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Biochimica et Biophysica Acta 1468 (2000) 175–186

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Physical properties of liposomes and proteoliposomes prepared from *Escherichia coli* polar lipids

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Received 30 August 1999; received in revised form 15 May 2000; accepted 1 June 2000

Abstract

Reconstituted proteoliposomes serve as experimental systems for the study of membrane enzymes. Osmotic shifts and other changes in the solution environment may influence the structures and membrane properties of phospholipid vesicles (including liposomes, proteoliposomes and biological membrane vesicles) and hence the activities of membrane-associated proteins. Polar lipid extracts from *Escherichia coli* are commonly used in membrane protein reconstitution. The solution environment influenced the phase transition temperature and the diameter of liposomes and proteoliposomes prepared from *E. coli* polar lipid by extrusion. Liposomes prepared from *E. coli* polar lipids differed from dioleoylphosphatidylglycerol liposomes in Young's elastic modulus, yield point for solute leakage and structural response to osmotic shifts, the latter indicated by static light scattering spectroscopy. At high concentrations, NaCl caused aggregation of *E. coli* lipid liposomes that precluded detailed interpretation of light scattering data. Proteoliposomes and liposomes prepared from *E. coli* polar lipids were similar in size, yield point for solute leakage and structural response to osmotic shifts imposed with sucrose as osmolyte. These results will facilitate studies of bacterial enzymes implicated in osmosensing and of other enzymes that are reconstituted in *E. coli* lipid vesicles.

Keywords: Lipid; Liposome; Proteoliposome; Light scattering; Membrane; Differential scanning calorimetry; Melting transition; Phospholipid; Membrane protein; *Escherichia coli*

1. Introduction

The analysis of catalytic mechanism and regulation for membrane enzymes presents particular challenges: biological membranes include diverse, interacting components (both lipids and proteins); membrane proteins are particularly difficult to purify and handle in vitro; and the activities of many membrane enzymes, particularly transporters, can be detected only in experimental systems in which the membrane acts as a boundary between two aqueous compartments. In addition, the activities of some membrane-associated enzymes may be regulated via effects of the solution environment on membrane structure [1]. Our particular interest is in membrane-based osmosensors, integral membrane pro-

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00255-8

teins whose catalytic activities are altered by changes in extracellular (or extra-proteoliposomal) osmolality [1,2].

Researchers who wish to understand the function and regulation of membrane enzymes frequently use proteoliposomes reconstituted from purified membrane proteins and purified lipids or lipid mixtures as experimental systems [3,4]. Diverse phospholipids, many available commercially, are used to prepare proteoliposomes. Since the activities of many membrane enzymes are phospholipid-dependent, phospholipids derived from the membrane that forms the native enzyme environment are often selected for this purpose, at least in the first instance [2,5,6].

Virtually all biological membranes that have been examined include a substantial proportion of phospholipids with a low lamellar to hexagonal or cubic phase transition temperature and hence a significant propensity to form an inverted phase under physiological conditions [7]. For example, like the membranes of other eubacteria and most eukaryotes, the cytoplasmic membrane of the Gram-negative bacterium *Escherichia coli* includes a high proportion (70–75%) of phosphatidylethanolamine (PE) [8]. This phenomenon and the lipid specificity of membrane enzymes have led researchers to propose that a membrane physical property correlated with lipid phase behavior (e.g. intrinsic curvature) may influence or even regulate enzyme activity [9].

The goal of our research is to learn how osmotic shifts and other changes in the solution environment influence the structures and membrane properties of phospholipid vesicles (including liposomes, proteoliposomes and biological membrane vesicles) and how those changes correlate with the activities of membrane-associated proteins. We have previously demonstrated that, when exposed to decreasing extraliposomal osmolality, liposomes prepared with dioleoylphosphatidylglycerol (DOPG) swell to a yield point beyond which solute leakage from the liposome lumen maintains a constant transmembrane osmolality gradient and a constant membrane strain ($\Delta r/r$, where r is liposome radius) [10,11]. Further, changes in membrane thickness and/or hydration, not changes in liposome size/shape, appeared to dominate changes in the optical properties of DOPG liposomes exposed to increasing extraliposomal NaCl concentrations [12].

Since polar lipid extracts from *E. coli* are commonly used in membrane protein reconstitution (e.g. [2,5,6]), and since our ultimate goal is to model the properties of native biological membranes, we have now extended our analysis to this particular preparation. Below, we demonstrate that the physical and optical properties of liposomes prepared from *E. coli* lipid differ from those of DOPG liposomes and that liposomes prepared from *E. coli* lipid serve as an appropriate physical model for proteoliposomes prepared from *E. coli* lipid. We also identify experimental conditions under which changes in liposome structure determined by light scattering spectroscopy can be correlated with changes in membrane enzyme activity determined using structurally analogous proteoliposomes. These results will facilitate studies of bacterial enzymes implicated in osmosensing [2,13,14] and of other enzymes that are reconstituted in *E. coli* lipid vesicles.

2. Materials and methods

2.1. Materials

The polar lipid extract derived from *E. coli* B-11303 and DOPG were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

For shipping, lipids were dissolved in methylene chloride and hermetically sealed in borosilicate glass ampoules under nitrogen. Ampoules were stored at −40°C immediately on arrival. 5(6)-Carboxyfluorescein (CF) and 3,3′-dipropylthiodicarbo-cyanine iodide (diS-C_3-(5)) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals were of reagent grade.

Protein ProP-$(His)_6$ was purified as described by Racher et al. [2]. Liposomes and proteoliposomes were prepared and analyzed in solutions based on MOPS buffer (20 mM 3-*N*- morpholinopropane sulfonic acid (MOPS) and 0.5 mM ethylene diamine tetraacetic acid (EDTA) adjusted to pH 7.4 with NaOH) or phosphate buffer (0.1 M potassium phosphate, 0.5 mM NaEDTA, pH 7.4). Buffer osmolalities were adjusted with NaCl or sucrose as indicated and measured using a vapor pressure osmometer (Wescor, Logan, UT, USA).

2.2. Preparation of liposomes and proteoliposomes

Lipids were quantitatively transferred from ampoules provided by Avanti Polar Lipids, Inc. (Alabaster, AL, USA) (typically 100 mg/4 ml methylene chloride) to a 100 ml round-bottomed flask and the $MeCl_2$ removed by rotary evaporation. The lipid film was dried under vacuum for at least 30 min, then rehydrated in the selected buffer by stirring for 30 min. Lipid dispersions were subjected to 10 freeze-thaw cycles, using liquid N_2 and a 37°C water bath, then stored as aliquots, under argon, at −20°C. All steps were performed under argon in order to limit lipid exposure to air.

Aliquots of frozen and thawed multilamellar vesicles (FATMLVs) were extruded essentially as described by Nayar et al. [15] using a hand held extrusion device [16] obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). DOPG lipid dispersions were passed 15 times through a 19 mm (diameter) Nucleopore polycarbonate filter (Costar, Cambridge, MA, USA) with 100 nm (diameter) pores. *E. coli* lipid dispersions were passed three times through a 19 mm (diameter) Nucleopore polycarbonate filter (Costar, Cambridge, MA, USA) with 400 nm pores and then 15 times through a 200 nm filter from the same source. For some light scattering experiments, the *E. coli* lipid liposomes were additionally passed through a 100 nm filter. Proteoliposomes, containing pure ProP-$(His)_6$, were prepared as described by Racher et al. [2] from *E. coli* FATMLVs (see above) in potassium phosphate buffer (pH 7.4) with or without 0.3 M NaCl, as indicated.

2.3. Analytical techniques

2.3.1. Phospholipid and fatty acid analyses

The composition of the polar lipid extract was analyzed by thin-layer chromatography. Polar lipids were identified by their chromatographic and staining properties [17]. For fatty acid analyses, the polar lipid extract was dried under a stream of nitrogen and saponified with NaOH (15% w/v) in methanol/water (1:1, v/v) for 30 min at 100°C. Fatty acids were methylated by addition of 6 N HCl in methanol/water (1:1, v/v) and incubated for 10 min at 80°C. The resulting fatty acid methyl esters were extracted with hexane/methyl tert-butyl ether (1:1, v/v). This extract was washed with 1.2% aqueous NaOH and analyzed by gas chromatography–mass spectrometry [18,19].

2.3.2. (Proteo)liposome characterization

For differential scanning calorimetry (DSC), extruded liposome samples were put into previously weighed aluminum pans, to a final mass of approximately 15 mg of solution. A similarly prepared pan containing the same mass of appropriate buffer was used as a reference. The pans were then hermetically sealed and the sample temperature was reduced to −30°C before equilibrating at −10°C. Thermograms were recorded from −10°C to +25°C at a 2°C/min heating rate using a Peltier cooled DSC 2910 differential scanning calorimeter (DuPont, Mississauga, Ont., Canada). A single scan was done up to 60°C with no sign of a transition to the hexagonal phase. Resulting endotherms were analyzed to determine the gel to liquid crystalline transition temperatures (T_m) using Thermal Analyst 2000 software (DuPont, Mississauga, Ont., Canada).

For dynamic light scattering (DLS), extruded (proteo)liposomes were diluted to achieve a concentration of approximately 0.06–0.1 mg lipid/ml buffer, transferred to square cuvettes (SO, Hellma, Concord, Ont., Canada), and analyzed by DLS, as previously described [20], using a Model 1096 Autocorrelator (Langley-Ford Instruments, Amherst, MA, USA), an Autosizer 4700 (Malvern, UK) or a BI9000AT Autocorrelator (Brookhaven Instruments Corp., Holtsville, NY, USA). Intensity distributions of (proteo)liposome diameters were determined using a non-negative least squares fitting routine [21]. Buffer refractive indices were measured using a sodium D-line refractometer (Atago, Japan). For phosphate-buffered solutions supplemented with sucrose, viscosities were measured using an Ostwald viscometer. In other cases, published values were used to estimate medium viscosity [22].

Initial rates of proline uptake mediated by proteoliposomal ProP were measured using a filtration assay as described by Racher et al. [2].

2.3.3. Solute release

Leakage of CF from liposomes upon hypotonic dilution was measured as previously described [10]. *E. coli* lipid liposomes were prepared, as above, in

CF buffer (20 mM MOPS, 0.5 mM EDTA buffer (pH 7.4) with 100 mM CF and NaCl added to adjust the osmolality to approximately 0.6 mol/kg). Extravesicular dye was removed from approximately 150 μl of liposome suspension by size exclusion chromatography on a 30 cm (height) by 1.5 cm (diameter) Sephadex G-50 column equilibrated with MOPS buffer to which NaCl was added to match the osmolality of the CF-loaded liposome preparation. The fluorescence of liposome suspensions was measured with a Hitachi F-2000 Fluorescence Spectrophotometer (Mississauga, Ont., Canada). Regression analysis of the linear region of the plot of %CF release data shown in Fig. 3 was performed using Sigma Plot scientific graphing software (Jandel Scientific, Corte Madera, CA, USA).

Leakage of K^+ from proteoliposomes and liposomes upon hypotonic dilution was measured using the membrane potential-sensitive fluorescent dye diS-C_3-(5) [23]. The response of dye fluorescence to imposed membrane potentials was first calibrated (with respect to the magnitude of the induced membrane potential across the vesicle membrane) as follows. For this experiment, both proteoliposomes and liposomes were prepared according to the protocol for proteoliposome preparation. Two μl of proteoliposomes (0.4 mg/ml protein, 58 mg/ml lipid) or liposomes (58 mg/ml lipid), prepared in 0.10 M potassium phosphate buffer containing 0.30 M NaCl, were diluted into 1.5 ml aliquots of isotonic buffers comprised of 0.10 M sodium phosphate, 0.8 μM diS-C_3-(5), 0.5 mM to 0.10 M KCl and enough NaCl to give a final combined (KCl and NaCl) concentration of 0.30 M. Fluorescence was measured before and after addition of 0.2 μM valinomycin (to induce a membrane potential) in a Hitachi F-2000 Fluorescence Spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 690 nm. The resulting calibration curve indicated that the degree of diS-C_3-(5) fluorescence quenching observed after valinomycin addition was linearly proportional to extravesicular K^+ concentrations between 1 and 45 mM at a constant intravesicular K^+ concentration of 161 mM. In this system, these K^+ gradients corresponded (according to the Nernst equation) to an induced membrane potential between approximately −30 and −110 mV.

To measure K^+ leakage upon hypotonic dilution, 2 μl of proteoliposomes or liposomes (same preparations as used above) were diluted into 1.5 ml 0.10 M sodium phosphate buffer (pH 7.4) containing 0.8 μM diS-C_3-(5), 3 mM KCl and concentrations of NaCl varying between 0 and 297 mM. Fluorescence was measured, as above, before and after the addition of 0.2 μM valinomycin. The calibration curve discussed earlier was used to convert observed fluorescence changes to changes in the intravesicular K^+ concentration (due to leakage) assuming that the extravesicular K^+ concentration remained at 3 mM.

2.3.4. Static light scattering

All buffers used for static light scattering experiments were filtered through a 0.2 μm Acrodisk (Prod. No. 4192, Gelman Sciences, Ann Arbor, MI, USA) into sterile vials and left overnight to reduce dust and bubbles, respectively. Static light scattering measurements were performed using a CCD camera-based small angle light scattering spectrometer (SAILs), previously described by Alexander et al. [24]. This instrument measures scattered light at angles from 8.8° to 33° to the incident beam. A 20 mW helium–neon laser (model LHRP-1701, Research Electro Optics, Inc., Boulder, CO, USA) is shone through the sample in a square cuvette (SO, Hellma, Concord, Ont., Canada). The intensity profile of light scattered by the sample is then focused onto a glass diffusing plate which is photographed by the CCD camera (Princeton Instruments, Inc., Trenton, NJ, USA). The camera and controller are run from a computer using WINVIEW software (Princeton Instruments, Inc., Trenton, NJ, USA) which receives the scattered intensity information as a bitmap file. WINVIEW then makes use of a program, written in BASIC, to convert the data to an ascii file with an average intensity measured at a series of previously determined angles [24]. Measured intensities and angles were then corrected as described previously [25].

3. Results

3.1. Preparation and properties of (proteo)liposomes prepared from an E. coli polar lipid extract

As previously reported [8,26], PE was the predominant lipid and phosphatidylglycerol (PG) and cardi-

olipin were more minor constituents of the *E. coli* polar lipid extract. Palmitic (C16:0) and *cis*-vaccenic (C18:1 *cis*11) acids were predominant, as is commonly reported, and approximately 25% of the fatty acids present in these preparations were cyclopropane fatty acids (CFAs) (17:0 cyclo and 19:0 cyclo) (Table 1). (CFA levels vary from strain to strain and with growth conditions from undetectable to 32% of fatty acids [27,28]). The ratio of unsaturated plus CFAs to saturated fatty acids for this material, 3:2, was consistent with previous data [28].

E. coli polar lipids formed large aggregates (visible to the eye) when dispersed in buffers containing more than 0.45 M NaCl and these dispersions could not be extruded. Extrusion of the lipid extract dispersed in buffer with up to 0.3 M NaCl through filters with 400 nm, then 200 nm (diameter) pores yielded liposomes with monomodal size distributions (e.g. Fig. 1A). Further extrusion through a filter with 100 nm (diameter) pores yielded small to negligible volumes of liposomes and did not significantly alter the resulting distribution of liposome sizes (data not shown). Therefore, the standard procedure for *E. coli* lipid liposome preparation was to hydrate with buffer containing no more than 0.3 M NaCl and extrude through filters with 400 nm, then 200 nm diameter pores.

Proteoliposomes were prepared in two different ways during this study. For solute leakage studies, proteoliposomes prepared as described by Racher et al. [2] were subjected to three cycles of freezing, thawing and sonication. The size distributions of the resulting proteoliposomes were trimodal (Fig. 1B). The particle size for the population that contributed the most scattering intensity was similar to that of the corresponding *E. coli* lipid liposomes (Fig. 1A), with a mean diameter of approximately 150 nm. The particle size of the second population was three times as large (diameter approximately 450 nm). A minor population of micelles or other particles with a mean diameter of only approximately 50 nm was also present. For light scattering experiments, proteoliposomes were prepared by extrusion. This treatment eliminated the population of very large particles (see Fig. 1C). For reasons described below (see Section 3.3), the very small particle population that remained was not expected to contribute significantly to light scattering. The proline uptake activities of representative proteoliposomes prepared

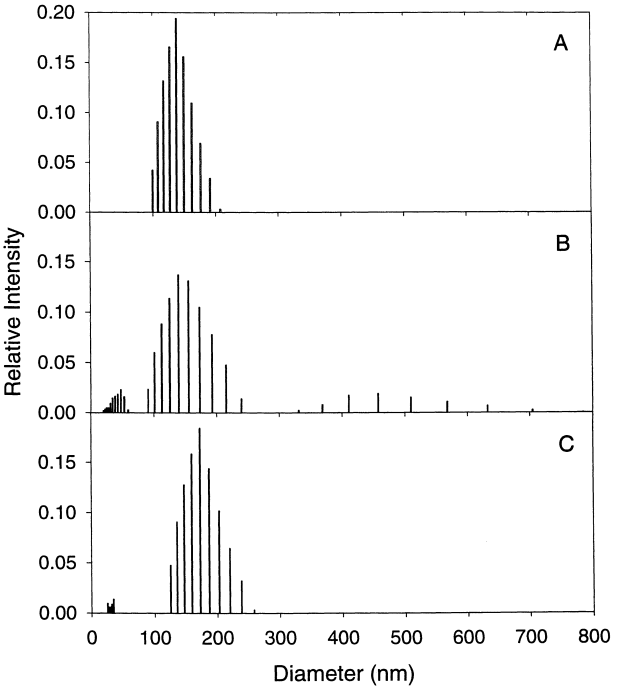

Fig. 1. Size distributions of (proteo)liposomes prepared from *E. coli* lipid. Size distributions were determined by DLS as described [21] (see Section 2). A: Liposomes prepared by extrusion of *E. coli* lipid in phosphate buffer. B: Proteoliposomes were prepared (see Section 2) in phosphate buffer and subjected to three cycles of freezing in liquid N_2, thawing at room temperature and bath sonication immediately prior to use. C: Proteoliposomes prepared as in B were extruded through one 400 nm filter and then through three 200 nm filters (20 passages per filter).

Table 1
Fatty acid compositions of polar lipid extracts from *E. coli* B-11303

Fatty acid	Percent of total lipid (w/w)	
	Lot 1	Lot 2
14:0	1.5	1.8
16:1 *cis*9	4.2	6.1
16:0	37.3	37.6
17:0 cyclo	18.2	19.7
18:1 *cis*11	31.1	31.0
18:0	0.9	trace
19:0 cyclo	6.7	3.9
Total unsaturated FA	35.3	37.9
Total cyclopropane FA	24.9	23.9

The fatty acid compositions of two different polar lipid extracts from *E. coli*, provided by Avanti Polar Lipids, Inc., were determined as described in Section 2.

as described in the legends to Fig. 1B (freeze-thaw sonication only) and Fig. 1C (freeze-thaw sonication plus extrusion) were 33 ± 5 and 55 ± 1 nmol/min/mg protein, respectively. (Values are cited ± standard deviation for three replicates, all measured at a proline concentration of 40 μM). Thus the transporter was retained and active in the final proteoliposome population illustrated in Fig. 1C.

Although MOPS buffer has been used for our previous studies of liposome properties [10–12], it is not possible to prepare proteoliposomes containing active ProP in that buffer system (K.I. Racher and J.M. Wood, unpublished data). It was therefore necessary to complete comparisons of liposomes and proteoliposomes using phosphate buffer, the system of choice for ProP reconstitution [2]. The mean liposome diameter depended on medium composition (Table 2). The addition of NaCl (0.30 M) to phosphate buffer resulted in an increase in liposome size as did the replacement of MOPS buffer with phosphate buffer. The addition of $MgCl_2$ (2 mM) to the buffer in which liposomes were prepared had no effect on liposome size (data not shown). Proteoliposomes and liposomes prepared in phosphate buffer were similar in size (Table 2).

DSC was used to examine the phase behavior of liposomes prepared from *E. coli* lipids in phosphate buffer (Fig. 2). The gel to liquid crystalline phase transition began just below 0°C and the average phase transition temperature (T_m) from six determinations was 2.6 ± 0.3°C. In the presence of 0.30 M NaCl, the average T_m was reduced to 1.5 ± 0.2°C. Transition temperatures were stable for the first 30–45 min after exposure of the liposome suspension to air, beyond which the T_m decreased. Scans done on lipid before extrusion (FATMLVs) gave similar results to those for extruded liposomes in the same buffer (data not shown).

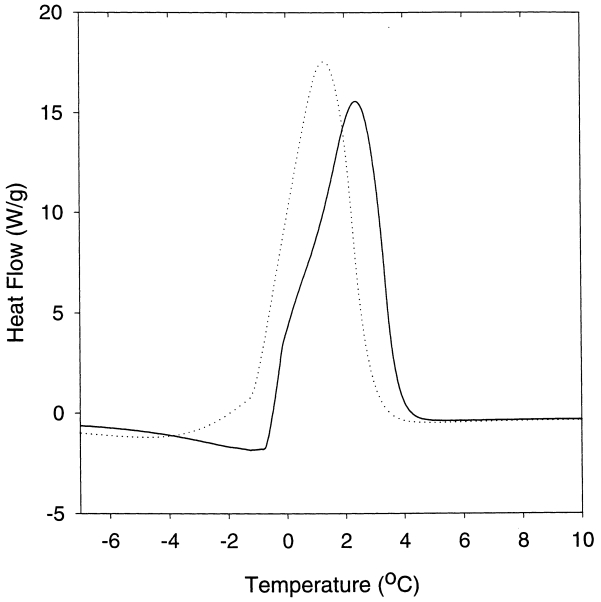

Fig. 2. Thermotropic phase transition of liposomes prepared with *E. coli* lipid. DSC was performed on liposomes prepared from *E. coli* lipid in phosphate buffer without (solid line) or supplemented with (dotted line) 0.30 M NaCl (see Section 2).

3.2. Mechanical properties of (proteo)liposomes: osmotically induced swelling and leakage

Upon dilution of their external medium, liposomes and proteoliposomes will swell and lumenal solutes may leak across the membrane to dissipate the imposed osmotic gradient ([10] and references therein). The dependence of membrane permeability on imposed osmotic gradients was determined by monitoring the release of CF that had previously been en-

Table 2
The effects of buffers on the sizes of (proteo)liposomes prepared from *E. coli* lipid

Buffer	NaCl (M)	Osmolality (mol/kg)	Average diameter (nm)	
			Liposomes	Proteoliposomes
MOPS	0.30	0.58	192 ± 4 (n = 6)	ND
KP_i	0.30	0.74	216 ± 11 (n = 3)	ND
KP_i	0	0.22	163 ± 5 (n = 6)	175 (n = 2)

Intensity-weighted mean diameters were determined by DLS spectroscopy as described in Section 2 and are reported ± standard error for numbers of determinations, n, ≥3. The averaged values for proteoliposomes, 173 nm and 176 nm, were derived from the major particle population within distributions like that represented in Fig. 1C. ND, not determined.

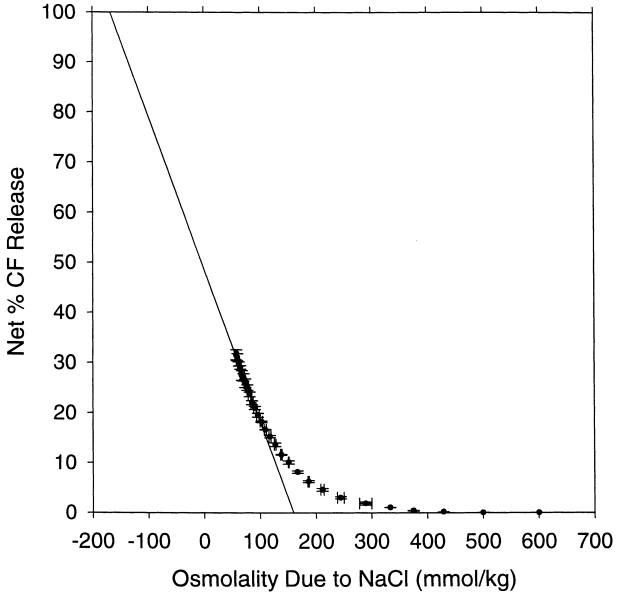

Fig. 3. Solute leakage in response to osmotic downshifts imposed on CF-loaded liposomes prepared with *E. coli* lipid. CF release from *E. coli* liposomes prepared in MOPS buffer with 0.30 M NaCl was determined by fluorescence spectroscopy (see Section 2) and analyzed as previously described [10,11].

trapped in liposomes prepared with *E. coli* lipid (Fig. 3). CF leakage was observed when an osmotic gradient of more than 0.16 mol/kg (or approximately 5 atm) was imposed by diluting the extraliposomal NaCl. Analysis of the resulting leakage profile, using the procedure of Hallett et al. [11] and a distribution width of 18, yielded a maximum lateral strain of 0.22 ± 0.02 ($\Delta r/r$) and a Young's elastic modulus of $1.29\ (\pm 0.08) \times 10^7$ N/m^2. Similar measurements performed previously on DOPG liposomes, with a distribution width of 29, resulted in values for maximum lateral strain and Young's elastic modulus of 0.18 ± 0.02 and $1.86\ (\pm 0.19) \times 10^7$ N/m^2, respectively [29].

Concerns over the effects of CF, at high concentrations, on proteoliposome reconstitution and protein function precluded the use of CF to measure solute leakage from proteoliposomes. Therefore, the dependence of liposome and proteoliposome membrane permeability on imposed osmotic gradients was determined by monitoring the collapse of the transmembrane K^+ gradient imposed by diluting (proteo)liposomes, prepared in potassium phosphate buffer, into sodium phosphate buffer. The fluorescence of diS-C_3-(5) is enhanced when this dye partitions into the hydrophobic core of phospholipid membranes and this fluorescence is quenched when a membrane potential (inside negative) is imposed. The magnitude of the membrane potential produced upon addition of valinomycin, a K^+ ionophore, is proportional to the log of the K^+ gradient across the membrane into which valinomycin partitions [23]. The quenching of diS-C_3-(5) fluorescence can therefore be used to determine the residual potassium gradient maintained by K^+-loaded (proteo)liposomes post-osmotic shift. The K^+ leakage profiles of proteoliposomes and liposomes, determined in this way, were similar (Fig. 4). Furthermore, the imposed osmotic gradient at which K^+ leakage was observed, approximately 0.20 mol/kg, was similar to that determined by CF fluorescence for liposomes (Fig. 3).

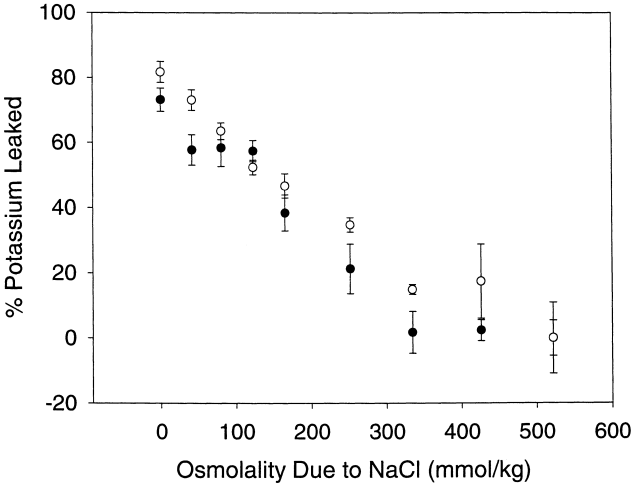

Fig. 4. Solute leakage in response to osmotic downshifts imposed on liposomes and proteoliposomes prepared with *E. coli* lipid. For these experiments, both proteoliposomes (closed circles) and liposomes (open circles) were prepared (see Section 2) in phosphate buffer supplemented with 0.30 M NaCl and subjected to three cycles of freezing in liquid N_2, thawing at room temperature and bath sonication immediately prior to use. Changes in the quenching of diS-C_3-(5) fluorescence due to valinomycin-induced membrane potential formation were used to estimate changes in the intra(proteo)liposomal concentration of K^+ ($[K^+_{in}]$) due to the prior imposition of osmotic downshifts (see Section 2). The percentage of potassium leaked from (proteo)liposomes was calculated as the difference between the $[K^+_{in}]$ before and after hypotonic dilution divided by the $[K^+_{in}]$ before hypotonic dilution, and expressed as a percent. The point at 0% potassium leaked corresponds to dilution of the (proteo)-liposomes into isotonic buffer. Each point represents a mean of either six or 12 determinations, from experiments performed on two different days.

3.3. Mechanical properties of liposomes: osmotic shifts and scattered light intensity

Changes in the intensity of light scattered at a single angle were analyzed as indicators of liposome structure (Figs. 5 and 6). The relationship between scattered light intensity (I_s) and liposome structure is given in Eqs. 1 and 2, where I_o is the incident intensity, k is the wave vector, λ is the laser wavelength, N is the number of scatterers and M is the mass of an individual scatterer. The contrast factor $((m^2-1)/(m^2+2))^2$ depends on m, which is equal to n_1/n_o where n_1 and n_o are the refractive indices of the membrane and medium, respectively. Q (dependent on λ) is the scattering vector and $P(Q)$ is the scattering factor. $P(Q)$ depends on the sizes and shapes of the scatterers.

$$\frac{I_s}{I_o} \propto k^4 N M^2 \left(\frac{m^2-1}{m^2+2}\right)^2 P(Q) \quad (1)$$

$$k = \frac{2\pi n_o}{\lambda} \quad (2)$$

For the experiments reported below, changes in scattered light intensity could in principle arise from changes to N, M, n_0, n_1 or $P(Q)$ induced by imposed osmotic shifts. To extract indications of osmotically induced changes to liposome structure from the light scattering data, it is necessary to first account for changes in scattered light intensity due to other factors (changes in N, M and/or n_0). In collecting the data presented in Figs. 5 and 6, liposomes were always diluted to the same lipid concentration (0.25 mg/ml) to render N and M constant. If aggregation (or fusion) were to occur, however, the scattered light intensity would decrease linearly with N and increase as the square of M (see Eq. 1). Since the effect of changing M would dominate, aggregation would cause dramatic increases in scattered light intensity.

Scattered light intensity was measured as osmotic shifts were imposed on liposomes with NaCl or sucrose as osmolyte. Little change in intensity was observed if the NaCl concentration remained low (*E. coli* lipid liposomes, Fig. 5, solid circles). However, a dramatic increase in intensity was observed when the NaCl concentration became high (osmolalities greater than 1 mol/kg), and the presence of aggregates was confirmed by DLS (Fig. 5, inset). This aggregation or fusion appeared to result from the high ionic strength of the NaCl-supplemented medium, since no increase in the scattered light intensity was observed when sucrose was added to raise the osmolality as high as 1.3 mol/kg (Fig. 5, open circles). Further analyses, reported below, represent data obtained under experimental conditions that did not induce such aggregation or fusion. Thus, observed changes in the scattered light intensity should arise from changes in parameters other than N and M.

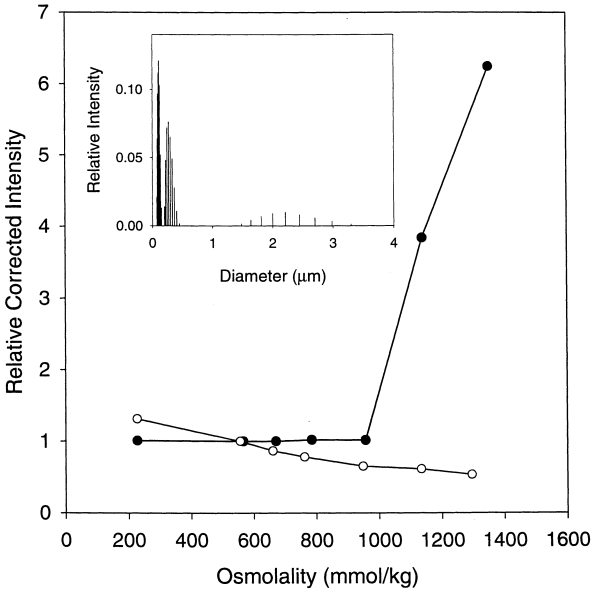

Fig. 5. Effects of osmotic shifts on light scattering by liposomes prepared with *E. coli* lipid. Liposomes were prepared in MOPS buffer adjusted to 0.6 mol/kg with NaCl or sucrose. Samples were prepared by diluting such preparations to a final lipid concentration of 0.25 mg/ml in MOPS buffers of higher or lower osmolality (adjusted by varying the NaCl (closed circles) or sucrose (open circles) concentration, respectively) to impose osmotic upshifts or downshifts, as previously described [12]. The relative intensity of light scattered at a Q of 3.66×10^{-3} nm^{-1} was determined, as described in Section 2. Inset: the intensity-weighted size distribution of liposomes diluted into buffer at approximately 1.1 mol/kg was determined by DLS spectroscopy as described in Section 2. The liposome size distribution prior to this treatment was comparable to that illustrated in Fig. 1A with a mean diameter comparable to that cited in Table 2.

As osmolyte concentration is raised, the refractive index of the medium (n_o) also increases. This alters both k and m (see Eqs. 1 and 2). It is possible to compensate for the effect on k by multiplying each

scattered light intensity (I_s) by the ratio of the measured value for the refractive index of the isotonic buffer to the measured refractive index of the buffer used to impose each osmotic shift, all raised to the fourth power (see Eq. 2). This has been done in the generation of Figs. 5 and 6. Any residual changes in relative corrected intensity should result from changes to the contrast term (through m) and/or the scattering factor ($P(Q)$).

Since osmolyte concentration determines medium refractive index (n_0) and m is the ratio of n_1 (the membrane refractive index) to n_0, the contribution of m to scattered light intensity changes with each osmotic shift. To correct for this effect on m, it is necessary to determine n_0 for each osmolality and estimate n_1. Based on published values for fatty acid refractive indices [30], it was estimated that the membrane refractive index would be between 1.42 and 1.46. The curves in Fig. 6 represent the changes in scattered light intensity that would occur due to these contrast effects alone (measured values for n_0; solid line, n_1 of 1.42; dotted line, n_1 of 1.46).

The refractive indices of sucrose-supplemented solutions approach the estimated values of n_1 more closely than do the refractive indices of iso-osmolal NaCl-supplemented solutions. For example, at an osmolality of 1.15 mol/kg, n_o for a NaCl-supplemented MOPS-buffered medium was 1.3398 while that for a sucrose-supplemented MOPS-buffered medium was 1.3751. Because of lower contrast, larger decreases in scattered light intensity were expected and observed with sucrose (Fig. 6B) than with NaCl (Fig. 6A,C) as osmolyte. The contrast term accounts well for the observed changes in scattering at small osmotic upshifts. At larger upshifts, significant differences appear. These differences are expected to result from osmotically induced changes in membrane refractive index (n_1) and/or $P(Q)$ (liposome shape or size).

Both osmolyte (Fig. 6A,B) and lipid composition (Fig. 6A,C) influenced the impact of osmotic upshifts on light scattering by liposomes. For *E. coli* lipid liposomes in NaCl-supplemented MOPS buffer, scattered light intensity remained essentially constant when osmotic shifts were imposed by dilution into NaCl-supplemented buffers (Fig. 6A). A slight, net increase in scattering due to liposome size or shape

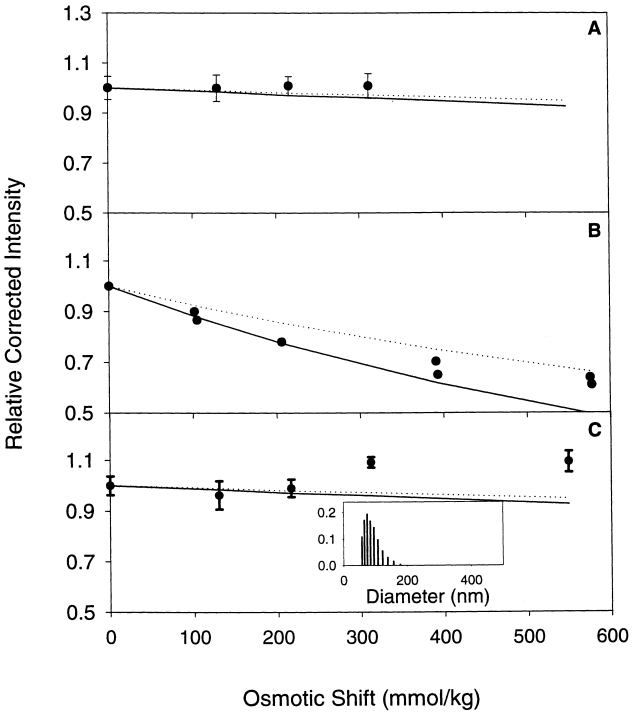

Fig. 6. Effects of osmotic shifts on light scattering by liposomes prepared with *E. coli* lipid and DOPG. Liposomes were prepared in MOPS buffer with 0.30 M NaCl (A, C) or 0.60 M sucrose (B) from *E. coli* lipid (A, B) or DOPG (C) and subjected to osmotic shifts using NaCl (A, C) or sucrose (B) as osmolyte, as described [12]. The intensity of light scattered at a Q of 3.66×10^{-3} nm^{-1} was measured as described in Section 2. Where error bars are shown, intensities were obtained from the analysis of three liposome preparations. Intensities were corrected (see Section 2), normalized to the corrected intensity for liposomes diluted into isotonic buffer and averaged. The origin of the lines is discussed in the text. Inset to C: the light scattering intensity-weighted size distribution of DOPG liposomes subjected to an osmotic upshift of approximately 0.55 mol/kg with NaCl-supplemented MOPS buffer was determined by DLS spectroscopy as described in Section 2.

changes could be inferred once the contrast effect was taken into account (compare data points and lines, Fig. 6A). For *E. coli* lipid liposomes in sucrose-supplemented MOPS buffer, scattered light intensity decreased when osmotic shifts were imposed by dilution into sucrose-supplemented buffers (Fig. 6B). Again, contrast effects could not fully account for this change at larger upshifts. The difference in net light scattering between liposomes in NaCl- and in sucrose-supplemented medium may reflect a difference in the shape adopted after osmotic upshifts (reflected in $P(Q)$) or a difference in changes to mem-

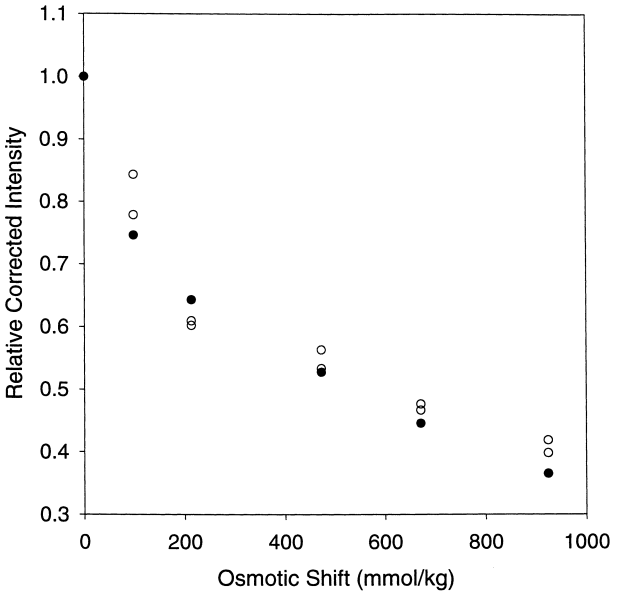

Fig. 7. Effects of osmotic shifts on light scattering by liposomes and proteoliposomes. Liposomes (closed circles) and proteoliposomes (open circles) were prepared as described for Fig. 1C. Samples were then diluted to 0.25 mg/ml lipid in 0.1 M sodium phosphate buffer, pH 7.4, supplemented with sucrose at the concentrations required to attain the indicated osmolalities. The intensity of light scattered at a Q of 3.66×10^{-3} nm^{-1} was measured as described in Section 2. Intensities were corrected, normalized to the corrected intensity for (proteo)liposomes diluted into isotonic buffer and averaged.

brane optical properties (reflected in the contrast term) [12]. However, the more intense light scattering by NaCl-treated liposomes may also have been due, at least in part, to incipient fusion or aggregation of the sort that became markedly more pronounced at higher NaCl concentrations (Fig. 5, closed circles).

Larger increases in the intensity of scattered light were observed when DOPG liposomes prepared in NaCl-supplemented MOPS buffer were subjected to osmotic shifts by dilution into NaCl-supplemented buffers (Figs. 6C and 7 of White et al. [12]) than had been observed with *E. coli* lipid liposomes (Fig. 6A). No sign of aggregation was detected by DLS, however (Fig. 6C, inset). Thus, for DOPG, the observed increase in the intensity of the scattered light could not be attributed to aggregation. Presumably the high ionic strength of the NaCl-supplemented buffers was more effective in promoting the aggregation of liposomes prepared with *E. coli* lipid because their net surface charge is low.

3.4. Comparison of liposomes and proteoliposomes

The experiments described above established the experimental conditions, data corrections and predictions appropriate for the comparison of liposomes and proteoliposomes by static light scattering spectrometry. Liposomes and proteoliposomes prepared from *E. coli* lipid in phosphate buffer were subjected to osmotic upshifts using sucrose as osmolyte. The resulting data were corrected for changes in k due to the varying sucrose concentration. Changes in scattered light intensity due to anticipated changes in contrast between membrane and medium were estimated as described above (see Section 3.3). Light scattering from the two preparations was indistinguishable in intensity (Fig. 7). Osmotic upshifts resulted in light scattering intensity decreases that were similar to those seen in MOPS buffer (Fig. 6B).

4. Discussion

Proteoliposomes reconstituted with purified membrane proteins play a prominent role in membrane enzymology. The dependence of membrane enzyme activity on phospholipid composition has long been recognized and documented. With some notable exceptions, however [31,32], neither the physiological relevance of that dependence nor the physiological rationale underlying the complexity of membrane lipid composition is well understood [8].

In an effort to avoid artefacts due to lipid composition and to extract physiologically meaningful data, investigators often prepare proteoliposomes using enzyme and phospholipid extracted from the same organism. Polar lipid extracts from Gram-negative bacteria such as *E. coli* include phospholipids derived from both leaflets of the cytoplasmic membrane as well as the inner leaflet of the outer membrane, each of which may possess a distinct composition [33]. At least to a first approximation, the proportions of these lipids in the outer and inner leaflets of the resulting (proteo)liposome membranes are expected to be similar to one another, and not necessarily to those of either the cytoplasmic or outer membrane from which they originated. In addition, proteins embedded in such mixed lipid systems may exist in lipid sub-domains formed by lateral phase separa-

tion. For *E. coli*, reported phospholipid head group and fatty acid compositions vary [8,26,33]. Since the phospholipid compositions of bacteria are strongly dependent upon both their genotype and their culture conditions, commercially available polar lipid extracts may or may not correspond in composition to the lipid of organisms under study in a particular laboratory.

The phospholipid and fatty acid compositions of *E. coli* polar lipid extracts provided by Avanti Polar Lipids, Inc., a material commonly used for studies of bacterial membrane enzymes, were consistent with previous reports ([8,26,28]). The mechanical properties of liposomes prepared from pure DOPG (Fig. 6 and [12]) and from *E. coli* lipids (Figs. 1, 3–7) were similar but not identical. Extrusion of DOPG through filters with 100 nm and 200 nm diameter pores yielded liposomes with mean diameters of approximately 100 nm and 200 nm, respectively ([10,12], this study). In contrast, extrusion of *E. coli* lipids or proteoliposomes prepared with *E. coli* lipids through filters with either 100 nm or 200 nm diameter pores yielded liposomes with diameters in excess of 160 nm (Table 2). The diameters of *E. coli* lipid liposomes depended on the composition of the extrusion medium, as previously reported for PG liposomes [34]. (The phospholipid phase transitions were also buffer-dependent (Fig. 2)). Nevertheless, liposomes and proteoliposomes prepared in the same medium were very similar in size. Since structural responses of vesicles to osmotic shifts are vesicle size-dependent [1,11], vesicle size variations must be taken into account when such responses are analyzed.

The maximum strain ($\Delta r/r$) created by imposing osmotic downshifts on liposomes prepared from DOPG and from *E. coli* lipid was similar (0.18 ± 0.02 [29] and 0.22 ± 0.02 (Fig. 3), respectively). The respective Young's elastic moduli differed slightly ($1.86\ (\pm 0.19) \times 10^7$ N/m^2 [29] and $1.29\ (\pm 0.08) \times 10^7$ N/m^2 (Fig. 3)). A smaller osmotic gradient would therefore be required to create equivalent strain in *E. coli* liposomes than in DOPG liposomes of the same diameter. Similar fractional volume changes (38% and 32%, respectively) were required to effect leakage of CF from *E. coli* lipid liposomes and from DOPG liposomes [11,29]. The optical properties of liposomes prepared with DOPG and with *E. coli* lipid also differed (Fig. 6).

In this study, the sizes and the size distributions of liposomes and proteoliposomes prepared with *E. coli* lipid were shown to be similar (Table 2 and Fig. 1A,C). The measured responses to osmotic shifts were also similar (Figs. 4 and 7). These observations substantiate the claim that liposomes are appropriate structural models for investigation of the mechanical properties of proteoliposomes. Osmotic upshifts imposed with NaCl elicited fusion and/or aggregation of liposomes prepared with *E. coli* lipid, whereas osmotic upshifts imposed with sucrose did not (Fig. 5). By replacing NaCl with sucrose, we eliminated (proteo)liposome aggregation and observed significant, osmotically induced changes in the intensity of light scattering by liposomes and proteoliposomes prepared from *E. coli* lipid (Figs. 6B and 7). The intensity changes at high upshift that exceeded values predicted by contrast changes are attributed to the changing structures of the (proteo)liposomes themselves. Further interpretation of these structural changes will be integral to mechanistic analyses of membrane-based osmosensors that have recently been purified and reconstituted in proteoliposomes [2,13].

Acknowledgements

We are grateful to Chris Schultz-Nielsen and Jordan Green for performing some DLS analyses, to the Department of Food Science, University of Guelph, for use of the differential scanning calorimeter, to Karlheinz Altendorf for his assistance and support, to the Natural Sciences and Engineering Research Council for Research and Collaborative Grants awarded to F.R.H. and J.M.W., and to the Fonds der Chemischen Industrie for financial support to A.L.

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