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Pyrenedodecanoylcarnitine and Pyrenedodecanoyl Coenzyme A: Kinetics and Thermodynamics of Their Intermembrane Transfer†

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ABSTRACT: The intermembrane transfer kinetics and transition-state thermodynamics of pyrenedodecanoylcarnitine (PDC), pyrenedodecanoyl coenzyme A (PDCoA), and pyrenedodecanoic acid (PDA) were measured by observing the time-dependent decay in pyrene excimer fluorescence. Probe molecules transferred more slowly with an increase in vesicle size. Rates of PDC and PDA transfer were increased from a liquid lipid phase when compared to a gel phase, while a saturated lipid phase had variable effects on the transfer kinetics when compared to an unsaturated lipid vesicle. Increasing vesicle surface charge by the introduction of phosphatidylserine (PS) into the vesicle matrix had two distinct effects: (i) a decrease in PDC transfer rates as the PS concentration increased and (ii) an initial increase in transfer rates of the amphiphilic anions PDA and PDCoA, followed by a decrease as the PS content increased. Transfer from natural membranes (cardiac and hepatic reticular and mitochondrial membranes) was markedly decreased (up to 35-fold) when compared to large phospholipid vesicles. These decreases in rates were accompanied by significant increases in the transition-state free energies. Finally, the pyrenedodecanoate esters had critical micelle concentrations similar to the natural long-chain esters, i.e., palmitate. In the presence of acceptor vesicles all probes showed only slight accessibility to quenching by the aqueous quencher nicotinamide.

The relationship between the biochemical mechanisms controlling the oxidation/esterification processes and the physical interaction of lipid metabolites with their natural matrix, i.e., cellular membranes, is important to the study of lipid metabolism. The amphipathic properties of active lipid metabolites including fatty acids, fatty acyl-CoA, and fatty acylcarnitine and the membrane-bound nature of the enzymes of their metabolism necessitate their interaction in specific cell membrane environments. The location and concentration of these molecules in cell membranes must be related to the metabolic state of the cell, amphiphile availability, and the probability of metabolite–membrane interaction. Therefore, a thorough description of fatty acid metabolism requires a delineation of the physical processes controlling the kinetics of movement and thermodynamics of association of fatty acid metabolites between the various intracellular membrane compartments.

Pyrene esters of fatty acids represent one group of probe molecules that allows direct observation of amphiphile movement via concentration-dependent excimer fluorescence (Doody et al., 1980; Roseman & Thompson, 1980). With this method the rates of intervesicular transfer of pyrenedodecanoic acid and its coenzyme A and carnitine derivatives can be measured. The thermodynamics of the activated state are derived from absolute rate theory. Differences in transfer kinetics can then be correlated with differences in the structure of the transferring amphiphile and the microenvironment from which it is transferred.

Materials and Methods

Materials. Pyrenedodecanoic acid was purchased from Molecular Probes Inc., Junction City, OR. Dicyclohexylcarbodiimide and 4-pyrrolidinopyridine were obtained from Aldrich Chemical Co., Milwaukee, WI. All phospholipids were obtained from Avanti Polar Lipids Inc., Birmingham, AL. Carnitine was obtained from Tridom-Fluka Inc., Hauppaugge, NY. Coenzyme A and nicotinamide were purchased from Sigma Chemical Co., St. Louis, MO, and Sephadex G-50 was obtained from Pharmacia Fine Chemicals Inc., Piscataway, NJ. All other reagents were of standard laboratory grade.

Synthesis of Probes. Pyrenedodecanoyl coenzyme A (PDCoA) and pyrenedodecanoylcarnitine (PDC) were synthesized from pyrenedodecanoic acid (PDA) by using the procedure of Patel et al. (1979) as previously described (Wolkowicz et al., 1982). Briefly, the fatty acyl anhydride was prepared in distilled tetrahydrofuran by mixing PDA (0.3 mmol) and dicyclohexylcarbodiimide (0.15 mmol). The dicyclohexylurea precipitate was removed by filtration and the filtrate added to coenzyme A or carnitine suspended in warm

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Me_2SO. Following complete mixing the catalyst 4-pyrrolidinopyridine was added, and the reaction was allowed to proceed for 4 h. The products were isolated according to the method of Bremer (1968). Both compounds gave a single fluorescent spot ($R_f = 0.55$) on thin-layer chromatographic analysis (Bremer, 1968; Pullman, 1973).

Preparation of Vesicles and Natural Membranes. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) donor vesicles were prepared by ethanol injection (Batzri & Korn, 1973). Following removal of the excess alcohol from the vesicle suspension by the method of Fry et al. (1978), the final phospholipid concentration was 2 mM. Large diameter POPC vesicles were prepared by cholate dialysis (Brunner et al., 1976). POPC acceptor vesicles, dipalmitoylphosphatidylcholine (DPPC), or 1-phosphatidylserine (POPS) donor vesicles were prepared from a 2 mM solution of multilamellar liposomes by sonication using a Branson Model 350 sonicator set at 50% full power. Sonication was carried out for approximately 30 min, followed by centrifugation of the vesicle suspension at 100000*g* for 60 min in a Beckman 60-Ti rotor. Titanium fragments and residual lipids were discarded, and the upper portion of the resulting supernatant was used in these studies. All vesicles were prepared in a standard solution of 100 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.2, 1.0 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM NaN_3.

Dog heart mitochondria were isolated by using polytron homogenization (McMillin-Wood et al., 1980; Palmer et al., 1977). Dog heart sarcoplasmic reticulum was prepared according to the procedure of Entman et al. (1973). Rat liver mitochondria were isolated as previously described (Wolkowicz & McMillin-Wood, 1980). Rat liver endoplasmic reticulum was obtained by centrifugation of the postmitochondrial supernatant at 100000*g* for 60 min (Moore et al., 1975). Protein was determined by using the biuret method (Layne, 1957). Membrane protein concentration was converted to membrane phospholipid concentration as previously described (Parsons et al., 1967; Weglicki et al., 1970).

Carnitine–palmitoyl-CoA transferase activity was determined on isolated mitochondria in the presence or absence of PDCoA or PDC (Bremer & Norum, 1967). The effect of PDCoA and PDC on ADP-stimulated mitochondrial respiration was determined as previously described (Wolkowicz et al., 1982).

Physical Measurements. Critical micelle concentration (cmc) was determined by measuring the loss of excimer fluorescence with decreasing pyrene probe concentration as described by Galla et al. (1979). The fluorescence of each derivative was measured on a Perkin-Elmer Model SP-40 spectrofluorometer. Samples were excited at 344 nm, and monomer emission (I_M) was recorded at 395 nm while excimer fluorescence (I_E) was measured at 475 nm. Slit widths were 2 nm for excitation and 5 nm for emission. All static experiments were conducted at room temperature.

Stern–Volmer Measurements. The accessibility of derivatives was determined by addition of either 200 nmol of vesicle phospholipid or membrane protein equivalent to this amount of phospholipid to 1 mL of standard solution. In experiments where rat liver mitochondria were the acceptor matrix, 100 nmol of phospholipid was used. Two nanomoles of probe molecule was then added from 1 mM stock solutions. Following a 5-min equilibration period, aliquots from a 1 M stock solution of the water-soluble fluorescence quencher nicotinamide were added, and the change in monomer fluorescence intensity was measured. Accessibility values were derived from

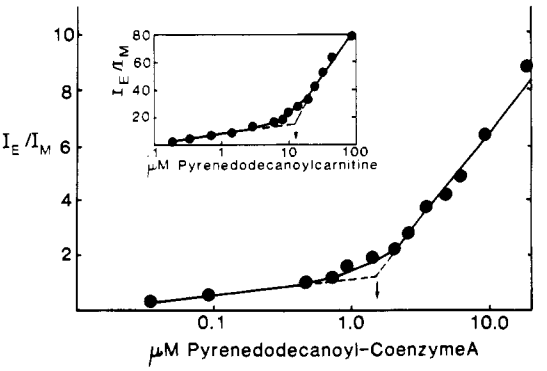

FIGURE 1: Critical micelle concentrations: pyrenedodecanoyl-CoA and pyrenedodecanoylcarnitine. Critical micelle concentrations of pyrenedodecanoyl-CoA and pyrenedodecanoylcarnitine (inset) are determined from pyrene excimer to monomer fluorescence intensity (I_E/I_M). All determinations were carried out at 23 °C and pH 7.2.

the Stern–Volmer constants as described by Lakowicz & Hogen (1980). Changes in solution volume equivalent to the maximum volume of nicotinamide added (i.e., 5%) caused no change in the I_M.

Kinetic Methods. Rapid kinetic measurements were made on a Durrum stopped-flow fluorescence spectrophotometer using an excitation wavelength of 344 nm and an emission cutoff filter of 475 nm, the excimer wavelength maximum. The slit widths were 3.5 nm. Between 5 and 10 decay curves were collected and their rate constants averaged.

In a typical experiment, a solution of 100–200 nmol of membrane phospholipids containing 2–4% probe molecule was rapidly mixed with an equal volume of a solution containing a 10-fold excess of unlabeled POPC acceptor vesicles. This concentration of acceptor vesicles was used to maximize signal change and to reduce the contribution of reverse transfer processes. The reaction temperature was maintained by a Neslab Endocal RTE 9DD constant-temperature circulating bath. Data were collected on a Biomation Model 805 waveform recorder and transferred to an Apple II Plus computer. The decay curves were analyzed according to an equation of the form

$$E_t = E_\infty + A \exp(-kt)$$

where E_t is the measured excimer fluorescence at time t, E_∞ is the excimer fluorescence at $t = \infty$, k is the first-order rate constant, and A is the difference between E_0 and E_∞. Activated-state parameters were obtained from linear regression analysis of the Arrhenius plots using the equations of Glasstone et al. (1941).

In experiments with isolated natural membrane systems, 2 mol % probe was added to 200 μM total membrane phospholipid as described under accessibility measurements. Rat liver mitochondrial phospholipid content was 100 μM due to solution capacity at higher concentrations. Low PDA excimer fluorescence in mitochondrial membranes prevented accurate assessment of transfer rates with this probe.

Statistical analysis was carried out by Student's t test for independent samples. The level of significance was set at $P < 0.05$. All values are expressed as means ± standard deviation.

Results

The critical micelle concentration (cmc) of both derivatives was determined by changes in the excimer to monomer ratio by extrapolation of the two linear portions of this ratio to their intersection point. The cmc for PDCoA is approximately 1.5 μM (Figure 1) and 16 μM for PDC (Figure 1, inset). These

Table I: Accessibility of PDA, PDCoA, and PDC to Nicotinamide Quenching[a]

probe	solution	POPC vesicles	rat liver mitochondria	dog heart mitochondria	endoplasmic reticulum	sarcoplasmic reticulum
PDA	100	2.1	1.0	1.0	1.1	0.5
PDCoA	100	4.4	1.5	0.6	6.4	1.3
PDC	100	2.6	0.3	0.2	0.6	0.4

[a] Accessibility values (expressed as percent) were obtained from the initial slopes of the Stern–Volmer plots. The concentration of probe used in the solution quenching experiments was 0.2 μM. All other values were obtained by using 2 μM probe and 200 μM phospholipid for all membranes except for rat liver mitochondria, where 100 μM phospholipid was used. Conditions were identical with the standard incubation conditions, and nicotinamide was added from a 1 M stock solution in standard solution (see Materials and Methods). Following a 1-min incubation at each nicotinamide concentration the fluorescence intensity at 395 nm (344-nm excitation) was recorded.

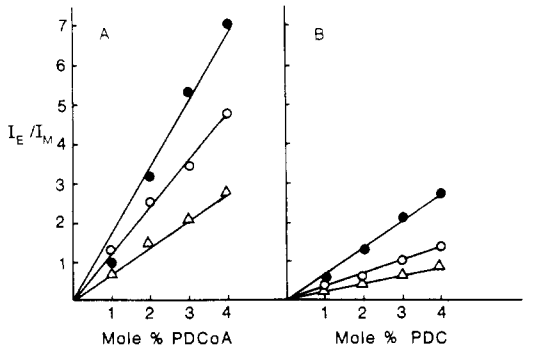

FIGURE 2: Relationship between excimer and monomer fluorescence intensity ratios (I_E/I_M) and pyrenedodecanoyl ester concentrations in artificial and natural membranes. Membrane solutions are composed of either 200 μM POPC (cholate) vesicles (●), 100 μM rat liver mitochondrial phospholipid (100 μmol of phospholipid/0.5 mg of protein) (○), or 200 μM rat liver microsomal phospholipid (200 μmol of phospholipid/0.5 mg of protein) (△). Solutions containing appropriate membranes are titrated with probes (PDCoA, left panel; PDC, right panel) as described under Materials and Methods.

values are consistent with the cmc values of the respective palmitate esters (Yalkowsky & Zografi, 1970; Zahler et al., 1968).

The biochemical activity of these compounds is similar to that reported for the pyrenebutyryl analogues (Wolkowicz et al., 1982). PDCoA is a competitive inhibitor of the carnitine–acyl-CoA transferase with a K_I of 2.74 ± 0.42 μM and of the adenine nucleotide translocase (K_I = 0.16 ± 0.03 μM). Palmitoylcarnitine-supported respiration in rat liver mitochondria is inhibited by PDC with an observed I_{50} of 2.2 ± 0.5 μM at 10 μM palmitoylcarnitine. This inhibition has been shown to occur at the carnitine–acylcarnitine translocase. PDC is not a substrate for respiration (Wolkowicz et al., 1982).

One of the experimental advantages of pyrene derivatives is the linear relationship between microscopic concentration and excimer fluorescence intensity (Pownall & Smith, 1973). Both PDCoA (Figure 2A) and PDC (Figure 2B) exhibit linear increases in excimer fluorescence over the experimental concentration range in both artificial and natural membranes. This linearity was also observed for PDA in all membrane systems studied. Therefore, measured changes in the excimer intensity are proportional to changes in the concentration of membrane-bound probe molecules.

Fluorescence Quenching. An assumption in the kinetic analysis of changes in excimer fluorescence intensity is the membrane-bound nature of the molecules studied. This may be tested by determination of Stern–Volmer quenching constants using a water-soluble quencher (e.g., nicotinamide) in the presence or absence of a membrane acceptor (Lakowicz & Hogen, 1980). In the presence of POPC vesicles only 2.6% of added PDC at concentrations well below its cmc is accessible to a water-soluble quencher (Figure 3 and Table I). With dog heart mitochondria the accessibility is even less (Figure 3). All three probes are essentially inaccessible to nicotinamide

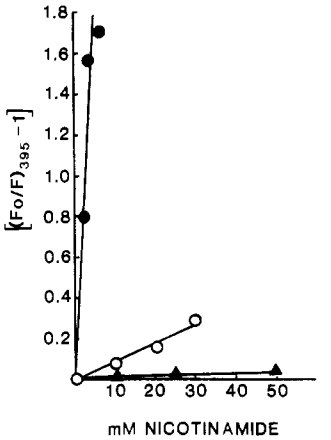

FIGURE 3: Accessibility of PDC to quenching by nicotinamide. PDC monomer intensity at 0.2 μM (●, solution) or 2 μM in the presence of 200 μM POPC vesicles (○) or dog heart mitochondria (0.53 mg of protein/200 μM phospholipid) (▲) is measured as a function of the nicotinamide concentration in solution. The probe is equilibrated for approximately 5 min at 23 °C with the membrane-containing solution, following which nicotinamide is serially added, mixed, and allowed to equilibrate 2–3 min, and then the change in monomer intensity is measured. In the case of solution quenching, nicotinamide is serially added and equilibrated and the change in monomer intensity measured. The Stern–Volmer relation is calculated as described by Lakowicz & Hogen (1980).

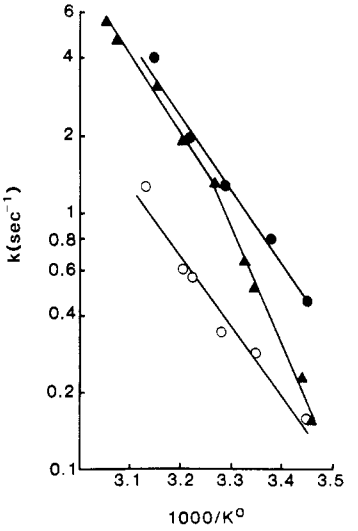

FIGURE 4: Arrhenius plots of intravesicular transfer of pyrenedodecanoylcarnitine. Donor vesicles at 200 μM concentration (●, POPC; ▲, DPPC; ○, POPC, cholate) containing 2 mol % PDC are mixed with an equal volume of 2.0 mM POPC acceptor vesicles. The decrease in excimer intensity is measured as described under Materials and Methods.

quenching in the membranes studied (Table I). These results indicate a membrane location for all three probes and satisfy one of the assumptions concerning the membrane microenvironmental origin of excimer fluorescence. Subsequent

Table II: Transition-State Thermodynamics of PDA, PDCoA, and PDC Intervesicular Transfer[a]

	PDA			PDCoA			PDC		
	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal/mol)
POPC (large)	+8.7	−34.4	+18.9 ± 0.08[b]	+12.3	−19.0	+18.0 ± 0.07[b]	+11.6	−22.0	+18.2 ± 0.10[b]
POPC (small)	+10.2	−27.7	+18.5 ± 0.05	+16.2	−4.8	+17.6 ± 0.04	+13.3	−14.1	+17.5 ± 0.08
DPPC (liquid)	+5.6	−42.1	+18.2 ± 0.05[b]	+11.3	−21.0	+17.5 ± 0.08	+11.8	−19.0	+17.4 ± 0.07
DPPC (gel)	+14.53	−13.7	+18.6 ● 0.06[c]	+15.6	−6.6	+17.6 ● 0.08	+20.1	+7.5	+17.9 ± 0.09[b,c]

[a] Transfer reactions were conducted as noted under Materials and Methods. Activated-state parameters were derived from previously described equations (Glasstone et al., 1941). Results represent the average of 5–10 determinations. [b] $P < 0.005$ when $\Delta G^{\ddagger}$ values are compared to small POPC vesicles. [c] $P < 0.0005$ when $\Delta G^{\ddagger}$ values of DPPC (liquid) and DPPC (gel) are compared. Other values are not significantly different.

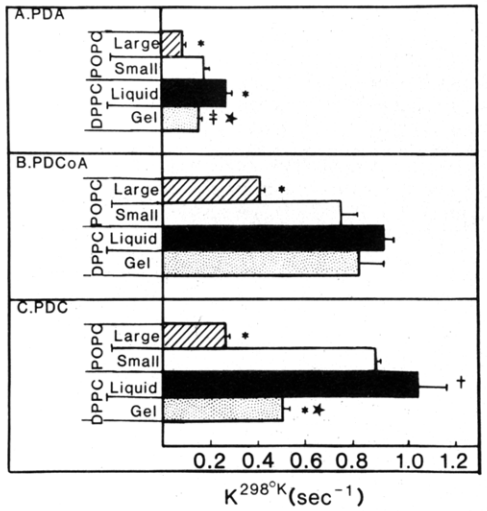

FIGURE 5: Effect of vesicle size, bilayer saturation, and fluidity on the rates of PDA, PDC, and PDCoA intervesicular transfer. Rates of probe intervesicular transfer at 25 °C are taken directly from the Arrhenius plots when 200 μM POPC as donor vesicles (2 mol % probe) is used with 2 mM POPC acceptor vesicles. Values reported are the average and standard deviation of 5–10 measurements. DPPC (liquid) rate constants are obtained by extrapolating to 25 °C from the best-fit line determined from the liquid region by linear regression analysis ($\bar{r} = 0.979$). *, $P < 0.0005$; †, $0.005 > P > 0.0025$; ‡, $0.01 > P > 0.005$; significance of transfer rates compared to small POPC vesicles. ★, $P < 0.0005$; significance of transfer rates between DPPC (liquid) and DPPC (gel).

changes in this parameter reflect changes in probe concentration in the membrane.

Transfer Kinetics. In all cases the observed transfer rates follow first-order kinetics. Typical Arrhenius profiles of probe transfer are shown in Figure 4. Rate constants of transfer (either determined at or extrapolated to 25 °C) for PDA, PDCoA, and PDC were derived from small and large POPC donor vesicles and DPPC vesicles in the gel or liquid-crystalline states (Figure 5). An increase in vesicle size significantly decreases the rate of probe transfer. The largest relative decreases are observed with PDC and PDA, followed by PDCoA (Figure 5). The free energies of activation ($\Delta G^{\ddagger}$) derived from the Arrhenius plots for these probes exhibit a consistent pattern of behavior (Table II). For all probe molecules the decreased transfer rates induced by a larger vesicle diameter are accompanied by significant increases in $\Delta G^{\ddagger}$. For PDC and PDA, transfer from a vesicle of larger diameter causes a decrease in both the enthalpy of activation ($\Delta H^{\ddagger}$) and the entropy of activation ($\Delta S^{\ddagger}$) toward more negative values. Likewise, PDCoA transfer from a vesicle of larger diameter is accompanied by substantial decreases in both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

The rate of PDA transfer from POPC vesicles is lower than that from DPPC (liquid) donor vesicles (Figure 5). This decreased rate of transfer is reflected in an increased value of $\Delta G^{\ddagger}$ of transfer when POPC and DPPC (liquid) donors are

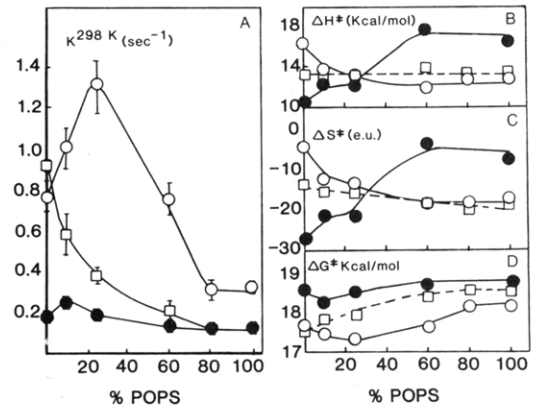

FIGURE 6: Effect of percent vesicle POPS concentration on the kinetics and transition-state parameters of PDA, PDC, and PDCoA intervesicular transfer. Rates of excimer fluorescence decay were determined with 200 μM donor vesicles of varying POPS composition containing 2 mol % PDCoA (○), PDC (□), or PDA (●) and pure POPC acceptor vesicles. Values represent the average and standard deviation of 5–10 measurements. The activated-state parameters are calculated according to Glasstone et al. (1941).

compared (Table II). In contrast to the POPC (cholate) results, decreasing the rate of PDA transfer, i.e., compare DPPC (liquid) to POPC (small), Figure 5 and Table II, causes an increase in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ to more positive values. Likewise, PDC undergoes a small but significant decrease in its rate of transfer when DPPC (liquid) and POPC vesicles are compared. However, the value of $\Delta G^{\ddagger}$ did not differ significantly between these two types of vesicles (Table II). In addition, the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for PDC transfer from DPPC (liquid) vesicles are similar in magnitude to the values observed with POPC–cholate vesicles although the rates of transfer differed 3-fold (Figure 5 and Table II). The rate or $\Delta G^{\ddagger}$ of PDCoA transfer was not significantly changed by the saturation state of the vesicle lipid matrix.

PDA and PDC transfer rates are markedly decreased by the change from a liquid-crystalline to a gel state using DPPC donor vesicles. PDCoA transfer rates are not significantly affected by this change in the donor vesicle matrix (Figure 5). However, the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of transfer for all three probes change toward more positive values independent of the change in the rates of probe transfer (Table II). For PDA and PDC the $\Delta G^{\ddagger}$ for the slower, gel-phase transfer is significantly increased over the more rapid liquid-crystalline-state transfer (Table II).

Natural membranes contain significant concentrations of negatively charged phospholipids (Innis & Clandinin, 1981; Farese et al., 1983). To determine the effect of increasing negative surface charge on the kinetics and thermodynamics of transfer, rates of probe transfer from POPS donor vesicles to pure POPC acceptor vesicles were determined. As vesicle POPS concentration is increased, PDC transfer rates show a continuous decline in value (Figure 6A). This decrease is accompanied by a decrease in $\Delta S^{\ddagger}$ at high POPS concentra-

Table III: Kinetics and Transition-State Thermodynamics of PDA, PDCoA, and PDC Intervesicular Transfer between Natural Membranes[a]

	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal/mol)	k (s^{-1}) at 25 °C	$t_{1/2}$ (s) at 37 °C
PDA					
+cholate	+8.7	−34.3	+18.9 ± 0.08	0.084 ± 0.009	4.47
+endoplasmic reticulum	+5.32	−46.4	+19.2 ± 0.04[b]	0.056 ± 0.003[b]	8.06
+sarcoplasmic reticulum	+9.2	−33.0	+19.1 ± 0.11	0.068 ± 0.012	5.06
PDCoA					
+cholate	+12.3	−19.0	+18.0 ± 0.07	0.40 ± 0.046	0.63
+endoplasmic reticulum	+4.9	−46.5	+18.7 ± 0.06[b]	0.116 ± 0.011[b]	4.20
+sarcoplasmic reticulum	+6.4	−42.7	+19.1 ± 0.05[b]	0.061 ± 0.005[b]	7.07
+rat liver mitochondria	+7.6	−40.0	+19.5 ± 0.05[b]	0.029 ± 0.003[b]	13.59
+dog heart mitochondria	+7.1	−41.7	+19.6 ± 0.02[b]	0.029 ± 0.001[b]	15.40
PDC					
+cholate	+11.65	−22.0	+18.2 ± 0.01	0.27 ± 0.029	1.22
+endoplasmic reticulum	+6.3	−42.9	+19.1 ± 0.06[b]	0.065 ± 0.007[b]	6.54
+sarcoplasmic reticulum	+10.3	−28.9	+18.9 ± 0.06[b]	0.086 ± 0.009[b]	4.56
+rat liver mitochondria	+7.5	−40.4	+19.6 ± 0.09[b]	0.029 ± 0.004[b]	13.60
+dog heart (liquid) mitochondria	+4.1	−51.3	+19.4 ± 0.05	0.035 ± 0.003[b]	13.60

[a] Transfer reactions were conducted as noted under Materials and Methods. Activated-state parameters were derived from previously published equations (Glasstone et al., 1941). Values for $t_{1/2}$ were determined from k (s^{-1}) values by using the first-order rate equation $t_{1/2}$ (s) = 0.693/k (s^{-1}). Results represent the average of 5–10 experiments. [b] $P < 0.0005$ when $\Delta G^{\ddagger}$ or K is compared to POPC–cholate vesicles.

tions (Figure 6C), while $\Delta G^{\ddagger}$ undergoes a steady significant increase in value (Figure 6D). PDCoA and PDA transfer rates are affected in a biphasic manner by POPS (Figure 6A). Both PDCoA and PDA show initial significant increases in their rates of transfer ($P < 0.001$ and $0.01 > P > 0.005$, respectively) followed by a decrease as the percent POPS vesicle composition increases. However, a higher amount of POPS is required for a decreased rate of PDCoA transfer to be observed. The enthalpies of activation demonstrate a reciprocal relationship between PDA and PDCoA (Figure 6B). Whereas $\Delta H^{\ddagger}$ increases for PDA transfer as a function of POPS, the $\Delta H^{\ddagger}$ for PDCoA transfer demonstrates a steady decline. The $\Delta S^{\ddagger}$ values decrease for PDCoA and increase (i.e., become less negative) for PDA as POPS composition increases (Figure 6C). The $\Delta G^{\ddagger}$ values initially decrease and then increase for both PDA and PDCoA as POPS composition increases (Figure 6D).

Natural membranes have a higher affinity for probe association than the artificial vesicles (Table I) and are physiologically more relevant to membrane–amphipath interactions. Therefore, the kinetics and thermodynamics of PDC, PDCoA, and PDA transfer from natural membranes were measured. The rate constants for probe transfer from all natural membranes are significantly lower than those observed in the large POPC vesicles (Figure 7 and Table III). The rates of PDC and PDCoA transfer are decreased by as much as 94%, e.g., PDCoA transfer from rat liver mitochondria. Transfer from endo- or sarcoplasmic reticulum is decreased when compared to POPC–cholate vesicles but to a lesser degree (Table III).

The activation energies derived from the Arrhenius profiles exhibit a pattern similar to that observed for the rate constants. In general, the activation energies for these transfers are decreased. The two exceptions are PDC transfer from the "gel" phase of dog heart mitochondria (data not shown, but see Figure 7) and PDA transfer from sarcoplasmic reticulum.

The transition-state parameters derived from the Arrhenius plots demonstrate a consistent phenomenon. If the transfer rate significantly decreases, in all but one instance, the $\Delta H^{\ddagger}$ values and $\Delta S^{\ddagger}$ are considerably lower than their POPC–cholate counterparts. The one exception is PDC in the "gel" phase from dog heart mitochondria where the transition-state parameters approximate the POPC–cholate values but the transfer rate is still suppressed. For PDA transfer from sarcoplasmic reticulum both the rates of transfer and the transition-state parameters are similar to the POPC–cholate values.

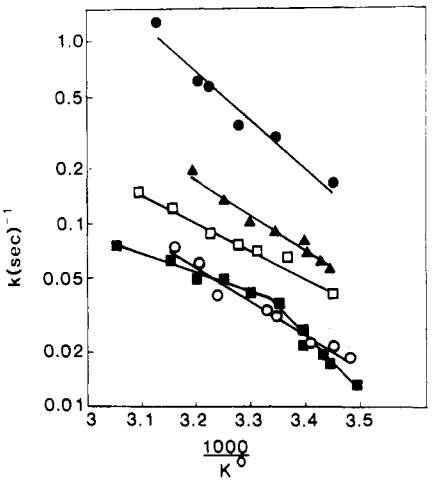

FIGURE 7: Arrhenius plots of PDC transfer between natural membranes or POPC (cholate) vesicles. All points are the average of 5–10 measurements. Donor membranes (200 μM phospholipid or 100 μM phospholipid in the case of rat liver mitochondria) are mixed with 2 mM POPC acceptor vesicles. Donor membrane protein to lipid ratios are previously given (see Figures 2 and 3) except for dog heart sarcoplasmic reticulum which is 0.3 mg of protein/200 μM phospholipid. Donor membranes contain 2 mol % PDC:POPC (cholate) vesicles (●), dog heart mitochondria (■), rat liver mitochondria (○), dog heart sarcoplasmic reticulum (▲), and rat liver endoplasmic reticulum (□).

Discussion

The location of amphipathic metabolites in the cell is regulated by their site of synthesis and by the affinity of these molecules for different intracellular membranes. Specific properties such as lipid unsaturation or fluidity, surface charge, and curvature are likely cellular factors that control amphiphile distribution. The pyrenedodecanoyl esters of carnitine and coenzyme A employed in this study are almost completely confined to the microenvironment of artificial and natural membranes as demonstrated by accessibility measurements. Since the excimer fluorescence of these pyrene molecules in the bilayer is a linear function of their concentration, the intensity of excimer fluorescence can be employed to follow changes in membrane concentration of the pyrenedodecanoate esters of coenzyme A and carnitine. The decay of excimer fluorescence is a first-order process and, therefore, is independent of pyrene concentration in either the artificial or natural bilayers employed. These measurements provide in-

formation concerning properties of bilayer structure that control the residence time of the natural analogues within any membrane microenvironment.

Transition-state kinetics have been employed to provide a quantitative theoretical model for amphiphile transfer from donor vesicles (Doody et al., 1980). Although the differences in $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ are difficult to assign, there is a relationship between $\Delta G^{\ddagger}$ and ΔG_t, the free energy of transfer of an amphiphile from a lipid environment to water (Pownall et al., 1983). Previous studies suggest that the activated state involved in amphiphile transfer is either in the aqueous bulk phase or in an interfacial aqueous region near the phospholipid head groups with properties similar to bulk phase water (Pownall et al., 1983). Therefore, an increase in aqueous phase solubility of the aliphatic esters should result in increased rates of transfer and a concomitant decrease in the transition-state free energy of transfer from a hydrocarbon environment to water. This effect results from formation of additional aqueous hydrogen bonds upon transfer of amphipaths to the water phase. This general principle is evident in the present observations where the rates of transfer of the more water soluble carnitine and CoA esters are 3.5–5 times more rapid than that of the free acid.

Further examination of the property of amphiphile solubility on rates of transfer of PDCoA and PDC predicts that the carnitine derivative should transfer more rapidly from donor vesicles by virtue of its approximate 10-fold higher water solubility. Since the dynamics of micellization is similar to the process of amphiphile transfer from a hydrophobic environment to water, then the free energy of transfer of an amphipath from a "hydrocarbon" environment to water may be approximated by $\Delta G_t = -RT \ln [i]$, where [i] is equivalent to the critical micelle concentration of the probe molecule in water (Pownall et al., 1983; Tanford, 1980). The free energy of transfer of PDCoA is anticipated to be at least 1.4 kcal higher than that of PDC concomitant with a decreased rate of transfer. However, no consistent pattern of the amphiphile transfer rates on ΔG_t is observed between the two derivatives.

Some differences in the association of PDCoA and PDC with the hydrophobic interior of the bilayer may contribute to the observed similar rates of transfer. We have previously demonstrated that the carnitine derivative is less readily removed from azolectin bilayers by bovine serum albumin than is pyrenebutyryl-CoA (Wolkowicz et al., 1982). Since carnitine exists as a zwitterion at neutral pH, it is likely that PDC is able to penetrate the hydrophobic interior of the membranes in a manner similar to that of the protonated form of the free acid. In this case, cocrystallization or association of PDC with the acyl chains of phospholipids could limit its rates of transfer and increase the $\Delta G^{\ddagger}$ for transfer. Conversely, smaller lipid-phase effects should be observed with the CoA ester, which is anchored in the water phase by virtue of its anionic head group. This speculation is supported by measurement of transfer rates and activation energies of the three probes from POPC and DPPC gel- or liquid-phase vesicles. Both the acylcarnitine and the free acid demonstrate rates of transfer in the gel phase that are significantly decreased from the rates measured in the liquid phase, whereas these rates are not significantly changed in the case of acyl-CoA. Values of $\Delta G^{\ddagger}$ have been reported to be higher for alkylpyrenes and protonated acylpyrenes as compared to the transition-state terms for amphiphilic pyrenes (Pownall et al., 1983). This suggests that the rates of transfer of PDC may be limited by localization of a significant proportion of these molecules to the interior of the bilayer.

In addition to effects of membrane fluidity on rates of transfer, the surfaces of biological membranes are heterogeneous with respect to phospholipid head group structure and charge. Cell membranes contain varying concentrations of negative phospholipids, which may contribute to the degree of amphiphile interaction with the bilayers as well as to the subsequent desorption process. PDCoA and PDA rates of transfer increase with low concentrations of POPS in POPC vesicles. The effect is larger for the CoA derivative than for the free acid. Since the free acid is transferred as an anion, and since CoA exists as an anion at the experimental pH, these enhanced rates of transfer may be a consequence of ionic repulsion. As the vesicle composition is increased to 100% POPS, the transfer rates of both pyrenedodecanoyl-CoA and the acid decrease to values below "control" rates (0% POPS). This biphasic effect on transfer may be due to multiple effects at the vesicle surface as a result of negative charge enrichment, i.e., decreased interfacial pH, increased ion–ion repulsion, decreases in surface hydration, formation of the ionic double layer, and possible changes in vesicle size with increasing POPS (Tsien & Hladky, 1982; Fernandez & Fromherz, 1977). The effects of increasing bilayer charge on transfer of the zwitterionic PDC cannot be directly interpreted, and significant, steady decreases in transfer are observed.

The organellar radii existing in the cell differ by 1–3 orders of magnitude from liposomes prepared by sonication or ethanol injection. It has been demonstrated that the rate of transfer of lipids from a membrane surface is inversely related to the radius of the donor vesicle (Charlton & Smith, 1982). Therefore, the influence of vesicle size on the transfer of pyrene-labeled amphiphiles is essential in correlative studies with isolated subcellular organelles. As anticipated, all derivatives transferred more slowly from vesicles prepared by cholate dialysis. However, in isolated natural membranes of comparable size (1000–2000 Å), the rates of transfer of all compounds were further reduced in comparison to the cholate rates. These data suggest that protein constituents of the membrane may exert significant additional effects on amphiphile transfer. A concomitant significant increase in the $\Delta G^{\ddagger}$ of transfer from natural membranes over that seen for cholate vesicles further implies restriction of interaction of these amphiphiles with bulk phase or interfacial water. Because of the high affinity of selected membrane proteins for these metabolites, e.g., the adenine nucleotide translocase and carnitine acylcarnitine translocase for the CoA and carnitine derivatives, respectively, it is probable that some specific protein interaction contributes to these observations. By comparison, only small effects on $\Delta G^{\ddagger}$ are observed with transfer of the free acid from cholate vesicles compared to reticular membrane. This suggests that PDA transfers from environments that are similar in both natural and artificial membranes. We would expect this environment to be the bulk membrane phospholipid.

In summary, we have demonstrated that phospholipid fluidity, composition, and charge play important roles in control of metabolic amphiphile association with membrane systems. It is also likely that, in addition to size effects on transfer rates, the nature of the ester group involved in intermediary metabolism is recognized by protein constituents of the natural membranes to limit its solubility in the aqueous phase of the cell.

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Registry No. PDA, 73451-05-5; PDCoA, 93255-33-5; PDC, 93255-34-6; POPC, 6753-55-5; DPPC, 2644-64-6.

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Erythrocyte Band 3 Protein: Evidence for Multiple Membrane-Crossing Segments in the 17 000-Dalton Chymotryptic Fragment†

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ABSTRACT: We have investigated the topology of the band 3 protein of the human erythrocyte membrane by a combination of chemical labeling and proteolytic cleavage. The N-terminal third of the membrane-bound domain of band 3 is a 17 000-dalton chymotryptic fragment that is known to traverse the membrane an odd number of times. At least three lysine residues on this fragment can be labeled by reductive methylation of intact cells, under conditions that cause labeling of exofacial, but not intracellular, lysine residues. One of the labeled lysines is the one that reacts with anionic aryl isothiocyanates, and another is very close to the C terminus of the fragment. Both these are on the C-terminal 11-kilodalton CNBr peptide. The third labeled lysine is on the 6-kilodalton N-terminal CNBr peptide, which had not been previously known to have an extracellular site. Control experiments using a stilbenedisulfonate derivative demonstrate that the labeled 6-kilodalton CNBr peptide is not a degradation product of the 11-kilodalton CNBr fragment. Also, the exofacial lysine on the 6-kilodalton peptide can be labeled by reductive methylation even when the stilbenedisulfonate site is occupied by 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate, which blocks band 3 mediated transport of BH_4 into the cells. This is further indication that the labeled lysine is accessible from the extracellular water. These data are the first direct evidence that the 17-kilodalton chymotryptic fragment spans the membrane more than once.

Band 3 is a 95 000-dalton polypeptide that constitutes over half the integral membrane protein of human red blood cells (Fairbanks et al., 1971). The protein consists of an N-terminal water-soluble cytoplasmic domain of M_r 43 000 (Steck et al., 1976; Fukuda et al., 1978; Steck et al., 1978) and a glycosylated membrane-bound domain of M_r 52 000 (Markowitz & Marchesi, 1981; Jenkins & Tanner, 1977; Steck et al., 1978). The function of the membrane domain is to transport anions (Cl and HCO_3 physiologically) by way of an obligatory one-

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