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Preferential association of membrane phospholipids with the human erythrocyte hexose transporter

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Abstract

This study reports the results of an investigation to determine to what extent the influence of membrane lipids on the human erythrocyte sugar transporter protein activity (Caruthers, A. and Melchior, D.L. (1988) Annu. Rev. Physiol. 50, 257–271) is related to lipid/protein associations in the membrane bilayer. Differential scanning calorimetry was carried out on the human erythrocyte transport protein reconstituted into artificial bilayers formed from preselected lipids. It was found that the transport protein displays a preferential and in some cases strongly preferential affinity for specific lipid types. This association is a function of lipid head group, backbone and hydrocarbon chain length. It appears that the affinity of the transport protein for various lipids can correlate with the lipid's ability to influence transporter activity. This study further suggests that certain lipids (in this case sphingomyelin) can induce an oligomeric association of HEST monomers in the bilayer.

Keywords: Protein-lipid association; Liposome; Erythrocyte sugar transport protein; DSC; Enzyme reconstitution; Lipid bilayer; Membrane protein; (Human)

1. Introduction

The activities of many membrane enzymes and transport proteins have been demonstrated to be governed by their bilayer lipid environment (for reviews see [1,2]). An important membrane protein displaying this characteristic is the human erythrocyte sugar transport protein [3]. This protein is a 55 kDa integral membrane glycoprotein [4] that facilitates the passive diffusion of D-glucose across the cell membrane [5]. The purified protein can be reconstituted into artificial bilayers of preselected lipid composition [6,7] and its transport characteristics determined as a function of bilayer composition and physical state [3].

Using this approach, we have demonstrated in artificial membranes that HEST transport activity is a function of the overall lipid composition and physical state of the membrane bilayer in which the HEST is embedded (for reviews see [8,9]). We have shown that in native membranes certain lipids, even in trace amounts (< 0.03 mol% membrane lipid), can significantly alter the activity of the sugar transporter [10,11].

More specifically, our studies to date have demonstrated that the catalytic activity of the human erythrocyte sugar transporter is influenced (in decreasing order of importance) by bilayer lipid class-'lipid head group' [12]; bilayer lipid acyl chain carbon number, saturation/unsaturation (possibly manifested as bilayer thickness) [13]; 'lipid backbone' [12]; and to the least extent bilayer order-'fluidity' [12–15]. In addition, we have demonstrated that cholesterol has certain marked effects on the activity of the transporter and that these are not related to the sterol's ability to modify 'membrane fluidity' [14,15].

An understanding of the precise molecular basis of these effects awaits the development of a detailed understanding of the relationship of how lipids interact with the transporter to determine transporter tertiary structure and consequent catalytic activity. An initial question in this direction is whether certain lipids have stronger associa-

Abbreviations: HEST, human erythrocyte sugar transporter; DSC, differential scanning calorimetry; T_n, turnover number; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMPG, dimyristoylglycerol; DMPA, dimyristoylphosphatidic acid; Sph, sphingomyelin.

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tions with the transporter than others. The native red blood cell membrane in which the HEST resides in vivo is comprised of numerous lipid species. A preferential association of the HEST with certain lipid species would be a factor in determining its in vivo activity. Further, the preference of the HEST for certain lipids could result in the transporter residing in putative lipid domains [16] (consisting of lipids for which it has a preferential association) in the native red blood cell membrane [3,15].

In this study, we report on the application of Differential Scanning Calorimetry (DSC) to investigate the preferential affinity of different lipid classes and species to the HEST in artificial membranes. The use of DSC to investigate protein/lipid interactions has many advantages [17]. Low amounts of protein are required, measurements can be made relatively rapidly, variables such as pH and ion concentration are easily controlled, no probes are involved and the interpretation of data is relatively straight forward.

We find large differences in the extent of association of different lipids with HEST. These affinities appear related to such factors as lipid headgroup, backbone and hydrocarbon chain length. This study demonstrates the competitive preferential association of the HEST for one lipid type over another in bilayers of mixed composition. Our results also indicate that certain lipid classes present in the red blood cell membrane may facilitate the aggregation of protein monomers into an oligomeric form.

2. Materials and methods

2.1. Analytical procedures

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (PAGE) was carried out on 8–10% gels as described by Laemmli [18]. Phospholipid phosphorus assays were as described by Bartlett [19]. Phospholipid purity was confirmed by DSC and thin-layer chromatography. Protein assays were as described by Lowry et al. [20] as modified by Yu and Steck [21]. Triton X-100 assays were performed according to the method of Garewal [22] with modification by Lukacovic et al. [23].

Differential scanning calorimetry (DSC) was carried out using Du Pont Instrument's 1090 Thermal Analyzer [24]. DSC pans were loaded with proteoliposomes (0.3–1.5 mg of lipid) and scanned at 3° C/min. Slower scans resulted in identical thermograms. Integration of thermograms, determination of onset temperatures, and decomposition of curves were performed by computer using the Du Pont general analysis utility program (Version 1.0).

2.2. Preparation of the HEST

The human erythrocyte sugar transport protein (protein band 4.5, a 55 kDa glycoprotein) was purified by a modification of the method described previously [12]. Briefly human erythrocyte ghosts were prepared as described by [25]. Integral membrane proteins (bands 3, 4.2, 4.5, 6 and trace amounts of 7) were eluted from the resulting ghosts by a 30 min agitation-free incubation at 1° C in a detergent medium consisting of 25 mM NaCl, 5 mM Tris-HCl, 0.2 mM EDTA, and 0.5% Triton X-100, pH 8 (5 volumes of detergent medium/1 volume of white ghosts) and collected as the supernatant fraction of the centrifuged material. Band 4.5 eluate was obtained by passing this supernatant (approx. 40 ml) over a DEAE-cellulose column (Whatman DE 52; 8 cm × 3 cm column equilibrated with detergent medium; flow rate 35 ml/h) and collecting 6 bed volumes of eluate. The remaining integral membrane proteins were eluted from the column by using detergent containing 1 M NaCl. The band 4.5 eluate was concentrated some 5–6-fold by ultrafiltration (Diaflo YM-10 membranes) and then treated with Bio-Beads (Bio-Rad SM2; prewashed with methanol followed by distilled water followed by NaCl medium; 1 volume of wet beads to 1 volume of protein solution) for at least 4 h at 4° C to remove detergent. The remaining suspension consists of band 4.5 protein (4–8) mg and minor protein contaminants (as verified by PAGE) and lipid (8–16 mg) in the form of particles of average diameter 0.1–0.6 μm as determined by a Coulter Model N4 Sub-Micron Particle Analyzer (Coulter, Hialeah, FL). These particles are osmotically inactive and, under phase contrast microscopy are amorphous in appearance.

In order to separate the band 4.5 protein from these lipid particles, the above suspension was pelleted by ultracentrifugation at 38 000 rpm for 60 min. Buffer containing 0.5% Triton X-100 was added (3 ml Triton X-100 buffer solution per 3 mg protein) and the suspension agitated for 30 min at 4° C. The resulting solution was layered on a 5–20% sucrose gradient and spun at 38 000 rpm in an ultracentrifuge for 18 h. This treatment resulted in a pellet at the bottom of the centrifuge tubes and a yellowish opaque band at the top of the tubes. Protein, lipid, and Triton X-100 assays were performed on the pellet and the top portion of the centrifuge tubes. The top portion of the gradient was found to contain lipid and Triton X-100. The pellet contained no detectable Triton X-100, approx. 66% of the original protein and lipid at a molar ratio of HEST to lipid of 1 to 10. The pellet was washed in buffer (10 mM Hepes (Sigma, St. Louis, MO), pH 7.4) to remove sucrose.

2.3. Transporter reconstitution

Proteoliposomes were prepared by the method of Doyle et al. (unpublished data). Briefly, 2 ml Hepes buffer were added to an appropriate amount of lipid (Avanti Polar Lipids, Birmingham, AL) and vortexed above its phase transition temperature. For the DMPC/DSPC bilayers, prior to the addition of buffer appropriate amounts of both these lipids were dissolved together in chloroform, the

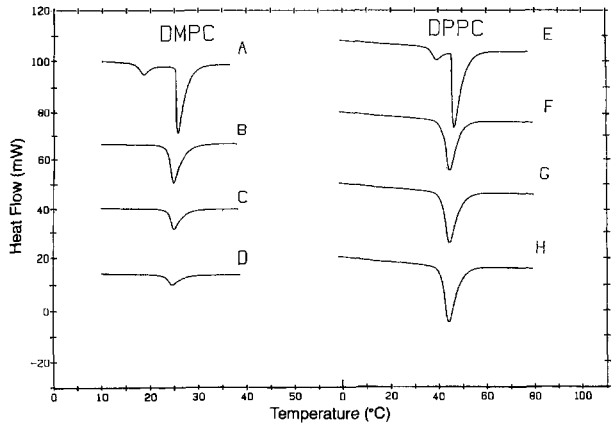

Fig. 1. Thermograms of bilayers formed from DMPC or from DPPC with varying concentrations of HEST. DMPC bilayers contain (A) 0, (B) 0.4, (C) 0.8, (D) 1.0 mol% HEST and DPPC bilayers contain (E) 0, (F) 0.2, (G) 0.6, (H) 1.2 mol% HEST.

chloroform evaporated under a stream of nitrogen and the co-crystallized lipids then put under vacuum (100 μmHg) for 1 h to remove residual solvent before the addition of buffer.

The lipid suspensions were added to the desired amount of purified band 4.5 and this mixture extruded 2–3-times through a French Press (SLM Instruments, Urbana, IL). The preparations were freeze-thawed three times to give larger vesicles in order to speed up centrifugation times. The freeze-thawed vesicles were spun at 100 000 $\times$ g for 1 h to concentrate the vesicles prior to loading in the DSC pans.

A single population of vesicles is formed by this procedure (Doyle et al.) Density ultracentrifugation in a 5–20% sucrose gradient gives a single band. DSC results in thermograms indicative of homogeneous lipid bilayers. FTIR spectra of transporter incorporated into extruded proteoliposomes gave spectra characteristic of the functional reconstituted transporter as employed in previous transport studies (Chasan and Melchior, unpublished results).

3. Results and discussion

In Fig. 1 are presented representative thermograms of proteoliposomes containing varying amounts of the HEST. In Fig. 1A, B, C and D are presented thermograms of proteoliposomes formed from dimyristoylphosphatidylcholine containing the HEST at bilayer protein to lipid ratios of 0, 0.4, and 0.8, and 1.0 mol%, respectively; E, F, G, and H are thermograms of proteoliposomes formed from dipalmitoylphosphatidylcholine containing the HEST at bilayer concentrations of 0, 0.2, 0.6, and 1.2 mol%. Pure DMPC bilayers have a $\beta \rightarrow \alpha$ a phase transition onset at 23° C and DPPC a $\beta \rightarrow \alpha$ phase transition onset at 42° C [26]. The presence of low amounts of protein serves to suppress the bilayer pretransition as is the case for most heterogeneous matter introduced into pure synthetic diacylphosphatidylcholine bilayers [24]. The diminution of the enthalpy of the main bilayer phase transition with increasing bilayer HEST content, however, reflects the association of bilayer lipid with protein molecules present in the bilayer [17]. Lipid associated with the HEST is unable to participate in the cooperative melting process characteristic of specific lipids in the bilayer conformation.

In order to determine if the ability of bilayer lipids known to affect HEST activity [3,8,9] results from tight molecular associations with the HEST, a DSC investigation was carried out using various sets of lipid molecules. These sets were designed to investigate the possible role of lipid headgroup, hydrocarbon chain length and backbone on the affinity of the lipid to the HEST.

Fig. 2 is a plot of the heats of transition for three bilayer types, each containing varying amounts HEST. Each bilayer type is composed of a different class of glycero-phospholipid. These lipid classes were chosen to vary in their head groups, but to all contain the same length acyl hydrocarbon chains (C14). Those lipids studied were dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidic acid (DMPA). The onset of the bilayer melt for pure bilayers of these lipids is 24° C, 21.5° C and 47.3° C respectively [12]. It was observed that diminution of the bilayer transition with increasing HEST concentration is

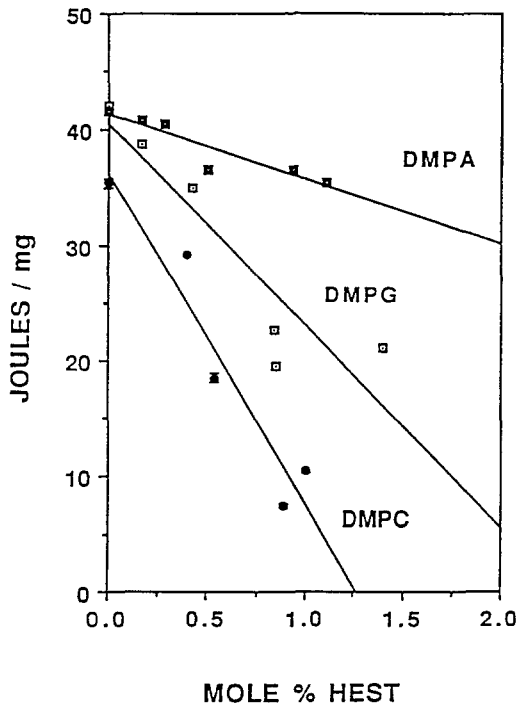

Fig. 2. Plot of transition enthalpies for various bilayers formed from different dimyristoyl-lipid classes as a function of increasing bilayer concentrations of HEST to bilayer lipid: DMPA, dimyristoylphosphatidic acid: DMPG, dimyristoylphosphatidylglycerol: DMPC, dimyristoylphoshatidylcholine.

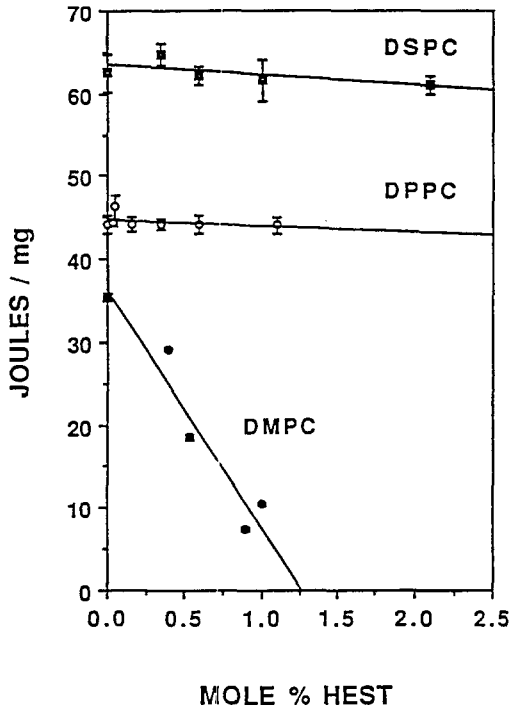

Fig. 3. Plot of bilayer transition enthalpies of various bilayers each formed from phosphatidyl choline with different fatty acyl chains as a function of the molar concentration of HEST to bilayer lipid.

most marked in DMPC bilayers, next most marked in DMPG bilayers, and least marked in DMPA bilayers. Bilayer enthalpy extrapolates to zero at 1.26 mol% HEST in DMPC, 2.34 mol% HEST in DMPG and 7.44 mol% HEST in DMPA.

These findings on the strength of association of various lipids with the HEST correlate with our earlier functional studies on the effect of bilayer lipid headgroup on HEST activity [12]. It was observed that protein mediated sugar turnover number (T_n) is inversely related to lipid head group charge and increases markedly in the order DMPC < DMPG < DMPA. We suggested that the turnover number might in some way be sensitive to bilayer surface potential or charge [27] which even at physiological pH increases in the same order as HEST T_n. In view of this current study where the affinity of bilayer lipids for HEST was found to be DMPA < DMPG < DMPC, it might be suggested that a tighter association of bilayer lipid with HEST is a factor in reducing HEST T_n.

Fig. 3 shows plots of the heats of transition for three bilayer types with varying amounts HEST, each bilayer type being formed from a different species of the same glycero-phospholipid, phosphatidylcholine. In this study, while the lipids comprising the different bilayers have the same headgroup, they vary in the length of their acyl hydrocarbon chains: dimyristoylphosphatidylcholine (DMPC) – (C14), dipalmitoylphosphatidylcholine (DPPC) – (C16), distearoylphosphatidylcholine (DSPC) – (C18). The onset of bilayer melt for pure bilayers of these lipids is 24° C, 42° C and 54° C, respectively [28]. Unlike the lipids presented in Fig. 2, the HEST appears to have an affinity only for only one of the lipids investigated, DMPC.

Fig. 4 shows the results of a study on the association of the HEST for lipids in two bilayer types. These bilayers are formed from either the phosphoglycerolipid, dimyristoylphosphatidylcholine (DMPC) or the sphingophospholipid, sphingomyelin (Sph). These two lipid species both posses the phosphatidylcholine head group but differ in backbone. For both types of bilayers, increasing concentrations of HEST up to about 0.5 mol% result in a very similar progressive reduction in transition enthalpy, presumably reflecting a progressive association of bilayer lipid with progressively increasing amounts of bilayer protein. Above 0.5 mol% protein, however, the two curves differ markedly. While in DMPC bilayers increasing concentrations of HEST continue to reduce the DMPC bilayer transition enthalpy, in Sph bilayers, increasing concentrations of HEST causes a reversal of the curve with an increasing transition enthalpy until at about 1.25 mol% bilayer HEST, the transition enthalpy reaches 30 J/mg and remaining at this value even for further increasing concentrations of HEST.

This reversal might result from HEST monomers beginning to aggregate at bilayer concentrations of HEST above about 0.5 mol%. In this hypothesis, the oligomeric form of the HEST would have a weak interaction with bilayer sphingomyelin. Concentrations of HEST at concentrations

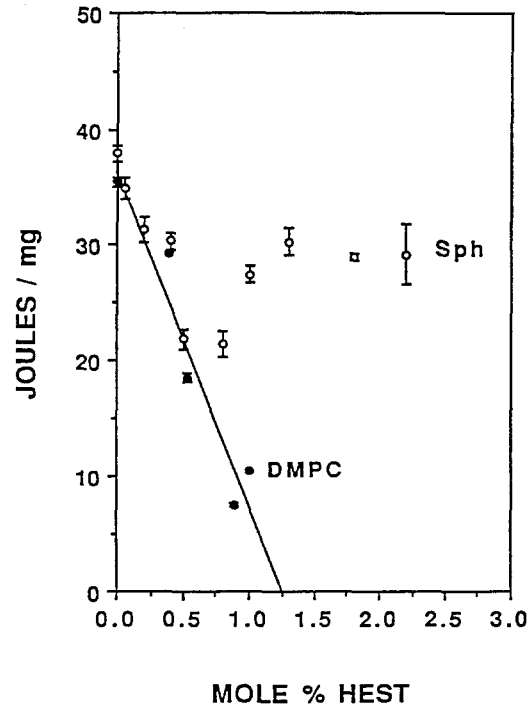

Fig. 4. Plot of bilayer transition enthalpies for bilayers formed from either of two different choline containing phospholipid classes as a function of molar concentrations of HEST to bilayer lipid: dimyristoyl phosphatidylcholine (DMPC) or sphingomyelin (Sph).

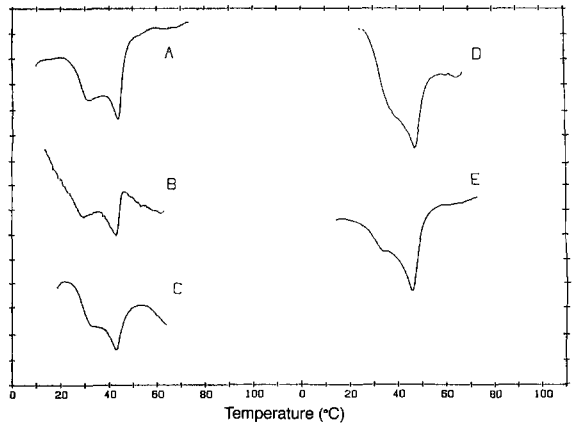

Fig. 5. Thermograms of bilayers formed from a 1:1 molar ratio of dimyristoyl phosphatidylcholine and distearoylphosphatidylcholine and containing different molar concentrations of HEST to bilayer lipid: (A) 0%, (B) 0.25%, (C) 0.5%, (D) 0.67%; (E) 1.0%.

above 1.25 mol% form only oligomers that minimally interact with sphingomyelin. The constant value of 30 J/mg for bilayer enthalpy from the original 38 might result from residual HEST monomers in the bilayer in equilibrium with their oligomeric form. The similarity of the DMPC and Sph curves up to 0.5% HEST suggests that HEST monomers interact similarly with both these phosphocholine containing molecules (compare with Fig. 2 showing lipids with different headgroups). The difference in the curves of DMPC and Sph then, would result from an aggregation of HEST monomers occurring in Sph bilayers but not DMPC bilayers.

Fig. 5 displays thermograms of a mixed lipid bilayer containing different molar concentrations of HEST to total bilayer lipid: (A) 0%, (B) 0.25%, (C) 0.5%, (D) 0.67%; (E) 1.0%. These bilayers were formed from a co-crystallized mixture of dimyristoylphosphatidylcholine and distearoylphosphatidylcholine at a 1:1 molar ratio. Due to nonideal mixing [29,30,28], two shoulders are seen in the thermogram of the bilayer melt. The lower temperature shoulder is primarily due to the melting of DMPC and the higher primarily due to the melting of DSPC. It is observed that while increasing concentrations of HEST reduces the lower temperature shoulder, the higher temperature shoulder is relatively unaltered by the HEST. This behavior confirms a preferential association of DMPC with the HEST over DSPC in mixed lipid bilayers. This behavior is consistent with our findings shown in Fig. 3, where the HEST shows a strong affinity for DMPC in DMPC bilayers and a minimal association with DSPC in DSPC bilayers.

This study demonstrates that the HEST displays a preferential and in some cases strongly preferential association with specific bilayer lipids. This association is seen to be a function of lipid head group, backbone or hydrocarbon chain length. In certain cases, the strength of this association correlates with the lipid's ability to modulate of HEST activity. This study further suggests, that certain lipids (in this case sphingomyelin) can induce an oligomeric association of HEST monomers in the bilayer.

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