Effect of Bilayer Cholesterol Content on Reconstituted Human Erythrocyte Sugar Transporter Activity*

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The influence of altered bilayer cholesterol content on the catalytic activity of the human red cell hexose transporter was examined by reconstitution of the transport protein (band 4.5) into bilayers of large unilamellar vesicles formed from dipalmitoyl lecithin and varying amounts of cholesterol. The physical state of the bilayers was characterized by differential scanning calorimetry. The major findings are as follows: 1) changes in bilayer phase behavior occur at membrane cholesterol levels of 15 to 20 mol % and 30 to 40 mol %; and 2) the catalytic activity of the reconstituted transporter (Vmax/transporter) correlates with bilayer phase behavior. In crystalline bilayers, this is seen as an abrupt, stimulation of activity at 15 mol % cholesterol (which is reversed at 17.5 mol %) and a gradual acceleration of activity between 30 to 40 mol % cholesterol. In fluid bilayers (where activity is high), activity is unaffected by 10, 20, and 30 mol % cholesterol. However, 12.5 and 17.5 mol % cholesterol reduce activity by 100-fold. These studies demonstrate that small changes in bilayer cholesterol content result in drastic alterations in transporter activity. Transporter sensitivity to cholesterol is a complex rather than monotonic function of bilayer cholesterol content and appears to be primarily determined by bilayer composition rather than by bilayer “fluidity.”

Recent calorimetric studies have suggested that changing the cholesterol content of membrane bilayers from below 15 to above 20 mol % cholesterol (1, 2) results in a major molecular reorganization within the bilayer. Over this narrow range of membrane cholesterol, the lateral mobility of bilayer lipids is drastically altered (3), an event demonstrated by dilatometry to coincide with an abrupt change in bilayer lipid packing (4). Although of potential biological importance, the possible consequences of this cholesterol-induced bilayer rearrangement on membrane enzyme activities have remained unexplored.

Progressive depletion of native erythrocyte membrane cholesterol content has been demonstrated to first accelerate then reduce hexose transfer activity (5). In various studies using a variety of bilayer probe molecules, the cholesterol depletion of erythrocyte membranes has been reported to either increase (6) or decrease (7) membrane fluidity. These findings emphasize problems routinely encountered in studying specific membrane enzymes in their native membrane; namely that using the complex, heterogeneous native membrane, it is neither possible to incisively interpret data in terms of specific lipid/protein interactions, nor in terms of altered molecular organization within the protein’s “microenvironment.” The approach we have adopted is to reconstitute the purified human erythrocyte hexose transport protein into the lipid bilayers of large unilamellar vesicles (LUVs) of preselected phospholipid and cholesterol composition (8). With such a system, bilayer physical state (crystalline/liquid) may be determined by differential scanning calorimetry (DSC) (9), a technique that monitors the heat required to melt the bilayers and the temperature over which this occurs. Protein activity (flux/transport protein) may be monitored by combined kinetic analysis of protein-mediated trans-bilayer sugar fluxes and reconstituted protein cytochalasin B binding activity (2, 8, 10). Both may then be expressed as a function of bilayer cholesterol content, and the relationships between cholesterol content, physical state, and protein-mediated hexose transport may then be directly addressed.

The use of an integral membrane protein to evaluate bilayer organization may be of unique value. Should the protein be sensitive to its membrane environment, then the importance of membrane lipid composition in the expression of integral membrane protein activity will be firmly established. Moreover, any perturbation of bilayer structure brought about by the protein is “natural” and thus accurately reflects the biological state. Our choice of protein, the human erythrocyte hexose transport protein (a 55-kDa bilayer-spanning glycoprotein), is based upon its physiological importance and well characterized kinetic (11) and structural (12, 13) properties, as well as its relative abundance (11), ease of purification (14), and ease of reconstitution into large unilamellar vesicles of homogeneous size distribution (8). In addition, as the protein facilitates the passive translocation of d-stereoisomers of hexose in the pyranose ring form, the assay of protein activity is uncomplicated by additional consideration of reactions and complex gradient dependencies typical of active transport systems such as cation-sugar cotransporters (15) or the (Na⁺K⁺)ATPase (16).

Here we report that the activity of the purified, reconstituted human erythrocyte hexose transport protein (band 4.5) is critically dependent upon the cholesterol content of its resident bilayer. Further, increasing bilayer cholesterol over the narrow range of 17.5 to 20 mol % abruptly transforms the transporter from an almost fully inhibited state to one of maximum catalytic activity. Our enzymatic studies are in full accord with the aforementioned physical studies of pure lipid bilayers and run counter to the view that progressively increasing membrane cholesterol content monotonically transforms the bilayer state to one of intermediate fluidity (6).
Materials and Methods
Solutions—NaCl medium consisted of 25 mM NaCl, 5 mM Tris-HCl, 2 mM EDTA, pH 7.4.

Purification of Human Red Cell Band 4.5 Protein—Purification of human erythrocyte band 4.5 protein (a 55-kDa glycoprotein) was carried out as reported previously (8, 10). This preparation consists of band 4.5 protein, red cell lipid (1 mol of lipid/0.75 ± 0.05 mol of protein), and band 7 protein (0.2 ± 0.03 mol of protein). The cytochalasin B binding activity of this preparation is 14 nmol/mg of protein (a 15-fold purification of native activity) and after correction for contamination by band 7 protein represents a ratio of binding sites/transport protein of 0.82:1.

Transport Determinations—Influx of D-glucose was monitored under conditions where the external sugar level is saturating (100 mM). This condition is, by convention, referred to as infinite-sis entry (11, 23). Vmax and Km for entry are then obtained by application of the classical linearized form of the integrated infinite-sis equation to the time course of equilibration of glucose-free vesicles with external sugar (8, 10, 23). Reconstituted LUV diameter was estimated using the Coulter N4 Submicron Particle Analyzer (8). In all cases, cholesterol neither modified the narrow LUV size distribution produced by reconstitution by reverse phase evaporation (8) nor the efficiency of protein reconstitution (60–66% here; see also Ref. 8). Sugar transport determinations were made by turbidimetry (8, 10, 19) and by use of 18O-labeled D-glucose (10). At low sugar concentrations (0.5–10 mM), D-[18O]glucose uptake was consistently described by a monoeponential process indicating the absence of polydispersity in LUV transporter content.

With turbidimetric measurements, LUVs containing NaCl medium were first preincubated in D-glucose-free medium (±0.1 mM phloretin and 1 mM Tris-HCl) at the temperature of interest then incubated (0.5 to 3 μl of LUVs—6.0 × 10^10 to 4.0 × 10^10 LUVs) into 400 μl of NaCl medium containing 100 mM D-glucose (±inhibitors). Mixing was complete within 100 ms and the time course of entry was monitored at sample intervals of 50 to 100,000 ms (2). The transport activity of the reconstituted systems was, in all cases, inhibited (>98%) by 100 μM phloretin and 50 μM cytochalasin B. Phloretin and cytochalasin B are potent competitive inhibitors of red cell D-glucose entry and exit, respectively (11, 19). The permeability coefficient for saturable (inhibitor-sensitive) D-glucose transport (Vmax/Km, 11) ranged from 7.7 ± 0.3 × 10^-9 cm·s^-1 in DPL:cholesterol (87.5:12.5 molar ratio) LUVs at 4°C to 16 ± 2 × 10^-9 cm·s^-1 in pure DPL LUVs at 55°C. The remaining component (permeability coefficient = 4 × 10^-10 (4°C, 12.5 mol % cholesterol) to 5 × 10^-9 cm·s^-1 (55°C, DPL LUVs)) was typical of diffusion across protein-free bilayers (2). The kinetic parameters for zero-trans D-glucose efflux (efflux into sugar-free medium) in human red cells were determined as described previously (17).

Analytical Procedures—Carry-through of red cell lipid into the reconstituted LUVs was assessed by fatty acid analysis of LUVs by gas chromatography (10). This contamination by red cell lipid amounted to less than 0.7 mol % in all cases and was without effect on the calorigraphic behavior of the LUVs. Cytochalasin B binding assays of Triton X-100 preparations were performed in the absence and presence of 500 mM D-glucose as described previously (8, 10). The glucose-displaceable component of steady state binding is presumed to represent binding to band 4.5 protein (11, 18, 19). Protein assays were as described by Lowry et al. (20) with modification by Yu and Steck (21). Triton X-100 assays, lipid extractions from LUVs, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were as previously reported (8, 10). The physical state of reconstituted LUV bilayers was determined by DSC using the DuPont Instruments 1090 Thermal Analyzer (2, 8, 10).

Results
Fig. 1 summarizes both the temperature dependence of D-glucose transport activity (Vmax/cytochalasin B binding protein reconstituted) and the phase behavior (melting properties) of LUVs formed from DPL, with varying cholesterol concentrations of up to 50 mol %, and the sugar transport reconstituted. Above 50 mol % cholesterol no longer interdigitates within bilayers (22). The calorigrometrically determined phase behavior of the reconstituted LUVs (Fig. 1a) is similar to that reported for protein-free systems (1–3). Reconstituted LUVs formed from pure DPL and transporter underg a liquid crystalline-to-fluid phase transition (melt) at 40.8°C (the onset temperature, Tm). This melt is complete at 44°C (completion temperature, Tc). Below 40.8°C the bilayers in a crystalline state and above 44°C are in a fluid state (1, 2, 4). This narrow transition is seen at all cholesterol levels examined except 15, 20, and 35 mol % where the transition is broadened (Tm is lowered and Tc is raised) and at 50 mol % where no thermal events are seen. The thermal behavior of this broad melt (which is first detected at 15 mol % cholesterol) is suggestive of a lipid phase distinct from pure DPL. The broad melt is absent at 17.5 mol % cholesterol, then reappears at 20 to 30 mol % cholesterol with much lower Tm, raised Tc, and diminishing heat as cholesterol content is raised. At low temperatures, therefore, pure, crystalline DPL lipid packets or domains appear to coexist with both fluid and
crystalline DPL-cholesterol phases, the relative proportions being dependent upon both temperature and cholesterol levels. DSC is insensitive to lipid domains in fully fluid bilayers.

Fig. 1b summarizes the dependence of transport activity upon bilayer cholesterol content at two temperatures, 10°C where pure DPL bilayers are in the crystalline state and 55°C where pure DPL bilayers are in the fluid state. At 10°C, no significant protein-mediated transport activity is detected in cholesterol-free bilayers; on melting the bilayers, however, activity increases (e.g. see 55°C). In the absence of translocase activity in crystalline DPL bilayers, D-glucose-sensitive cytochalasin B binding to the reconstituted protein is readily discernible (Kf for inhibition of binding by D-glucose = 10.5 ± 1.3 mM; n = 12; Kf for transport between 33 and 59°C = 11.4 ± 1.8 mM to 16.9 ± 0.9 mM, n = 87) indicating that translocation, not substrate binding, may be the step inhibited in crystalline bilayers. At 10°C, further increases in bilayer cholesterol to 12.5 mol% are without effect on transport. However, activity abruptly increases at 15 mol% cholesterol then disappears at 17.5 mol% cholesterol. This behavior parallels the phase behavior of the bilayers and suggests that the transport protein is sensitive to the presence of the DPL-cholesterol phase detected at 15 mol% cholesterol. Further increases in cholesterol at 10°C is without effect on activity until 30-40 mol%. Thereafter, activity increases. The cholesterol dependence of transport activity at 55°C is more complex. Vmax/site at 6 and 10 mol% cholesterol are high. At 12.5, 15, and 17.5 mol% cholesterol, triphasic behavior is observed which again parallels the phase behavior of the bilayers. At 20 and 30 mol% cholesterol, activity is indistinguishable from that observed in pure DPL LUVs and thereafter falls with increasing bilayer cholesterol content. At 50 mol% cholesterol, activity at 10 and 55°C are identical. The full temperature dependence of Vmax/reconstituted protein at all cholesterol levels studied is shown in Fig. 2. While this figure is somewhat complex, it is apparent from a cursory inspection that bilayer cholesterol content can alter transport activity by many orders of magnitude over a wide range of temperatures, in addition to those temperatures displayed as examples in Fig. 1. These data emphasize that at around 15 mol% bilayer cholesterol content, reconstituted sugar transporter activity shows an abrupt deviance from its behavior at cholesterol levels of between 0 and 30 mol%. Moreover, activity at 15 mol% cholesterol is similar to that of cholesterol-rich bilayers (40 and 50 mol% cholesterol) in both its absolute value and its lack of strong temperature dependence. The greatest increase in transport activity in pure DPL LUVs is observed following the small pretransition characteristic of lecithin bilayers and prior to the main bilayer transition. This sensitivity to the DPL bilayer pretransition was also observed in our previous studies (8). Included in this diagram are data obtained from transport studies with the human red cell. Vmax/site was calculated assuming 3 × 10⁶ cytochalasin B binding sites/red cell (11, 19) and indicates the close correspondence between Vmax/site for transport in native and reconstituted membranes.

The temperature dependence of transport activity in these various bilayers is summarized in Fig. 3, a and b. These are plots of activation energy (Ea) and Arrhenius constants (A) obtained from Arrhenius plots of the data shown in Fig. 2. The marked curvatures in the plots of Fig. 2 (e.g. cholesterol mol% 0, 10, 20, and 30) were omitted from these analyses. As the parameter measured is Vmax/site, e-EdRT provides an estimate of the fraction of carrier-substrate complexes with sufficient energy to be committed to translocation at any point in time at a given temperature. For enzyme systems, A is related to the product of the probability of the reaction occurring when the reactants collide and the collision frequency of the reactants (24). Fig. 3 illustrates that both Ea and A are severely attenuated between 12.5 and 17.5 mol% cholesterol, recover to values typical of pure DPL between 17.5 and 20 mol% cholesterol, then at levels greater than 30 mol% cholesterol again fall markedly.

**Fig. 2.** Temperature dependence of Vmax/cytochalasin B binding protein in DPL/cholesterol bilayers. Ordinate, Vmax/cytochalasin B binding site (mol/site%), Abscissa, temperature (°C). These systems are those described in Fig. 1. The various cholesterol levels are 0 (○), 10 (□), 12.5 (×), 15 (□), 17.5 (□), 20 (△), 30 (+), 40 (V), and 50 (A) mol%. Also included are data for transport in native red cell membranes (△). Vmax/site was calculated assuming 3 × 10⁶ D-glucose-sensitive cytochalasin B binding sites/red cell (11, 29). The cholesterol content of human red cell membranes is 42 ± 3 mol% of total lipid (2). Where the curve is dropped vertically to the x axis, this indicates that zero transport activity was detected below this temperature. Each point consists of at least five determinations/reconstitution, the number of reconstitutions being three or more. Variation between reconstitutions was less than ±25%.

**Fig. 3.** Cholesterol dependence of the thermal behavior of reconstituted transport. A, ordinate, activation energy (Ea) in kcal/mol/K; abscissa, mol% cholesterol. B, ordinate, Arrhenius constant (A); abscissa, as in A. The data of Fig. 2 were plotted as an Arrhenius graph, and the line of best fit was calculated by the method of least squares. As the measured parameter is Vmax, the Arrhenius equation takes the form Vmax = A e-EdRT where R is the gas constant and T the absolute temperature. Data for 48 mol% cholesterol were obtained from previous studies (8). Ea for transport in 45 mol% cholesterol was obtained from runs in which cytochalasin B binding assays were not performed.
**DISCUSSION**

The results of our DSC and transport studies are in agreement with physical studies of synthetic membranes indicating that significant reorganization of bilayer lipid packing occurs at membrane cholesterol levels both between 15 and 20 mol % and 30 and 40 mol % (1–4). These findings run counter to the view that cholesterol monotonically transforms the bilayer into a state of intermediate fluidity (6). This study also confirms and significantly extends our previous findings showing that the absolute activity of the reconstituted hexose transfer protein is governed by the lipid composition of its resident bilayer (8). This is not unexpected because, by analogy with water-soluble enzymes, we might expect that integral membrane proteins would be sensitive to the “state” of their solvent, the lipid bilayer.

A number of previous studies have suggested models to account for the influence of membrane lipids on membrane protein activity (6, 25). The fluidity hypothesis suggests that substrate-induced conformational changes within the transporter molecules are rate-limited by the fluidity of the membrane bilayer (25). Thus as membrane fluidity increases, so transport activity is stimulated. However, recent observations have suggested that while altered membrane fluidity may indeed modify the activity of integral membrane proteins, more important bilayer determinants of protein activity appear to be bilayer lipid acyl chain length and lipid class (8, 26). Studies with the reconstituted sarcoplasmic reticulum (Ca"²-Mg"²) -dependent ATPase found no consistent relationship between bilayer fluidity and ATPase activity (26). Rather, activity was directly related to bilayer lipid acyl chain length. Similarly, the absolute activity of the human red cell hexose transfer protein reconstituted into lecithin bilayers was found to be primarily dependent upon lecithin acyl chain length and saturation/unsaturation in a manner independent of the fluidity of the resultant bilayers (8). Nevertheless, the activity of the reconstituted hexose transporter in bilayers formed from a single lecithin was found to increase during the liquid crystalline-to-fluid phase transition (8).

The results of this present study are in keeping with the findings described above. The behavior of the protein in cholesterol-rich DPL bilayers (40 to 50 mol % cholesterol) is consistent with the fluidity hypothesis. Cholesterol at these levels fluidizes crystalline DPL bilayers while condensing fluid DPL bilayers (4). Accordingly, these high cholesterol levels serve to inhibit activity in fluid membranes while stimulating activity in crystalline membranes. The behavior of the protein in DPL bilayers containing 12.5 to 20 mol % cholesterol, however, is not explained by the fluidity hypothesis thus suggesting that the activity of the protein is sensitive to other membrane factors, possibly compositional or steric. Moreover, detailed analysis of the effects of high cholesterol levels on transport activity indicates that activity may be independent of cholesterol’s effects on lipid packing (8, 29).

In view of the 100-fold variation in measurable protein activity over the cholesterol levels employed in this study and the ubiquity of cholesterol in eukaryotic cells, these data give rise to the real possibility that alterations in bilayer cholesterol could be used in nature to regulate membrane enzyme activities. This could occur both in intracellular membranes where cholesterol levels are within the range of 15 to 20 mol % (27) and in the plasma membrane within cholesterol-rich or -poor domains (28). Our data also suggest that relatively small changes in the cholesterol content of various tissues of an organism (brought about by factors such as diet, exercise, drug therapy, etc.) may alter membrane functions to a much greater extent than has hitherto been allowed.

*Note Added in Proof—An extended study of the changes in reconstituted sugar transport activity brought about by altered bilayer cholesterol content is currently in press (29).*

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