Conformational Order of Specific Phospholipids in Human Erythrocytes: Correlations with Changes in Cell Shape†

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ABSTRACT: Acyl chain perdeuterated dimyristoylphosphatidylcholine (DMPC-d_{54}) and dimyristoylphosphatidylserine (DMPS-d_{54}) were incorporated into human erythrocytes. Light microscopy demonstrated that erythrocytes incubated with an equimolar mixture of DMPC-d_{54}/DMPS or DMPC/DMPS-d_{54} remained mostly discocytic whereas cells incubated with either DMPC-d_{54} or DMPS-d_{54} alone became echinocytic or stomatocytic, respectively. Cells in which the aminophospholipid translocating protein was inhibited became echinocytic when incubated with DMPS-d_{54}. Fourier transform infrared (FTIR) spectroscopy was used to monitor conformational order in the acyl chains of the incorporated phospholipid, as detected through the asymmetric CD_2 stretching vibrations in the intact cells. In cells incubated with equimolar mixtures of DMPC-d_{54}/DMPS or DMPC/DMPS-d_{54}, the deuterated species exhibited no thermotropic phase transitions but revealed chain order intermediate between the gel and liquid-crystal states. In contrast, DMPS-d_{54} incorporated into the outer leaflet of echinocytic erythrocytes was conformationally ordered while the same species incorporated into the inner leaflet of stomatocytic erythrocytes was highly disordered at all temperatures studied. Finally, DMPC-d_{54} incorporated into the outer leaflet of echinocytic erythrocytes exhibited a phase transition, suggesting that this species persists in domains. These data indicate that the acyl chain conformational order of specific phospholipids in the intact human erythrocyte is changed with alterations in cell morphology.

The asymmetric distribution of lipids across the human erythrocyte membrane is well documented (Schroit & Zwaal, 1991; Zachowski, 1993; Devaux, 1991). However, the functional consequences of this phenomenon are not entirely understood. Lipid asymmetry is maintained, at least in part, by an ATP,Mg^{2+}-dependent integral membrane protein ("aminophospholipid translocase") which transports phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) across the plasma membrane (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Devaux, 1992).

Asymmetry may also be manifest in the plane of the bilayer through the occurrence of lipid and protein domains (Welti & Glaser, 1994; Tocanne et al., 1994). These are usually observed by co-localization of fluorescent probes detected by fluorescence microscopy (Rodgers & Glaser, 1993), or by restrictions to lateral diffusion detected by techniques such as single-particle tracking (Simson et al., 1995). As in the case of transbilayer asymmetry, the functions of these domains are mostly undetermined, although it has been suggested that proteins such as phospholipase A_2 (Honger et al., 1996) and protein kinase C (Yang & Glaser, 1995) require specific lipid environments for optimal function. The general conclusion from the above investigations is that the traditional view of the fluid mosaic model presenting the bilayer as a homogeneous, fluid solvent for membrane proteins must be modified, so that various impediments to free diffusion must be incorporated into models of membrane structure and dynamics (Tocanne et al., 1994; Kinnunen et al., 1994).

The acquisition of structural and dynamic information about the molecules that constitute the domains requires a nonperturbative approach that can be applied to intact cell membranes. Toward this end, FTIR spectroscopy provides unique advantages and has been applied by our laboratory and others to study membrane lipids in intact cells (Moore & Mendelsohn, 1994; Moore et al., 1996; Naumann et al., 1996).

Characteristic alterations in erythrocyte morphology may be induced by incorporation of specific exogenous phospholipids into either or both of the erythrocyte monolayers (Ferrell et al., 1985; Daleke & Huestis, 1989), and are easily observed using interference contrast microscopy (ICM). There are three basic cell shapes which appear upon phospholipid incorporation. Discocytic morphology (char-

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[1] Abbreviations: DMPC-d_{54}, acyl chain perdeuterated dimyristoylphosphatidylcholine; DMPS-d_{54}, acyl chain perdeuterated dimyristoylphosphatidylserine; FTIR, Fourier transform infrared; GPS, glycerophosphoserine; ICM, interference contrast microscopy; MI, morphological index; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; T_m, gel→liquid-crystal phase transition temperature; $\nu_{asym}CD_2$, asymmetric CD_2 stretching frequency.

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acteristic of normal erythrocytes) is maintained when equimolar levels of PS and PC are co-incubated. Echinocytic morphology results from the expansion of the outer membrane leaflet relative to the inner and is readily achieved by incorporation of short-chain (12−15) disaturated PC's. Stomatocytic morphology results from the expansion of the inner membrane leaflet such as occurs with the incorporation of short-chain PS's. The extent of the change in cell shape away from discocytes is dependent upon the relative expansion of the inner or outer membrane leaflet (Daleke & Huestis, 1989), and is semiquantitatively determined by ICM.

Incorporation of perdeuterated phospholipids produces the same characteristic shape changes in erythrocytes as proteated phospholipids (Moore et al., 1996). As noted in our previous study, the use of deuterated phospholipids permits the detection of the CD_2 stretching modes of the incorporated lipids by FTIR spectroscopy. As these modes are sensitive to lipid acyl chain conformational order, it is possible to acquire molecular conformation information concerning the single incorporated phospholipid species. The previous study examined two extreme morphologies: DMPC-d_{54}-induced echinocytosis and DMPS-d_{54}-induced stomatocytosis. The current work extends this technique to examine acyl chain conformational order of DMPS-d_{54} and DMPC-d_{54} in intact discocytic cells as well as DMPS-d_{54} selectively located in the outer membrane leaflet of echinocytes. The results clearly indicate that the conformational order of specific phospholipids changes with erythrocyte morphology.

MATERIALS AND METHODS

Materials. Phospholipids were from Avanti Polar Lipids (Alabaster, AL). All other chemicals, including glycerophosphoserine(GPS) and *N*-ethylmaleimide (NEM), were from Sigma (St. Louis, MO).

Erythrocytes. Human erythrocytes were collected from healthy adult volunteers by venipuncture into heparin. Cells were washed 3 times with phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4), and the buffy coat was removed.

Vesicle Preparation. All lipid samples were dried from chloroform solution with nitrogen followed by pumping under vacuum and then suspended in PBS (1.5 mg/mL). These lipid suspensions were then sonicated above T_m in a bath sonicator for 30 min.

Erythrocyte−Vesicle Incubations. Packed, washed erythrocytes (1 mL) were mixed with an equal volume of sonicated vesicles (1.5 mg/mL) to give a final cell concentration of 50%. For overnight incubation periods, the PBS was supplemented with additional glucose and penicillin. In all experiments, a parallel control incubation (minus only the lipid vesicles) was included. Three different experiments for aminophospholipid translocase inhibition were performed (Martin & Pagano, 1987; Connor & Schroit, 1988; Daleke & Huestis, 1985; 1989). First, cells were deprived of glucose, thereby preventing ATP synthesis and resulting in the shutting-down of energy-dependent processes, including translocase function. In these experiments, the erythrocytes were initially depleted of glucose by a 6 h preincubation in glucose-free PBS, and then incubated in glucose-free PBS with DMPS-d_{54} vesicle addition as described above. Second, the pump function was inhibited with *N*-ethylmaleimide (NEM), a relatively nonspecific sulfhydryl group alkylating agent which chemically modifies the translocase. For these studies, cells were preincubated for 1 h in 2 mM NEM/PBS solution. Lipid vesicles were then added. Finally, the translocase was specifically inhibited with glycerophosphoserine (GPS). For these studies, a GPS stock solution was added to the cells before the DMPS-d_{54} vesicles, giving a final GPS concentration of 0.1 mM.

Erythrocyte Morphology. At the end of the incubation period, an aliquot of cells was fixed in 1% glutaraldehyde in PBS. These fixed samples were then examined with ICM to determine erythrocyte morphology. The morphologic index of each sample was determined as described (Moore et al., 1996). Discocytes were given a score of 0, stomatocytes were scored from −1 to −4, and echinocytes from +1 to +5. The MI was determined from averaging 200 cells by a single observer working with blind-coded samples.

Methods and Sample Preparation for FTIR Spectroscopy. Washing and preparation of post-incubation erythrocyte samples for spectroscopy were exactly as described (Moore et al., 1996). The final washed erythrocyte samples were placed between two CaF_2 windows separated with a 50 μm spacer and held in a thermostated transmission cell (Harrick Scientific, Ossining, NY). The temperature was controlled with a circulating water bath (Fisher Scientific, Springfield, NJ) and monitored with a thermocouple. Spectra were routinely acquired over a range of 20 °C at 1 °C intervals. All spectra were acquired on a Mattson RS-1 spectrometer by co-addition of 1024 interferograms collected from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution under continuous N_2 purge. The interferograms were apodized with a triangular function and Fourier-transformed with two levels of zero filling.

Processing of FTIR Spectra. The advantages and disadvantages of various data reduction protocols for this experimental approach were discussed in detail in a recent paper (Moore et al., 1996). In this work, as in our previous report, the precise frequency of $\nu_{asym}CD_2$ was determined from (inverted) second-derivative spectra.

RESULTS

The photographs in Figure 1 depict typical erythrocyte samples after overnight incubation at 37 °C. The cells in Figure 1A are discocytes incubated in PBS with an equimolar mixture of DMPC/DMPS-d_{54}. Erythrocytes incubated with DMPS-d_{54} are shown in Figure 1B; these cells are stomatocytic, indicating that the DMPS-d_{54} has been incorporated into the inner monolayer of the cell membrane. The echinocytic cells in Figure 1C depict erythrocytes in which the aminophospholipid translocase has been inhibited with GPS followed by incubation with DMPS-d_{54} vesicles. The echinocytic morphology demonstrates that the DMPS-d_{54} is retained primarily in the outer monolayer of the cell membrane. The average MI's for the DMPS-d_{54}/DMPC, DMPS-d_{54} (no glucose), and DMPS-d_{54} (GPS treated) erythrocytes were 0.57, −1.74, and 2.28, respectively. Typical values for normal discocytes were close to or slightly below zero.

The thermotropic behavior of the asymmetric CD_2 stretching frequency is plotted in Figure 2A for DMPS/DMPC-d_{54} vesicles and in Figure 2B for DMPS/DMPC-d_{54} incorporated into intact cells. The gel → liquid-crystal phase transition of the DMPC-d_{54} component of the DMPS/DMPC-d_{54} vesicles is centered at 29−30 °C. No phase transition is detected for DMPC-d_{54} incorporated into the outer cell

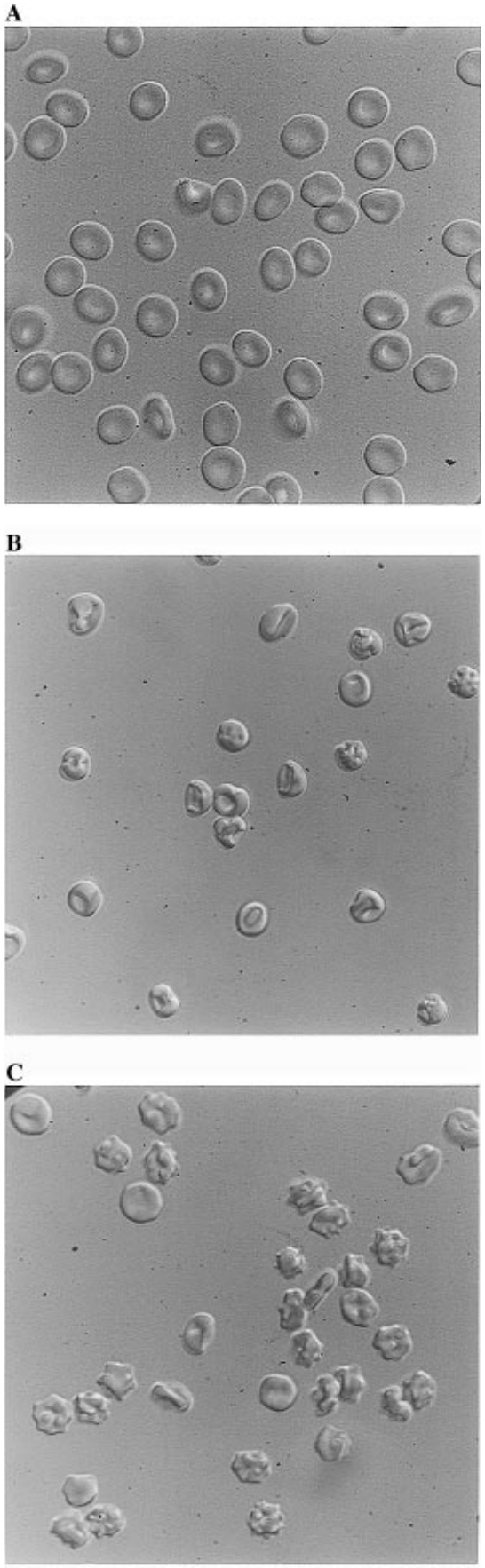

FIGURE 1: Shape characteristics of human erythrocytes incubated with phospholipids as follows: Discocytic cells after incubation with DMPC/DMPS-d_{54} vesicles (A); stomatocytic cells after incubation with DMPS-d_{54} vesicles (B); echinocytic cells after incubation with glycerophosphoserine and with DMPS-d_{54} vesicles (C).

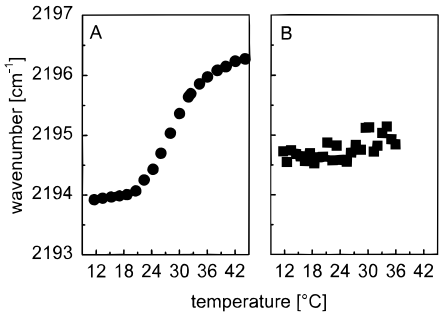

FIGURE 2: Temperature response of the CD_2 asymmetric stretching mode of DMPC-d_{54} in mixed 1:1 vesicles of DMPS/DMPC-d_{54} is plotted in panel A. The temperature response of the CD_2 asymmetric stretching mode frequency of DMPC-d_{54} incorporated into intact discocytic erythrocytes (by incubation with DMPS/DMPC-d_{54} vesicles) is plotted in panel B.

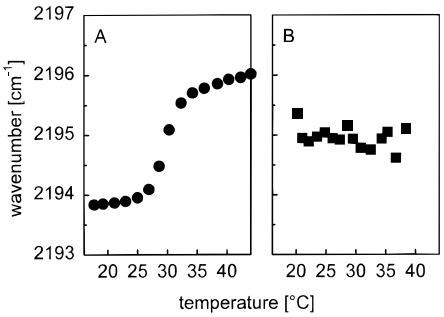

FIGURE 3: Temperature response of the CD_2 asymmetric stretching mode frequency of DMPS-d_{54} in mixed 1:1 vesicles of DMPC/DMPS-d_{54} is plotted in panel A. The temperature response of the CD_2 asymmetric stretching mode frequency of DMPS-d_{54} incorporated into intact discocytic erythrocytes (by incubation with DMPC/DMPS-d_{54} vesicles) is plotted in panel B.

monolayer of these discocytic erythrocytes. The CD_2 asymmetric stretching mode of the incorporated DMPC-d_{54} increases slightly from ~2194.6 to 2195.1 cm^{-1} (precision ~0.1−0.2 cm^{-1}) over the temperature range 12−37 °C. These frequency values lie about halfway between those for the gel (2193.8 cm^{-1}) and liquid-crystal (~2196 cm^{-1}) phases (Figure 2A) for the lipid in the binary mixture.

In Figure 3, the thermotropic behavior of $\nu_{asym}CD_2$ is plotted for DMPC/DMPS-d_{54} vesicles (Figure 3A) and for DMPC/DMPS-d_{54} incorporated into intact cells (Figure 3B). The gel → liquid-crystal phase transition of the DMPS-d_{54} component of the DMPC/DMPS-d_{54} vesicles is centered at 31 °C. As for the DMPC-d_{54} component (Figure 2B), no phase transition can be detected for DMPS-d_{54} incorporated into the inner cell monolayer of these discocytic erythrocytes. $\nu_{asym}CD_2$ of the incorporated DMPS-d_{54} is approximately constant at ~2195 cm^{-1}, halfway between the gel and liquid-crystal values in the vesicles (Figure 3A).

The thermotropic responses of $\nu_{asym}CD_2$ for pure DMPS-d_{54} vesicles, glucose-deprived erythrocytes with DMPS-d_{54} incorporated, and GPS-treated erythrocytes with DMPS-d_{54} incorporated are plotted in Figures 4A, 4B, and 4C, respectively. The highly cooperative gel → liquid-crystal phase transition of pure DMPS-d_{54} appears at 34 °C. In Figure 4B,C, no phase transitions can be detected; the CD_2 stretching frequency assumes a low value, similar to that of the ordered gel phase of the pure vesicles at all temperatures studied.

Figure 5 displays the temperature response of the exogenous and endogenous lipids in echinocytic erythrocytes. The thermotropic response of $\nu_{asym}CD_2$ in pure DMPC-d_{54}

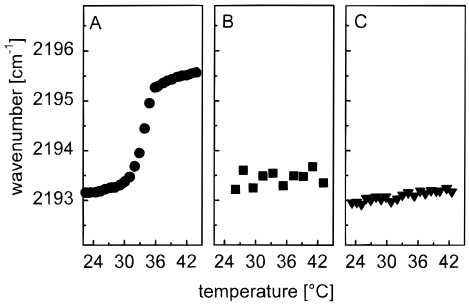

FIGURE 4: Panel A displays the temperature response of the asymmetric CD_2 stretching mode frequency in pure DMPS-d_{54} vesicles. Panel B shows the temperature response of the CD_2 asymmetric stretching mode frequency of DMPS-d_{54} in erythrocytes incubated without glucose. Panel C displays the temperature response of the CD_2 asymmetric stretching mode frequency of DMPS-d_{54} in erythrocytes incubated with both glucose and glycerophosphoserine.

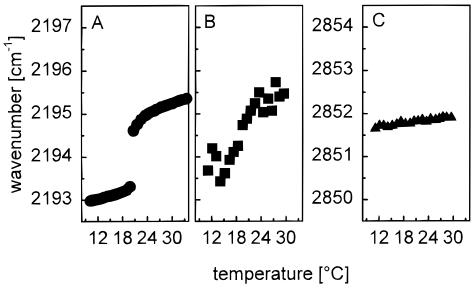

FIGURE 5: Panel A displays the temperature response of the asymmetric CD_2 stretching mode frequency in pure DMPC-d_{54} vesicles. Panel B shows the temperature response of the asymmetric CD_2 stretching mode frequency of DMPC-d_{54} in echinocytic erythrocytes. The temperature response of the symmetric CH_2 stretching frequency (arising from all the endogenous phospholipids) is plotted in panel C.

vesicles is plotted in Figure 5A. In Figure 5B, $\nu_{asym}CD_2$ for DMPC-d_{54} incorporated into echinocytic erythrocytes is plotted. A phase transition with cooperativity reduced from pure DMPC-d_{54} vesicles (Figure 5A) but centered at a similar transition temperature (∼20 °C) is observed. Figure 5C displays the thermotropic behavior of the symmetric CH_2 stretching frequency of the entire endogenous membrane phospholipid population in the same sample of echinocytic cells.

DISCUSSION

Although the phenomenon of membrane phospholipid asymmetry is well documented, the structures of particular lipid species resulting from either partial (PC, PE, SM) or essentially complete (PS) transbilayer segregation or from domain formation in half of the bilayer remain unknown. The elegant techniques used to date to measure either lipid transverse asymmetry or lateral domain formation are designed to report with as high sensitivity as possible on their existence, but produce limited information concerning the molecular conformation of the lipid classes involved in these processes. Determination of the conformational order of a given lipid species in either the inner or the outer monolayers of the cell membrane may offer clues as to its interactions under a given set of conditions.

The IR method described here and in our previous report offers the advantage of utilizing reporter molecules (acyl chain perdeuterated phospholipids) which possess structural, phase transition, and miscibility characteristics that are very similar to their proteated counterparts (Klump et al., 1981). The relatively low signal intensity in the IR spectral region of interest can be overcome by careful spectral subtraction or derivative spectroscopy.

The most interesting result of the current set of experiments is the observation of altered conformational order in the exogenous lipids for different erythrocyte morphologies and for altered locations of the incorporated DMPS-d_{54}. The discussion that follows is based on the well-established correlation between methylene stretching frequencies and acyl chain conformational order (Snyder et al., 1978; 1982; MacPhail et al., 1984; Dluhy et al., 1985). The frequency of the asymmetric CD_2 stretching vibration near 2193 cm^{-1} increases by 3−4 cm^{-1} when acyl chain perdeuterated phospholipids undergo their gel → liquid-crystal phase transition. This parameter is qualitative, in the sense that the mathematical relationship between the increase in frequency and the extent of disorder induced in the chains is unknown. This mode is the strongest in the spectrum of the incorporated lipids, and provides the only peak that can be precisely monitored under the current experimental conditions (intact cells, where the inserted species comprises ∼1−3% of the membrane phospholipid).

When discocyte shape is maintained upon incubation of erythrocytes with a 1:1 mixture of DMPC/DMPS-d_{54}, the acyl chain conformation of the PS is essentially one of intermediate disorder, characterized by a value for $\nu_{asym}CD_2$ that lies between those of the gel and liquid-crystal phases in the binary lipid mixture. In contrast, DMPS-d_{54} present (through aminophospholipid translocase inhibition) in the outer monolayer of echinocytic erythrocytes exhibited high conformational order ($\nu_{asym}CD_2$ of 2193.0−2193.5 cm^{-1}) independent of whether the inhibition was caused by glucose deprivation of the cells (Figure 4B) or by incubation with either GPS (Figure 4C) or NEM (data not shown for the NEM). This level of chain order approaches that in the gel phase of pure DMPS-d_{54} vesicles. In our recent prior report, a $\nu_{asym}CD_2$ value of 2195.1−2195.9 cm^{-1} (depending on the temperature) was observed for DMPS-d_{54} incorporated into the inner monolayer of stomatocytic erythrocytes, suggestive of a disordered acyl chain conformation (Moore et al., 1996). In neither the present study nor our earlier report was there evidence for a cooperative phase transition for this lipid species. These differences in chain conformational order in cells with altered shapes evidently report upon different environments for the DMPS-d_{54}.

The outer monolayer phospholipids in erythrocytes are composed mostly of unsaturated PC's (molecular species 16:0−18:1, 16:0−18:2, and 18:0−18:1) and sphingomyelins (molecular species 18:1−16:0, 18:1−24:0, and 18:1−24:1) (Myher et al., 1989). The miscibility of DMPS-d_{54} with these species is unknown but is presumably quite low based on studies of other similar PC-containing mixed systems (Silvius, 1982). The level of unsaturation of the PC suggests that this lipid class will be disordered in the outer monolayer, while the higher transition temperatures for the sphingomyelins might suggest more ordered acyl chains. However, the high levels of cholesterol in the membrane will tend to produce a state of intermediate chain disorder [the so-called "liquid ordered phase" (Ipsen et al., 1987)] assuming the miscibility of cholesterol. In the current experiments, the exogenous DMPS-d_{54} (observed to be highly ordered in the echinocytic cells) is evidently immiscible with the PC and/or cholesterol components, since if the relatively small

proportion of exogenous PS were indeed miscible, it would disorder upon mixing with the much high proportions of these species. Yet the exogenous DMPS-d_{54} cannot be segregated into a relatively pure lipid phase of its own, as in that case it would be expected to undergo a phase transition at ~34 °C. It is thus suggested that DMPS-d_{54} interacts strongly with proteins in the outer monolayer, although association with the sphingomyelin cannot be completely ruled out. When DMPS-d_{54} is incorporated into the inner monolayer (stomatocytes), the high level of disorder may reflect miscibility of this species with the remainder of the PS class. The latter contains mostly 18:0 and 20:4 chains (Myher et al., 1989).

The incorporated DMPC-d_{54} behaves quite differently in the outer monolayers of discocytic compared with echinocytic erythrocytes. In the former, the acyl chains exist in a conformational state quite similar to that of DMPS-d_{54}, i.e., one of intermediate disorder. This suggests substantial miscibility with the PC/cholesterol/sphingomyelin phases. $\nu_{asym}CD_2$ increases monotonically from 2194.6 to 2195.1 cm^{-1} as the temperature is increased from 12 to 37 °C, with no evidence of a thermotropic transition. In contrast, DMPC-d_{54} in echinocytic erythrocytes (see Figure 5 and our previous report) exhibits a clear phase transition at 18−20 °C, i.e., at the same temperature as in pure vesicles of this substance. The range of change in $\nu_{asym}CD_2$ is about the same (it varies slightly with the morphologic index) in intact cells compared to pure DMPC-d_{54} vesicles while the transition is broadened. As noted in our previous report, the most likely explanation for the phase transition is the existence of domains of relatively pure DMPC-d_{54}. The observed broadening of the transition is probably the result of small domain size and/or inclusion of additional membrane components.

Overall, it is clear that incorporation of exogenous lipids resulting in echinocyte formation produces substantial alterations in the molecular conformation and phase characteristics of the inserted phospholipid compared with the situation where phospholipids are incorporated with retention of the discocyte shape in the cells (compare Figures 2 and 4).

The current FTIR method may be extended to include a variety of phospholipid species, acyl chain lengths, and levels of unsaturation. Chain lengths of C14 (i.e., DMPS-d_{54} and DMPC-d_{54}) were selected for these initial investigations because of their ease of insertion into erythrocytes (Ferrell et al., 1985; Daleke & Huestis, 1985; 1989) and because the thermotropic phase transitions of the particular lipid classes occur at temperatures where the cells are viable. Utilization of longer chain lengths to investigate the miscibility and phase properties of the inserted species presents some technical difficulties with respect to the extent and time scale of incorporation and the extraction of membrane components. Some shorter chain lengths of these and other species may be feasible for study.

The current approach permits a direct evaluation of chain conformational order and thermotropic behavior of the incorporated species. The phase behavior of the latter may differ dramatically from the average conformational order of all chains in the membrane. This is demonstrated in Figure 5, where the CH_2 symmetric stretching frequency arising from all endogenous lipids in intact DMPC-d_{54}-incorporated echinocytic erythrocytes is shown. A slight monotonic frequency increase in this parameter (~0.2 cm^{-1}) with temperature is noted (Figure 5C). This behavior is quite different from the phase transition (frequency increase of ~2 cm^{-1}) observed for the exogenous DMPC-d_{54} (Figure 5B). Along the same lines, the morphology-dependent differences in behavior, as discussed above, of the exogenous PC compared with the PS highlight the need for techniques that can monitor specific lipids within the heterogeneous distribution found in the cell membrane. The FTIR approach offers the unique possibility of acquiring information about molecular level structural changes that accompany phenomena occurring on larger distance scales.

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