Cell to Vesicle Transfer of Intrinsic Membrane Proteins: Effect of Membrane Fluidity†

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ABSTRACT: Transfer of intrinsic membrane proteins between intact human erythrocytes and phosphatidylcholine vesicles has been reported. The relative fluidities of the cell and vesicle membrane phospholipids have profound effects on the rate, extent, and direction of such protein transfer. Two types of experiments were conducted to examine separately the effects of the donor (cell) and recipient (vesicle) membrane fluidity on these parameters. First, binary mixtures of saturated phosphatidylcholines were used to generate phospholipid vesicles in liquid-crystalline-, gel-, or mixed-phase states at a single temperature. This permitted continuous variation of the fluidity of the recipient membrane while the properties of the donor membrane were held constant. Changing the phase state of the recipient membrane had significant effects on the rate and equilibrium extent of protein transfer. Transfer to gel-phase vesicles was 10^3 times slower than to liquid-crystalline vesicles, while mixed-phase vesicles exhibited intermediate rates strongly dependent on the relative fractions of solid and fluid lipid present. The fraction of the membrane protein transferred to phosphatidylcholine vesicles was affected similarly by vesicle phase state, decreasing markedly with decreasing vesicle lipid fluidity. The second type of experiment examined the effects of the donor membrane fluidity on rate and extent of protein transfer. The fluidity of the cell membrane was altered by changes in the temperature of incubation, while the phase states of the recipient membranes were held constant by variation of their phospholipid composition. Protein transfer from erythrocytes to gel, liquid-crystalline, and mid-transition-range vesicles was studied at temperatures between 10 and 37 °C. Transfer to liquid-crystalline-phase vesicles was rapid and extensive at all temperatures examined, while transfer to mid-transition and gel-phase vesicles was favorable only at temperatures below 35 and 25 °C, respectively. These findings are consistent with the proposal that transfer of proteins between membranes is facilitated where the recipient membrane is more fluid than the donor. Those membrane proteins susceptible to transfer may exist in equilibrium between phosphatidylcholine bilayers, their distribution being a sensitive function of the relative fluidities of the available membranes. In these experiments, the intrinsic erythrocyte membrane protein acetylcholinesterase was used as the protein transfer marker. Phase states of the phospholipid vesicles were characterized by using the fluorescence intensity and anisotropy of incorporated fluorescent membrane probes, *trans*-parinaric acid and *cis*-parinaric acid methyl ester.

Several intrinsic membrane proteins have been shown to transfer spontaneously between artificial phospholipid membranes (Enoch et al., 1977, 1979; Holloway et al., 1977) and between intact cells and artificial lipid aggregates (Bouma et al., 1977; Huestis, 1977; Bierbaum et al., 1979). Strittmatter and his co-workers showed that cytochrome oxidase can transfer from one artifical membrane to another, provided that the protein is bound to the membrane in a configuration that does not span the bilayer (Enoch et al., 1979). Huestis and co-workers demonstrated transfer of five polypeptide species from intact human erythrocytes to sonicated phosphatidylcholine vesicles (Bouma et al., 1977) or to lysophosphatidylcholine micelles (Bierbaum et al., 1979). None of these polypeptides could be dissociated from the cells efficiently by hypertonic solutions, but two of them could be iodinated from the cytoplasmic side of the membrane by impermeant reagents (S. R. Bouma, unpublished results). The studies described here are part of a general investigation of membrane properties that influence the rate, extent, and direction of such intrinsic protein transfer. These studies employ the intact human erythrocyte as the donor membrane and a well-defined one- or two-component sonicated phospholipid vesicle as the recipient membrane.

The protein used as a transfer marker in these studies is acetylcholinesterase (AChE; EC 3.1.1.7),[1] the best characterized and most easily assayed of the transferred proteins. Erythrocyte AChE is an intrinsic protein solubilized readily by detergents but not by hypertonic solution (Sihotang, 1974; Ott et al., 1975). Its mode of association with membranes resembles that of brain and smooth muscle AChE (Hall, 1973; Massoulie & Rieger, 1969), in contrast with the extrinsic AChE of eel electroplax (Silman & Karlin, 1967; Dudai & Silman, 1974; Rosenberry & Richardson, 1977). When erythrocytes are incubated with sonicated dimyristoylphosphatidylcholine (DMPC) vesicles at 37 °C, more than 90% of the cell AChE activity is extracted into the vesicle fraction. More than 98% of the extracted enzymatic activity is found to be bound to lipid aggregates large enough to be excluded from Sepharose 4B, and the active site of the transferred enzyme remains accessible to impermeant substrates (Bouma et al., 1977). Thus, the AChE appears to transfer from one membrane to another in native orientation.

One membrane property which might be expected to influence protein insertion and transfer is phospholipid phase state or fluidity. Varying the incubation temperature, which should change the fluidity of both donor and recipient mem-

† From the Department of Chemistry, Stanford University, Stanford, California 94305. *Received October 23, 1979.* This work was supported by the National Institutes of Health (HL 18660 and HL 23787) and by the American Heart Association (Grant-in-Aid 77-828).

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[1] Abbreviations used: AChE, acetylcholinesterase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; *trans*-PnA, *trans*-parinaric acid; *cis*-PnA-Me, *cis*-parinaric acid methyl ester.

0006-2960/80/0419-4601$01.00/0

branes, produces nonlinear changes in transfer kinetics that are not readily interpretable. The contributions of donor and recipient membranes to these changes have been examined separately in experiments where the fluidity of one participant was held constant while that of the other was varied. In the case of the recipient membrane, a synthetic phospholipid vesicle, this was accomplished by varying the phospholipid composition of the vesicle so that its phase state was altered from gel to liquid crystalline at a fixed temperature (Lentz et al., 1976b). Altering the fluidity of the natural membrane is more complex; it can be achieved by changes in cholesterol content or by addition of chemical agents such as chlorpromazine (Fujii et al., 1979) or *cis*-vaccenic acid (Hanski et al., 1979), but the effects of such measures on protein–lipid interactions are problematic. A noninvasive approach to changing cell membrane fluidity is temperature variation. In the temperature range of interest (10–37 °C), the phase state of the recipient vesicle membrane could be dictated by appropriate choice of phospholipid composition. Thus, vesicle lipid compositions were selected to produce constant phase states as the incubation temperature was varied to change the donor membrane properties.

Selection of appropriate vesicle phospholipid compositions for both types of studies entailed characterization of the phase behavior of mixed composition sonicated vesicles. The phase properties of vesicles used in these studies were characterized by using the fluorescence intensity and anisotropy properties of two polyene membrane probes, *trans*-parinaric acid (*trans*-PnA; 9,11,13,15-*all-trans*-octadecatetraenoic acid) and the methyl ester of *cis*-parinaric acid (*cis*-PnA; 9,11,13,15-*cis*,*trans*,*trans*,*cis*-octadecatetraenoic acid). *trans*-PnA partitions preferentially into the solid phase of mixed-phase lipid aggregates, where its enhanced quantum yield is a sensitive indicator of the onset of the liquid-crystalline- to gel-phase transition (Sklar et al., 1977). *cis*-PnA-ME, in contrast, distributes approximately equally between gel and liquid-crystalline phases in mixed-phase membranes. Since it is present in both phases of a membrane throughout the phase-transition temperature range, the temperature dependence of its fluorescence anisotropy can be used to detect onset and completion of phase transitions in phospholipid dispersions (Sklar et al., 1979). *cis*-PnA-ME is particularly useful for constructing phase diagrams of mixed phospholipid systems and sonicated dispersions where transitions may occur over temperature ranges of 10 °C or more (Lentz et al., 1976a,b).

Materials and Methods

Phospholipid Vesicles. Dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine (DMPC, DPPC, and DSPC, respectively) were purchased from Sigma Chemical Co. and used without further purification. Phospholipid mixtures were prepared by dissolving weighed samples in ethanol and evaporating the solvent with a stream of dry nitrogen. The lipids were dispersed in buffer (23.6 μmol of lipid/mL) by vortexing at temperatures above their phase transition ranges. Unilamellar vesicles were prepared by sonication as described (Bouma et al., 1977). Fluorescence measurements were conducted in 310 mOsM phosphate-buffered saline of the following composition: 140 mM NaCl, 5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.38. Incubation of vesicles with erythrocytes was conducted in the above buffer containing 50 mM sucrose to suppress cell osmotic lysis (Huestis, 1977).

Fluorescence Spectroscopy. Fluorescence experiments were performed with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a variable-temperature sample compartment. The sample compartment used for fluorescence depolarization measurements was fitted with a fixed-mount Glan-Taylor UV transmitting polarizer in the exciting beam and a film polarizer in the emission beam. The two-position analyzing polarizer could be oriented either parallel or perpendicular to the polarization of the exciting beam. Stray light was reduced with a UV bandpass filter in the exciting beam and a 390-nm cutoff filter in the emission beam.

trans-PnA and *cis*-PnA-ME were gifts of Dr. Robert Simoni and Dr. Charles Berde. Stock solutions (2 mM in ethanol) were stored under nitrogen in the dark at −20 °C. Samples were prepared for fluorescence measurements by pipetting 42 μL of the freshly sonicated vesicle suspension into 3 mL of deoxygenated buffer. A stock solution of *trans*-PnA (1 μL) or *cis*-PnA-ME (5 μL) was added, giving molar ratios of 500:1 or 100:1 phospholipids to probe. The sample was then cooled to the desired temperature.

In measurements of the temperature dependence of fluorescence, temperature was varied at a rate not exceeding 1 °C/min by a circulating water bath and monitored by a copper–constantan thermocouple in the sample cuvette within a few millimeters of the exciting beam. The thermocouple output was used to drive the x axis of a Mosely 7030A X–Y recorder, while the y axis registered the output of the fluorometer. $I_\parallel$ and $I_\perp$ were measured during temperature scans by rotating the analyzing polarizer at half-minute intervals.

The excitation wavelengths for *trans*-PnA and *cis*-PnA-ME were 320 and 324 nm, respectively. Fluorescence was monitored at 420 nm for both probes.

Incubation of Vesicles with Erythrocytes. Human erythrocytes were obtained from adult volunteers and separated from the serums and washed as described (Bouma et al., 1977). Protein-transfer experiments were initiated within 36 h for collection of the cells. Vesicle suspensions were mixed with equal volumes of packed erythrocytes at the temperatures of subsequent incubation. Incubation temperatures were regulated ±1 °C with a water bath. During extended incubations, cell–vesicle suspensions were agitated on a vortex mixer every 3–6 h. Aliquots of cell–vesicle mixtures were removed after specified intervals and the cell and vesicle fractions separated by brief centrifugation at 3000g. The vesicle fractions were assayed for AChE activity (Ellman et al., 1961).

Results

Temperature Studies of Protein Transfer to DMPC Vesicles. Protein transfer from erythrocytes to pure DMPC vesicles is shown as a function of incubation time at several temperatures in Figure 1. After a time lag of varying duration, AChE activity appears associated with the vesicle fraction of the suspension at all temperatures between 10 and 37 °C. Decreasing the incubation temperature affects protein transfer in three ways. The time interval before protein transfer (onset time) increases, the rate of transfer (Δ(AChE)/Δt) decreases, and the amount of enzyme transferred at equilibrium changes. Figure 2 shows the effect of temperature on the rate and equilibrium extent of protein transfer. Although substantial scatter is introduced into the rate data by use of cells from many donors, a statistically significant break is apparent in the slope of the ln (rate) vs. temperature function. The data are fit best by two lines that intersect in the vicinity of 25 °C (Figure 2A). Similarly, the amount of AChE transferred to DMPC vesicles increases between 37 and about 25 °C but decreases at temperatures below 22 °C (Figure 2B).

The effects summarized in Figures 1 and 2 presumably reflect changes in both donor and recipient membranes due

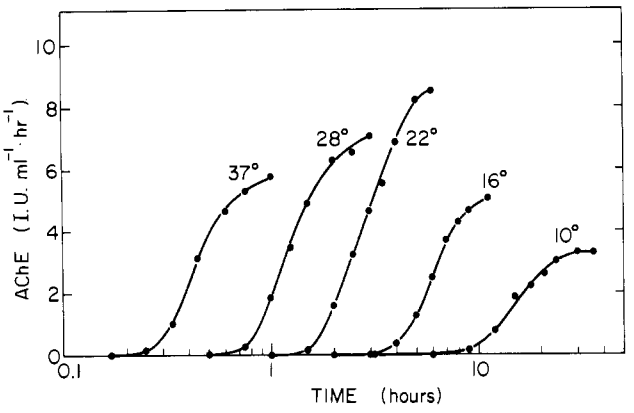

FIGURE 1: Appearance of AChE in DMPC vesicles incubated with human erythrocytes at five temperatures.

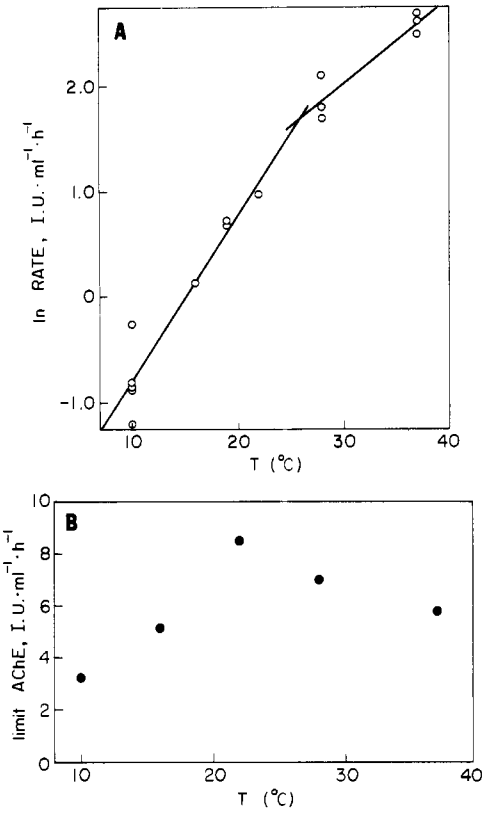

FIGURE 2: (A) Plot of ln (rate) vs. temperature. Rate is expressed as units of AChE per mL transferred per hour of incubation. Above 25 °C the slope is 0.081 ± 0.003 (AChE units·mL^{-1}·h^{-1})·°C^{-1} (90% confidence limit). Below 25 °C, the slope is 0.157 ± 0.036 ln (AChE units·mL^{-1}·h^{-1})·°C^{-1} (90% confidence limit). The slopes above and below 25 °C are significantly different ($P < 0.005$). Each symbol represents an independent observation. (B) Equilibrium level of transferred AChE (AChE units·mL^{-1}) vs. temperature (°C). Data shown were obtained by using cells from a single donor. Data from four other donors show qualitatively consistent behavior.

to temperature changes. Experiments employing mixed composition vesicles were conducted to examine separately the contributions from these membranes.

Phase Transition Behavior of Mixed Phosphatidylcholine Vesicles. Binary mixtures of saturated phosphatidylcholines differing by two carbons in alkyl chain length form ideal mixtures whose phase transition properties are intermediate between those of the pure lipid components (Lentz et al., 1976b). The vesicles used in these studies were mixtures of DMPC/DPPC or DPPC/DSPC, which have this property. Phase-transition temperature ranges for sonicated mixtures of these phospholipids were determined by using the fluorescence probes *trans*-PnA and *cis*-PnA-ME. Figure 3 shows the temperature dependence of *trans*-PnA fluorescence intensity (I) in vesicles of DMPC, DPPC, and a 1:1 DPPC/DMPC

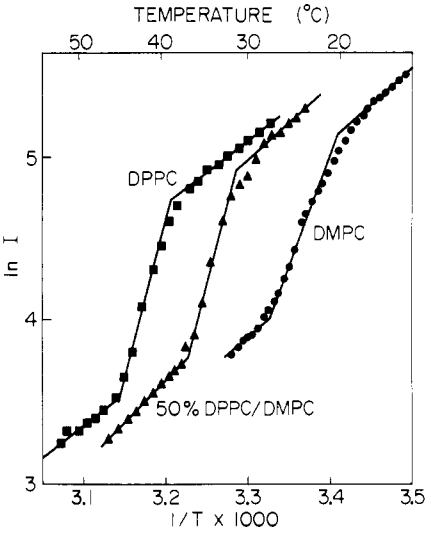

FIGURE 3: Fluorescence intensity of *trans*-PnA in phosphatidylcholine vesicles, as a function of temperature. (■) DPPC, (●) DMPC, (▲) 50% DPPC in DMPC.

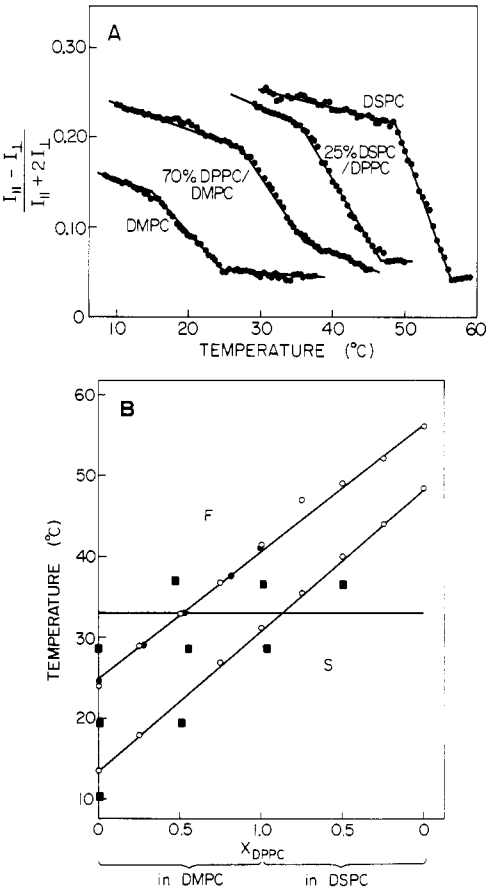

FIGURE 4: (A) Fluorescence anisotropy of *cis*-PnA-ME in phosphatidylcholine vesicles of the indicated compositions as functions of temperature. (B) Pseudo phase diagram for DMPC/DPPC and DPPC/DSPC sonicated vesicles derived from fluorescence anisotropy (○) of *cis*-PnA-ME and fluorescence intensity (●) of *trans*-PnA. Square symbols designate representative composition-temperature combinations used for fixed-phase state recipient membrane studies. The chosen compositions lie on three diagonal lines: one is 3–4 °C above the fluidus line (fluid), one is midway between the fluidus and solidus lines (midrange), and one is 3–4 °C below the solidus line (solid).

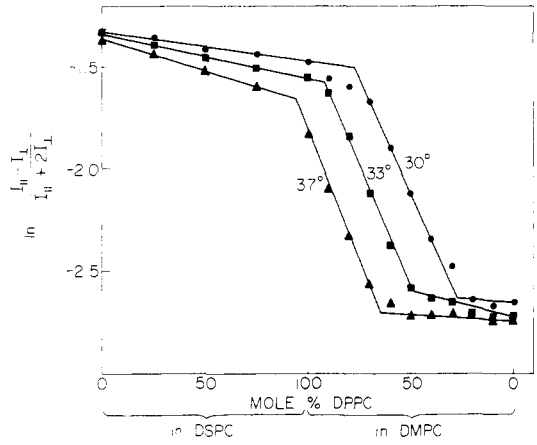

FIGURE 5: Fluorescence anisotropy of *cis*-PnA-ME as a function of vesicle phospholipid composition at 30 (●), 33 (■), and 37 °C (▲).

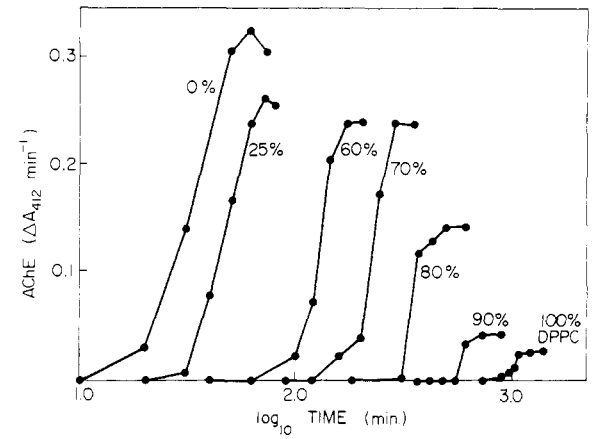

FIGURE 6: Appearance of AChE in phospholipid vesicles of various DMPC/DPPC compositions as a function of time of incubation with erythrocytes at 37 °C.

mixture. The temperature of inflection (T_c^{trans}) of these curves is a linear function of the mole fraction of higher melting lipid in the vesicle (Figure 4B, closed circles). These data give no evidence of nonideal behavior such as solid-phase immiscibility, indicating that all phases should be homogeneous mixtures of the two components.

The fluorescence intensity of *trans*-PnA is a useful indicator of phase-transition onset; however, it is insensitive to phase changes in membranes which are more than 50% solid phase because of preferential distribution of the probe into solid phase. In mixed composition bilayers and in sonicated vesicles of any composition, phase transitions generally are broadened to occur over temperature ranges of 10 °C or more (Lentz et al., 1976b). The lower bound of such broadened transitions can be detected by measuring the temperature dependence of the fluorescence polarization ratio, P, or the polarization anisotropy, r, of *cis*-PnA-ME incorporated into the lipid aggregate (Sklar et al., 1977). These quantities are defined as

$$P = I_{\parallel}/I_{\perp}$$

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{P - 1}{P + 2}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities viewed through a polarizer oriented parallel and perpendicular to the polarization plane of the excitation beam. Figure 4A shows the temperature dependence of r for *cis*-PnA-ME in PC vesicles of four compositions. The polarization anisotropy is relatively insensitive to temperature changes in ranges where the lipids are entirely fluid or solid. In the range of the phase transition, the anisotropy parameter decreases rapidly with increasing temperature. The inflections in this function define the limits of the phase transition range for each vesicle composition. The upper limits of these transition ranges are approximately the same for sonicated vesicles and for liposome dispersions (Sklar et al., 1977), but the lower limits are lowered by about 10 °C relative to liposomes of the same composition, and pretransition behavior is obscured. These results are in qualitative agreement with other measurements of phase transitions in sonicated vesicles (Lentz et al., 1976a). A pseudo phase diagram generated from these anisotropy data is shown in Figure 4B (open symbols) for mixtures of DPPC with DMPC or DSPC.

From Figure 4B, a set of phospholipid compositions could be chosen to yield solid-, fluid-, or mixed-phase vesicles at any single temperature between 25 and 45 °C. For example, at 33 °C (tie line, Figure 4B), DMPC/DPPC vesicles containing up to 50% DPPC are fluid, 90% DPPC vesicles are approximately midway through their phase transition,[2] and DPPC/DSPC mixtures are entirely solid. Thus, the "fluidity" of phase state of the vesicles can be varied continuously at a fixed temperature by varying the phospholipid composition. Figure 5 shows the effects of vesicle phospholipid composition on fluorescence depolarization of incorporated *cis*-PnA-ME at 30, 33, and 37 °C. The anisotropy of the probe is insensitive to phospholipid composition in vesicles which are fully solid or fluid but changes dramatically with mole fraction in composition ranges where both fluid and solid phases are present. The discontinuities in slope of this function occur at those vesicle compositions at the upper and lower bounds of their transition ranges at that temperature, consistent with the phase diagram.

Effects of Vesicle Phase State on Protein Transfer at Fixed Temperature. A series of vesicle samples was prepared which would exhibit a range of phase states at a chosen incubation temperature. Protein transfer to such vesicles is shown in Figure 6 for DMPC/DPPC mixtures at 37 °C. After a time lag of varying duration, AChE activity appears associated with the vesicle fraction of the suspension. Eventually, an equilibrium is reached after which no further transfer is observed. Changing the phospholipid composition at a fixed temperature has three effects on these events: the delay time before transfer, the rate of transfer after onset (Δ(AChE)/Δt), and the amount of protein transferred at equilibrium are all altered significantly. Figure 7 shows the effect of vesicle composition on the rate of protein transfer at two temperatures, 33 and 37 °C. As the mole fraction of the more fluid (lower melting) phospholipid increases, the transfer rate increases generally but not continuously. At 33 °C, DSPC/DPPC mixtures and pure DPPC vesicles take up protein at similar slow rates. At 10% DMPC in DPPC, the transfer rate increases abruptly and continues to increase sharply as the mole fraction of DMPC increases. At 50% DMPC/DPPC, a second discontinuity in this function is observed, and further increase in the fraction of DMPC has less effect on transfer rate. According to the fluorescence anisotropy data for vesicles at this temperature (Figure 7A, square symbols), these rate discontinuities appear at vesicle compositions at the upper and lower boundaries of the phase transition range.

By comparison, if the experiment is carried out at 37 °C, the latter rate discontinuity appears at a lower mole fraction of DMPC. At the higher temperature protein transfer is more

[2] Since the fluorescence lifetime of the probe *cis*-PnA-Me probably is different in solid and fluid phases, the steady-state measurements reported here do not yield quantitative information on the fraction of solid and fluid present in intermediate cases.

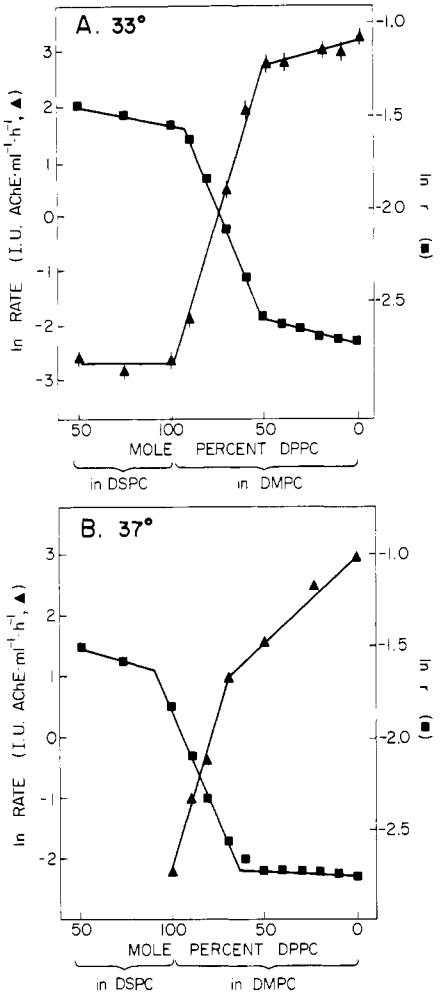

FIGURE 7: Dependence of protein transfer rate (▲) and fluorescence anisotropy (■) on vesicle composition at (A) 33 and (B) 37 °C.

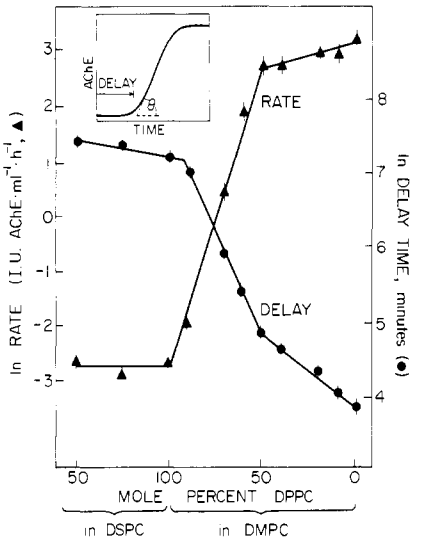

FIGURE 8: Dependence of the time lag before AChE transfer (●) and transfer rate (▲) on vesicle phospholipid composition, at 33 °C. The inset shows the relationship between the lag time and transfer rate (Δ(AChE)/Δt; tan θ).

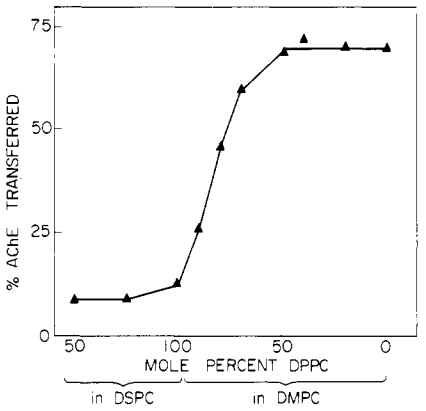

FIGURE 9: Fraction of total cell AChE transferred to vesicles at equilibrium, as a function of vesicle phospholipid composition. Data were obtained at 33 °C.

rapid for all vesicle compositions, and the rate becomes less sensitive to DMPC content at a lower mole fraction—about 40% DMPC/DPPC. This composition corresponds closely to the upper bound of the phase transition at 37 °C (Figure 7B), i.e., it is the composition at which solid phase appears at that temperature.

The effect of solid-phase formation is apparent in the time lag before protein uptake is observed as well as in the rate of transfer after onset. Figure 8 compares the composition dependence of the onset time and the rate (Δ(AChE)/Δt) at 33 °C. These parameters show coinciding discontinuities at the phase transition boundaries, with steep composition dependence in the transition range.

The phase state of the recipient membrane has similar effects on the amount of enzyme transferred at equilibrium. Figure 9 shows total AChE transferred from erythrocytes to vesicles at 33 °C as a function of vesicle phospholipid composition. At this temperature approximately 70% of total cell AChE was transferred to fluid vesicles, irrespective of their composition. Solid vesicles (25% and 50% DSPC/DPPC vesicles) took up 8–10% of total cell AChE, or about 10% as much as fluid vesicles. Vesicles of intermediate composition and phase state took up a fraction of the protein proportional to the mole fraction of the more fluid lipid. Thus the protein appears to partition between cell and vesicle membranes with a distribution coefficient tenfold greater for fluid than for solid membranes. Transfer to membranes containing gel and fluid phases reflects the mole fraction of the recipient membrane in each phase state, and the apparent distribution coefficient in such cases is a sensitive function of membrane composition.

Temperature Studies of Protein Transfer to Fixed Fluidity Membranes. The effects of the donor membrane fluidity can be separated from the overall response to temperature by varying the recipient membrane composition to keep its phase state constant at different temperatures. Vesicle compositions were chosen to yield a desired phase state (solid, fluid, or mid-transition range) at each temperature studied. On the pseudo phase diagram in Figure 4B, the closed square symbols designate the phospholipid compositions used to generate solid, fluid, and midrange vesicles at the temperatures chosen for study. The compositions selected for "fluid" and "solid" vesicles were such that the membranes would be 4 °C above or 4 °C below their transition ranges at the temperature of incubation.

Figure 10 shows the effects of temperature on protein transfer to fixed-phase vesicles. The rate of protein transfer and the total protein transferred at equilibrium are plotted as functions of temperature for recipient membranes of equivalent phase states. Transfer to fluid-phase vesicles is rapid and extensive, and neither rate nor equilibrium extent of transfer is affected greatly by a 9 °C change in temperature. For solid or midrange vesicles, however, changing temperature has more complex consequences. Vesicles in their transition range, which should contain both solid and fluid phases, take up protein

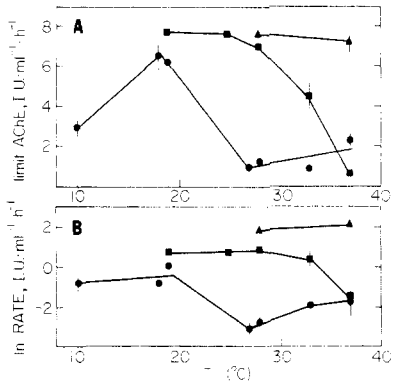

FIGURE 10: Changes in protein transfer parameters as functions of incubation temperature for fluid (▲), midrange (■), and solid (●) recipient membranes. (A) Amount of AChE transferred at equilibrium (AChE units·mL^{-1}·h^{-1}); (B) ln rate (AChE units·mL^{-1}·h^{-1}). Error bars represent standard error.

more rapidly and to a greater extent with decreasing temperature. Decreasing the incubation temperature from 37 to 25 °C produces a tenfold increase in transfer rate and in the amount of transferred protein. Fully solid vesicles exhibit similar behavior; the rate and extent of transfer increase as the temperature is decreased below 25 °C.

Discussion

Facile transfer of intrinsic membrane proteins from one phospholipid bilayer to another is a phenomenon which has interesting implications for the control and recognition functions served by cell surface proteins. The studies described here are part of a general investigation of the physical parameters that govern rate, extent, and selectivity of this process, using a well-defined model membrane as one partner in the transfer.

One critical physical property of lipid membranes which would be expected to influence protein insertion and transfer is "fluidity", defined operationally as that property which affects the distribution or mobility of some incorporated probe molecule such as a lipophilic spin or fluorescent label. Although natural membranes rarely exhibit extreme alterations in this property (such as the sharp thermotropic phase transitions of pure phospholipids), there is evidence from many sources to suggest that maintenance of controlled phase properties is critical for many membrane protein functions. We find that measures that alter this membrane property have profound effects on the rate and extent of protein transfer. Decreasing the fluidity of both donor and recipient membranes by decreasing the temperature produces a decrease in transfer rate with a slight discontinuity at about the upper temperature bound of the recipient membrane phase transition (Figure 2A). Temperature changes have a biphasic effect on the amount of protein transferred to single-component vesicles at equilibrium. For DMPC vesicles, the fraction of AChE transferred is greatest around 25 °C, the upper bound of the DMPC transition range. The fraction transferred decreases markedly at both higher and lower temperatures. This behavior suggests that decreasing fluidity in the donor membrane enhances protein transfer to liquid-crystalline-phase membranes but that the formation of gel phase in the recipient membrane inhibits transfer to an extent that counteracts the more gradual changes in the natural donor membrane.

For exploration of these effects in greater detail, two types of experiments were conducted to study donor and recipient membrane changes separately. The first was an isothermal study in which the donor membrane was maintained at constant temperature while the fluidity of the recipient membrane was varied through composition changes. The rate and extent of protein transfer were found to be sensitive functions of the phase state of the recipient membrane (Figure 6). Transfer to solid-phase vesicles is three orders of magnitude slower than to fluid ones, and transfer rate is very sensitive to lipid composition when both phases are present at the incubation temperature. Similarly, the phase state of the recipient membrane exerts significant effects on the amount of protein transferred at equilibrium (Figure 7). At the cell-to-vesicle ratio used in these studies, approximately 70% of the total cell AChE is transferred to fluid-phase vesicles, irrespective of composition. Solid vesicles take up only 8–10% of the total cell AChE, and mixed-phase-state vesicles take up a fraction of the protein proportional to the mole fraction of their more fluid lipid.

The second type of experiment employed temperature to change the fluidity of the cell membrane, while the composition of the vesicle membrane was varied to keep its phase state constant. We found that protein transfer to fluid-phase vesicles is rapid, extensive, and insensitive to temperature in the 28–37 °C range. Transfer to solid- or midtransition-range vesicles is predictably slower and less extensive at 37 °C, but becomes more favorable in both respects as the temperature decreases (Figure 10). This produces the striking result that the rate of transfer increases nearly tenfold as the temperature is decreased below 30 (for midrange vesicles) or 19 °C (for solid vesicles). Presumably this reflects effects of decreased fluidity in the donor membrane, consistent with the notion that protein transfer occurs most readily where the recipient membrane is more fluid than the donor. As for the rate, the fraction of cell AChE transferred at equilibrium increases tenfold below 30 and 19 °C for midrange and solid recipient membranes, respectively.

These observations are consistent with the proposal that proteins subject to intermembrane transfer distribute themselves between membranes at a rate and to an extent governed by the relative fluidity of the membranes. Such proteins partition preferentially into the most fluid membrane available, exhibiting high sensitivity to the phase state of their environment. These findings indicate that the rapid transfer events observed with fluid vesicles are unlikely to occur between naturally occurring membranes. While intermediate-phase-state membranes accept protein at much slower rates and to smaller extents, transfer of proteins can be observed on a time scale of hours even for vesicles that are in pure gel phase at 37 °C. Of course, a number of other membrane properties (particularly net surface charge and glycolipid composition: S. L. Cook & W. H. Huestis, unpublished results) are likely to play an important part in membrane contacts which might lead to protein transfer. Moreover, the behavior of AChE may not be representative of all intrinsic membrane proteins (Enoch et al., 1979). The generality and physiological implications of this phenomenon cannot be defined without further study, but it may be said that from fluidity considerations alone transfer of intrinsic proteins between natural membranes is not forbidden.

Acknowledgments

We thank Dr. Christina Harbury, Department of Medicine, Division of Hematology, Stanford Medical School, for supplying the erythrocytes used in this work. We also thank Dr. Paul Wolber for helpful discussions and Dr. Joseph Berry of the Carnegie Institute for the generous loan of Glan-Taylor polarizing filters.

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Electrochemical Proton Gradient across the Cell Membrane of *Halobacterium halobium*: Effect of *N,N'*-Dicyclohexylcarbodiimide, Relation to Intracellular Adenosine Triphosphate, Adenosine Diphosphate, and Phosphate Concentration, and Influence of the Potassium Gradient†

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ABSTRACT: The proton motive force across the cell membrane of halobacterial cells has been estimated and compared to intracellular values of ATP, ADP, and inorganic phosphate concentrations with respect to the chemiosmotic hypothesis. The accumulation of ^{14}C-labeled indicator substances, triphenylmethylphosphonium for the membrane potential and 5,5-dimethyloxazolidine-2,4-dione for the pH difference between the cell interior and the medium, has been measured in the cells. Values up to 270 mV for the proton motive force have been found in cells pretreated with *N,N'*-dicyclohexylcarbodiimide (DCCD, 10^{-4} M, 30 °C, 12 h). Upon illumination a high membrane potential is generated, which is then gradually replaced by a large pH difference. Cells treated with lower DCCD concentrations show only an enhancement of membrane potential upon illumination; the pH difference remains at a low level. Under anaerobic dark conditions, untreated cells maintain a proton motive force of 120–140 mV, which is equilibrated with the intracellular levels of ATP, ADP, and inorganic phosphate. The pH gradient is 1 unit at pH 6 but 0 at pH 8. The membrane potential is low (60–80 mV) at pH 6 and high (120–130 mV) at pH 8. We propose that the proton translocating ATPase compensates for the lowered pH difference at high external pH values by enhancing the membrane potential. The concentration difference of the potassium ions influences the proton motive force and the intracellular ATP levels, apparently via its action on the membrane potential. When the difference of the chemical potential of the potassium ion, expressed in millivolts, exceeds the preexisting membrane potential, the intracellular ATP level is enhanced. When the difference of the chemical potential of the potassium ion (millivolts) is smaller than the membrane potential, the ATP level is decreased.

During the last years halobacteria have been of much interest in biochemical and biophysical research. Under oxygen-limited growth conditions they synthesize a retinal–protein complex known as bacteriorhodopsin (Oesterhelt & Stoeckenius, 1971), which mediates light energy conversion (Oesterhelt & Stoeckenius, 1973).

In intact cells light has been found to drive ATP synthesis (Danon & Stoeckenius, 1974; Oesterhelt, 1974; Hartmann & Oesterhelt, 1977), to inhibit respiration (Oesterhelt & Krippahl, 1973), and to drive the uptake of amino acids (Hubbard et al., 1976) and potassium (Wagner et al., 1978). This light energy conversion of halobacteria is best explained by the chemiosmotic hypothesis (Mitchell, 1966, 1968): bacteriorhodopsin acts as an electrogenic proton pump by releasing protons at the outside of the cell membrane and taking up protons from the inside (Oesterhelt & Stoeckenius, 1973). This process creates an electric potential difference ($\Delta\psi$)[1] across the cell membrane, the so-called membrane potential,

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[1] Abbreviations and symbols used: DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium ion; $TPMP^+_i$ and $TPMP^+_o$, intra- and extracellular $TPMP^+$; DCCD, *N,N'*-dicyclohexylcarbodiimide; $\Delta\psi$, electric potential difference across the cell membrane; pH_o, extracellular pH; pH_i, intracellular pH; ΔpH, pH_o minus pH_i; *n*, number of protons translocated per molecule of ATP synthesized or hydrolyzed; $\Delta\bar{\mu}_{H^+}/F$, electrochemical potential difference of the proton across the membrane or the proton motive force (expressed in millivolts); basal salt, 4.3 M NaCl, 27 mM KCl, and 81 mM $MgSO_4$; potassium basal salt, 2.7 M KCl, 1.6 M NaCl, and 81 mM $MgSO_4$.

0006-2960/80/0419-4607$01.00/0