain crystallization, i.e., the possible freezing out of an impurity (pyrenyl-PC) (Doody et al., 1980), must also attribute to the difference in ΔE_a. The dissociating lipid should move perpendicularly to the phospholipid surface, such that the hydrophobic fatty acyl chain must pass through the polar head-group region which contains bound water and ions. The unfavorable interactions between the hydrophobic acyl chains and this interfacial region probably cause the activation energy to be high and to depend on the size of the hydrophobic part of the molecule. The amount of bound water in DMPC vesicles is significantly less in the gel state (at 20 °C, the bound water is 0.053 ± 0.063 g/g of lipid) as compared to the liquid-crystalline state (at 30 °C, the amount of bound water is 0.580 ± 0.037 g/g of lipid) (Watts et al., 1978) such that a decrease in the size of the interfacial region below T_c should cause a decrease in ΔE_a, in agreement with the experimental results. Other factors which contribute to ΔE_a would be the activation energy for diffusion of a monomeric molecule in solution that occurs in diffusion-controlled processes (Aniansson et al., 1976; Phillips et al., 1980) and the activation energy for the lateral diffusion of a molecule into the vacancy left by the dissociated phospholipid (Phillips et al., 1980). However, neither of these processes should be affected by the size of the hydrophobic region of the molecule.

Several lipophilic compounds such as pyrene (Charlton et al., 1976; Algrem, 1980), cholesterol (Kao et al., 1977; Phillips et al., 1980; McLean & Phillips, 1981), fatty acids (Doody et al., 1980; Sengupta et al., 1976), diglycerides (Charlton et al., 1978), micelle-forming surfactants (Aniansson et al., 1976), and phospholipids (Roseman & Thompson, 1980) appear to transfer between micelles, vesicles, or lipoproteins by a mechanism of dissociation from the lipid surface into the aqueous phase, where net transfer occurs when the lipid rapidly associates with another lipid aggregate. However, the half-time of transfer can vary from less than milliseconds to days for these lipids. Experimental data suggesting that the rate of transfer depends upon the aqueous solubility of the lipid can readily explain this phenomenon. Lipids such as fatty acids with a high aqueous solubility will transfer rapidly whereas lipids with a low solubility could be extremely slow (Israelachvili et al., 1980). Thus, in a physiologic setting, the sparingly soluble lipids such as fatty acids, cholesterol, and lysolecithin would be expected to transfer as monomers via the aqueous phase, whereas the more insoluble lipids such as cholesteryl ester, most phospholipids, and triglycerides probably require specific exchange proteins. The activities of exchange proteins for phospholipids (Ihm et al., 1980), cholesteryl esters (Pattnaik et al., 1978), and triglycerides (Rajaram et al., 1980) have been detected in plasma. Because spontaneous transfer can result in a net flux that is a function of the chemical structure of the lipid and the lipid–protein surface from which the lipid dissociates, it could be an important mechanism in the distribution of unsaturated or short-chain PC whereas longer chain or saturated PC should require an exchange protein.

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Mechanism of Phosphorylation Catalyzed by Chloroplast Coupling Factor 1. Stereochemistry†

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ABSTRACT: The reaction mechanism and substrate specificity of soluble chloroplast coupling factor 1 (CF_1) from spinach were determined by using the purified isomers of chromium–nucleotide complexes either as substrates for the enzyme or as inhibitors of the Ca^{2+}-dependent ATPase activity. The isolation of CrADP([^{32}P]P_i) formed upon the addition of the enzyme to [^{32}P]P_i and Λ-bidentate CrADP and the observation that the Λ-bidentate CrADP epimer was 20-fold more effective in inhibiting the Ca^{2+}-dependent ATPase activity than was the Δ epimer suggest that the substrate of phosphorylation catalyzed by CF_1 is the Λ-bidentate metal ADP epimer. Tridentate CrATP was hydrolyzed by soluble CF_1 to CrADP(P_i) at an initial rate of 3.2 μmol (mg of CF_1)$^{-1}$ min^{-1}, indicating that the tridentate metal ATP is the substrate for ATP hydrolysis. From these results a mechanism for the phosphorylation of ADP catalyzed by coupling factor 1 is proposed whereby the bidentate metal ADP isomer associates with the enzyme, phosphate inserts into the coordination sphere of the metal, and the oxygen of the β-phosphate of ADP attacks the inorganic phosphate by an S_N2 type reaction. The resulting product is the tridentate ATP ligand.

Soluble coupling factor 1 (CF_1),[1] the extrinsic membrane protein from spinach chloroplasts, neither has the ability to phosphorylate ADP nor will it catalyze appreciable rates of ATP–P_i or ADP–ATP exchange following isolation (Carmeli & Racker, 1973). Verification that CF_1 is responsible for energy transduction in higher plants has relied, therefore,

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CrADP(P_i), (phosphato)chromium adenosine 5′-diphosphate; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Chl, chlorophyll; PEI, poly(ethylenimine); RuBP, ribulose bisphosphate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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