Interaction of P-Glycoprotein with Defined Phospholipid Bilayers: A Differential Scanning Calorimetric Study†

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ABSTRACT: One of the major causes of multidrug resistance in human cancers is expression of the P-glycoprotein multidrug transporter, which acts as a drug efflux pump. P-Glycoprotein is a member of the ABC superfamily of membrane proteins, and is composed of 12 hydrophobic membrane-spanning segments and 2 cytoplasmic nucleotide binding domains. Membrane lipids are known to play an important role in the function of P-glycoprotein. In the present study, purified P-glycoprotein of high specific ATPase activity was reconstituted into defined bilayers of dimyristoylphosphatidylcholine (DMPC), and its effects on lipid thermodynamic properties were then investigated using differential scanning calorimetry. P-Glycoprotein had a large perturbing effect on DMPC bilayers, even at relatively high lipid:protein ratios. The gel to liquid-crystalline phase transition temperature, T_m, was lowered on inclusion of P-glycoprotein in the bilayer, and the cooperativity of the transition was markedly reduced. The phase transition enthalpy, ΔH, declined in a linear fashion with increasing P-glycoprotein content for lipid:protein ratios between 63:1 and 16:1 (w/w). Evaluation of these data using two different analytical methods indicated that P-glycoprotein perturbed either 375 or 485 phospholipids, withdrawing them from the phase transition. The ΔH value for those lipids undergoing melting was similar to that of pure DMPC, which implies that their thermodynamic properties are essentially unchanged in the presence of P-glycoprotein. At lipid:protein ratios below 16:1 (w/w), transition enthalpy increased with higher P-glycoprotein content, until the ΔH value reached that of pure DMPC. However, the lipid remained highly perturbed, as indicated by a very broad phase transition peak. This behavior may arise from either aggregation/oligomerization of P-glycoprotein within the bilayer or changes in the interaction of the transporter with the membrane at high density.

One of the major causes of multidrug resistance (MDR)[1] in human cancers is expression of the P-glycoprotein multidrug transporter (Pgp), which acts as an efflux pump for a diverse range of hydrophobic compounds [for reviews, see Leveille-Webster and Arias (1995) and Gottesman and Pastan (1993)]. One promising clinical approach to circumvention of Pgp action involves the use of compounds which reverse MDR, known as chemosensitizers, as adjuncts to chemotherapy treatment of cancer patients (Georges et al., 1990; Ford, 1996). Pgp is a member of the ABC superfamily of membrane proteins (Doige & Ames, 1993; Higgins, 1992), and is predicted to comprise 12 hydrophobic membrane-spanning segments and 2 cytoplasmic ATP binding domains, arranged in 2 homologous halves.

Two types of Pgp genes exist. Class I and II Pgps are drug exporters, and confer MDR upon transfection into drug-sensitive cells. Class III Pgp is expressed exclusively at the apical surface of bile canalicular cells in the liver, and appears to function as a specific flippase for export of phosphatidylcholine (PC) into the bile (Ruetz & Gros, 1994; Smit et al., 1993). Purification and reconstitution of the class I isoform of Pgp into lipid bilayer model systems have demonstrated that it carries out active, concentrative transport of natural products, chemotherapeutic drugs, and hydrophobic peptides, powered by ATP hydrolysis (Sharom et al., 1993, 1996; Sharom, 1995; Shapiro & Ling, 1995). Pgp is an unusual transporter in that it is highly promiscuous, and has high constitutive ATPase activity in the apparent absence of substrates. The ATPase activity of Pgp is further stimulated or inhibited by the addition of substrates or chemosensitizers [see, for example, Sharom et al. (1995a,b)]. Little is known about the mechanism by which Pgp transports such a large number of structurally unrelated compounds, and the means by which this transport is coupled to ATP hydrolysis.

The compounds transported by Pgp are generally hydrophobic. This has led to the suggestion that substrates gain access to Pgp from the lipid bilayer itself, rather than the aqueous compartment (Raviv et al., 1990; Homolya et al., 1993). The high degree of sequence homology between the class I and III Pgps supports the proposal that the multidrug transporter acts as drug flippase, moving substrates from the inner to the outer leaflet of the plasma membrane (Higgins & Gottesman, 1992). Given the close association that must exist between Pgp, its hydrophobic substrates, and the

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; DTE, dithioerythritol; MDR, multidrug resistance; PC, phosphatidylcholine; Pgp, P-glycoprotein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; QELS, quasi-elastic light scattering.

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membrane bilayer, it is not surprising that lipids appear to play a major role in modulating the function of the transporter [reviewed in Sharom (1997)]. Phospholipids are absolutely required for Pgp ATPase activity, and also appear to protect the protein against denaturation (Doige et al., 1993). Addition of various membrane phospholipids to the purified transporter resulted in changes in ATPase activity; some lipids produced a large, concentration-dependent increase in activity, whereas others caused inhibition (Doige et al., 1993; Sharom et al., 1995a). Urbatsch and Senior (1995) have also reported that the pattern of ATPase stimulation or inhibition by various MDR drugs is highly dependent on the lipid environment, which suggests that drug binding to Pgp may be modulated by lipids. The transport function of Pgp also appears to be regulated by the lipid environment. A 2−4-fold decrease in the rate of transport of daunorubicin and vinblastine was observed on addition of membrane fluidizers (Sinicrope et al., 1992), and the phase state of the bilayer affected both colchicine transport and the kinetics of ATP hydrolysis in a reconstituted system (Sharom, 1997).

Membrane lipids clearly have important modulatory effects on various aspects of Pgp function. However, there is currently no information concerning the influence of Pgp on the behavior of the membrane lipids surrounding it. The effects of integral membrane proteins on the thermodynamic properties of the host lipid bilayer can be assessed using differential scanning calorimetry (DSC). Details of the physicochemical properties of phospholipid at the lipid−protein interface have been characterized using this technique for only a few transporters, including the sarcoplasmic reticulum Ca^{2+}-ATPase (Lenz et al., 1983), the human erythrocyte band 3 anion exchanger (Chicken & Sharom, 1984), and the human erythrocyte hexose transporter (Naderi et al., 1995). To date, there has been no examination of a member of the ABC superfamily of transporters. The present work reports the results of a DSC study of the interactions of highly purified reconstituted Pgp with defined bilayers of a simple phospholipid. Membrane lipid behavior was examined over a wide range of lipid:protein ratios, and various important thermodynamic parameters of the phase transition are determined.

MATERIALS AND METHODS

Materials. Dimyristoyl-L-α-phosphatidylcholine (DMPC), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and dithioerythritol (DTE) were purchased from Sigma Chemical Co. (St. Louis, MO). Di[1-^{14}C]palmitoyl-L-α-phosphatidylcholine (DPPC; specific activity 111 mCi/mmol) was obtained from Amersham Canada (Mississauga, ON).

MDR Cell Lines and Pgp Purification. The MDR Chinese hamster ovary cell line CH^RB30 (Ling & Thompson, 1974) was grown as described previously (Loe & Sharom, 1993, 1994). Plasma membrane vesicles were isolated from the MDR cells using nitrogen cavitation (Doige & Sharom, 1991; Liu & Sharom, 1996), and the vesicles were stored at −70 °C for no longer than 3 months before use. Pgp was purified to high purity using a procedure involving a differential two-step extraction of CH^RB30 plasma membrane with CHAPS, followed by removal of contaminant glycoproteins by affinity chromatography on concanavalin A−Sepharose. A modification of a previously published procedure (Liu & Sharom, 1996) was used. Following the initial extraction of plasma membrane with CHAPS, the pellet was solubilized in buffer containing 8 mM CHAPS and 20% glycerol, at a final protein concentration of 1 mg/mL. Following centrifugation, the supernatant (S_2 fraction) was passed through a column of concanavalin A−Sepharose (Pharmacia Canada, Dorval, QC). The final product consisted of 90−95% pure Pgp, at a concentration of 0.1−0.2 mg/mL, in 8 mM CHAPS/50 mM Tris-HCl/0.15 M NH_4Cl/5 mM $MgCl_2$/1 mM DTE/0.02% (w/v) NaN_3, pH 7.4. The Pgp preparation was kept on ice and reconstituted into phospholipid bilayers the same day. SDS−PAGE was performed as described previously (Sharom et al., 1995a).

Protein Assay. The protein content of plasma membrane was determined by a microplate adaptation of the Bradford assay (Bradford, 1976). For purified Pgp and reconstituted proteoliposomes, protein was quantitated by the method of (Peterson, 1983). In both assays, bovine serum albumin (crystallized and lyophilized, fraction V, Sigma) was used as a standard.

Reconstitution of Purified Pgp into Proteoliposomes at Different Lipid:Protein Ratios. Reconstitution of purified Pgp was carried out by a modification of the procedure previously reported for partially purified protein (Sharom et al., 1993). For all reconstitution experiments, Pgp was purified in batches of ∼250 μg, to ensure that purity and ATPase activity were consistent. The freshly purified Pgp was then combined with varying amounts of DMPC, to give samples with final lipid:protein weight ratios in the range 63:1 to 6:1.

A stock solution of DMPC was prepared in $CHCl_3$/MeOH and stored at −20 °C. The required amounts of DMPC, plus [^{14}C]DPPC tracer (see below), were dispensed into a small glass tube, evaporated to dryness under a stream of nitrogen and then pumped *in vacuo* for 45 min to remove all traces of organic solvent. Dried DMPC (2−10 mg) was dissolved in 250 μL of 200 mM CHAPS in 50 mM Tris-HCl/100 mM KCl/5 mM $MgCl_2$/1 mM DTE/0.02% (w/v) NaN_3, pH 7.4, by warming to 37 °C, and the appropriate amount of purified Pgp solution was then added. The final detergent:lipid mole ratios of the reconstitution mixtures fell in the range 3.4:1 to 17:1. After periodic mixing on ice for 30 min, the mixture was passed through a column of Sephadex G-50 (1 × 25 cm), which was equilibrated and eluted with 50 mM Tris-HCl/100 mM KCl/5 mM $MgCl_2$/1 mM DTE/0.02% (w/v) NaN_3, pH 7.4, at 4 °C. Turbid fractions containing proteoliposomes were collected, and vesicular structures were harvested by centrifugation at 14000*g* for 15 min at 4 °C. Proteoliposomes were resuspended in sample buffer [10 mM Tris-HCl/0.25 M sucrose/5 mM $MgCl_2$/0.02% (w/v) NaN_3, pH 7.4] using a fine gauge needle and kept at 4 °C until ready to use. The size distribution of the proteoliposomes was assessed by QELS as previously described (Chicken & Sharom, 1983). DMPC proteoliposomes containing no Pgp were prepared using detergent removal by gel filtration, applying the same protocol used for Pgp reconstitution. Multilamellar liposomes of DMPC were also generated, by suspension of dried DMPC in sample buffer using a vortex mixer.

Phospholipid recovery was measured for each DMPC−Pgp sample by inclusion of tracer quantities of [^{14}C]DPPC

(0.025 μCi) in the reconstitution mixture, followed by liquid scintillation counting of the recovered proteoliposomes. Reconstitution of Pgp samples with varying lipid:protein ratios was carried out using independently isolated glycoprotein preparations.

The fraction of Pgp molecules with their ATP binding domains facing the vesicle lumen was estimated by determination of Pgp ATPase activity before and after permeabilization of the proteoliposomes with increasing concentrations of CHAPS (Sharom et al., 1993).

CHAPS Assay. Residual CHAPS in the Pgp proteoliposomes was measured using a modification of the method of Irvin et al. (1944) for the determination of cholates in bile and blood. Briefly, 600 μL of 16 N sulfuric acid and 100 μL of 1% 2-furaldehyde were added to duplicate 100 μL aliquots of reconstituted proteoliposomes (0.1−0.2 mg of lipid, resuspended in purified water) in 1.5 mL microcentrifuge tubes. Reagent and lipid blanks were also prepared, containing 100 μL of purified water, and 100 μL of 1−2 mg/mL DMPC in purified water, respectively. Tubes were heated at 65 °C for 13 min and cooled rapidly to room temperature, and glacial acetic acid (500 μL) was added to each. The absorbance at 620 nm was measured, and after making a small correction for turbidity using the lipid blanks, the CHAPS concentration in the proteoliposome samples was measured by comparison with CHAPS standards (0.25−4.0 mg/mL).

Differential Scanning Calorimetry. Calorimetric data were obtained using a Microcal MC-2 high-sensitivity differential scanning calorimeter (MicroCal Inc., Northampton, MA). Reconstituted vesicles (0.7 mg of DMPC) in a total volume of 1.5 mL of sample buffer were analyzed at a scanning rate of 1.5 °C/min. Samples were prewarmed above the gel to liquid-crystalline phase transition temperature for DMPC prior to calorimetric analysis, and then cooled to 4 °C. Each sample was scanned twice up to 35 °C, with highly reproducible results. Scanning over this temperature range avoids inactivation of Pgp ATPase activity, which takes place during prolonged incubation at temperatures above 37 °C (Y. Romsicki and F. J. Sharom, unpublished observations).

Data Analysis. The calorimetric data were analyzed using Microcal Origin Scientific software (MicroCal Software Inc.). The enthalpy change of the phase transition, ΔH, was obtained from the area under the peak and the mass of phospholipid in each sample. The phase transition temperature, T_m, was defined as the temperature at the peak maximum, and the sharpness of the transition was expressed as the width at half-maximum height, $\Delta T_{1/2}$. The van't Hoff enthalpy, ΔH_{vH}, was calculated from T_m and $\Delta T_{1/2}$ using the relationship

$$\Delta H_{vH} \approx 4RT_m^2/\Delta T_{1/2}$$

and the value of the cooperative unit, CU, was calculated as $\Delta H_{vH}/\Delta H$ (Mabrey & Sturtevant, 1978). The calorimetric parameters were averaged for the two scans carried out on each sample.

RESULTS

Reconstitution of Pgp into DMPC Proteoliposomes. In order to carry out a calorimetric investigation of the interactions of Pgp with phospholipid bilayers, it was necessary to reconstitute the protein into a defined synthetic lipid. DMPC was chosen as the defined lipid because it is stable to oxidation, and its gel to liquid-crystalline phase transition temperature, T_m, is in a convenient range to prevent inactivation of the ATPase activity of Pgp during DSC scanning of the sample. Pgp of very high purity (90−95%) was isolated from MDR CH^{R}B30 cells using a two-step extraction with the zwitterionic detergent CHAPS, followed by removal of contaminating glycoproteins by lectin affinity chromatography on concanavalin A−Sepharose (Liu & Sharom, 1996). Pgp isolated by this procedure has the highest basal level of ATPase activity reported to date [V_{max} of up to 2.9 μmol·min^{-1}·(mg of protein)$^{-1}$; Liu & Sharom, 1996, 1997]. The purified Pgp was immediately reconstituted into proteoliposomes of DMPC, using detergent removal by gel filtration. This rapid technique was previously employed in our laboratory to reconstitute partially purified Pgp into lipid bilayer vesicles (Sharom et al., 1993). Pgp reconstituted in this way retained both basal and drug-stimulated ATPase activity, and the proteoliposomes displayed a high level of ATP-dependent transport activity for specific MDR spectrum drugs (Sharom et al., 1993). SDS−PAGE analysis (Figure 1) showed that a 170 kDa band corresponding to Pgp was the major protein component in both the purified preparation (lane 3) and the reconstituted proteoliposomes (lane 4).

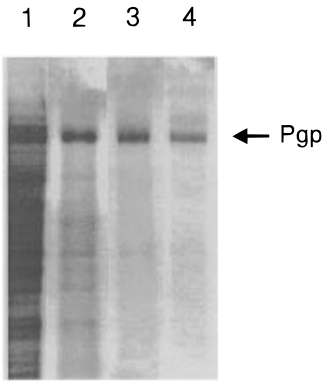

FIGURE 1: Purification and reconstitution of Pgp from MDR CH^R-B30 cells. Purified Pgp in CHAPS buffer was dialyzed against 50 mM ammonium bicarbonate and lyophilized before electrophoresis, whereas proteoliposomes containing purified Pgp were analyzed directly. SDS−PAGE was carried out without boiling the samples, followed by staining with Coomassie Blue. Lane 1, CH^{R}B30 plasma membrane, 19 μg of protein; lane 2, CHAPS extract (partially purified Pgp), 6 μg of protein; lane 3, concanavalin A−Sepharose run-through (highly purified Pgp), 5 μg of protein; lane 4, DMPC proteoliposomes containing reconstituted purified Pgp, 2.9 μg of protein. The Pgp band at 170 kDa is indicated by an arrow.

Lipid:protein ratios of the final reconstituted DMPC−Pgp preparations were determined following harvesting of the proteoliposomes by centrifugation. The lipid recovery was estimated by using a radioactive [^{14}C]DPPC tracer, and the Pgp content was determined by protein assay. The overall recovery of DMPC remained relatively constant, in the range 45−55%, for lipid:protein ratios in the range 36:1 to 6:1 (w/w) (Table 1). Inclusion of lower amounts of protein led to greatly reduced lipid recovery. For the sample with a lipid:protein ratio of 63:1, the DMPC recovery dropped to 24%, and for DMPC alone, recovery was only 6%. This effect likely arises because the incorporation of Pgp in the DMPC

Table 1: Summary of Lipid and Protein Recoveries for DMPC−Pgp Proteoliposomes

final L:P ratio (w/w) (%)[c]	P:L[a] mole ratio ($\times 10^4$)	DMPC recovery (%)[b]	Pgp recovery (%)
DMPC alone	0.000	6.0	−
63:1	0.633	24	51
36:1	1.11	43	56
35:1	1.14	50	53
25:1	1.60	55	61
24:1	1.66	44	61
22:1	1.81	54	59
20:1	1.99	55	51
16:1	2.49	45	53
14:1	2.85	50	56
10:1	4.20	54	64
6:1	6.65	52	77

[a] Protein:lipid (P/L) mole ratio was calculated assuming a Pgp molecular mass of 170 kDa. [b] Estimated based on recovery of [^{14}C]D-PPC tracer included in the reconstitution mixture, which was determined by scintillation counting. [c] Estimated by protein assay using the method of Peterson (1983).

bilayers increases their density, so that the liposomes sediment more easily.

Incorporation of purified Pgp into the proteoliposomes was in the range 51−77% for lipid:protein ratios from 63:1 to 6:1 (w/w) (Table 1). Protein recovery tended to increase slightly with decreasing lipid:protein ratio of the liposomes, although from 36:1 to 10:1, Pgp incorporation fell in a narrow range from 53 to 61%. The specific ATPase activity of Pgp in reconstituted DMPC proteoliposomes was typically around 0.45 μmol·min^{-1}·(mg of protein)$^{-1}$. Only the ATPase activity of inward-facing Pgp is measurable in a sealed vesicle system, since outward-facing protein does not have access to ATP (Sharom et al., 1993). Trial reconstitution experiments carried out above the phase transition temperature for DMPC, at 26 °C, showed substantially lower Pgp incorporation into the proteoliposomes (23−29%), and ATPase specific activity was also reduced by ∼15% compared to reconstitution performed at 4 °C. For this reason, reconstitution of purified Pgp was routinely carried out at 4 °C, below the DMPC phase transition temperature.

Residual detergent can affect the thermotropic properties of reconstituted proteoliposomes. A sensitive chemical assay for CHAPS was carried out using both 50:1 and 6:1 (w/w) DMPC:Pgp reconstituted proteoliposome samples. Results showed that residual CHAPS in the proteoliposomes was below the level of detection, and we estimate that <0.02% of the detergent originally added remains in the sample after reconstitution.

Our previous studies showed that proteoliposomes of 1:1 (w/w) egg PC−DPPE containing Pgp (lipid:protein ratio 45:1 w/w) prepared using this reconstitution method were largely unilamellar, forming a bimodal population with mean diameters of 0.24 and 0.80 μm (Sharom et al., 1993). QELS measurements of size distribution were carried out on reconstituted proteoliposome samples with both high (50:1 w/w) and low (6:1 w/w) DMPC:protein ratios. As shown in Figure 2A, the proteoliposomes with a high lipid:protein ratio formed a bimodal population, with mean diameters of 0.58 and 1.76 μm, respectively. Proteoliposomes with a low lipid:protein ratio formed a single population with a mean diameter of 0.86 μm (Figure 2B). These results indicate that even at very high Pgp contents, the vesicles remain relatively homogeneous, and are probably unilamellar. The fraction

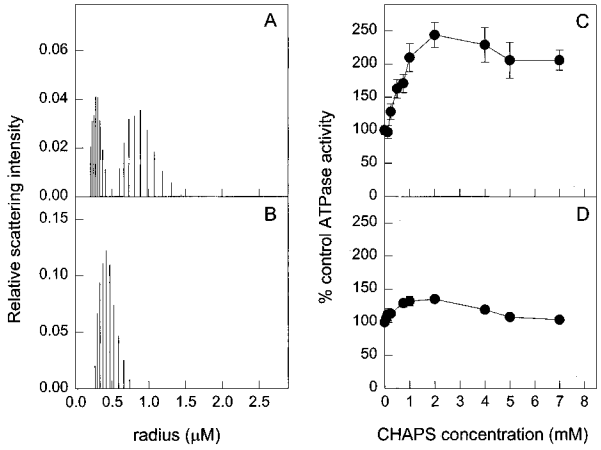

FIGURE 2: Characterization of reconstituted Pgp proteoliposomes with DMPC:protein ratios of 50:1 w/w (A, C) and 6:1 w/w (B, D). QELS measurements of size distribution were carried out (A, B), and the fraction of Pgp molecules with ATP binding domains facing the vesicle lumen was assessed by permeabilization with increasing concentrations of the detergent CHAPS (C, D). The data points in panels C and D are presented as the mean ± SE for triplicate measurements.

of Pgp molecules with their ATP binding domains facing the vesicle lumen can be estimated by permeabilization of the proteoliposomes with increasing concentrations of the detergent CHAPS (Sharom et al., 1993). In the proteoliposomes of high lipid:protein ratio, ATPase activity increased by 140% on addition of CHAPS (Figure 2C), suggesting that 42% of the Pgp molecules are reconstituted with their ATP sites facing the vesicle exterior (inward-facing), and 58% are reconstituted with their ATP sites facing the vesicle lumen (outward-facing). In contrast, the low lipid:protein ratio proteoliposomes showed an increase in ATPase activity of only 35% on permeabilization (Figure 2D), indicating that 74% of the Pgp molecules are inward-facing, and 26% are outward-facing, with their ATP binding domains facing the lumen.

Differential Scanning Calorimetry of DMPC Bilayers Containing Reconstituted Pgp. DSC scans of the gel to liquid-crystalline phase transition of DMPC−Pgp proteoliposomes at different lipid:protein ratios are shown in Figure 3. Repeated scans gave identical heat capacity profiles, indicating that the reconstituted system was at equilibrium, and the samples were stable over the time course of the experiments. The phase transition profile of unilamellar liposomes of DMPC alone, prepared by gel filtration detergent removal in the same way as the reconstituted Pgp proteoliposomes, was similar to that of multilamellar vortexed DMPC liposomes (data not shown). Values of T_m were 25.15 and 24.80 °C, and $\Delta T_{1/2}$ was 1.06 and 1.08 °C, for DMPC liposomes prepared by gel filtration and by vortexing, respectively.

The incorporation of Pgp into the proteoliposomes had a marked effect on the DMPC gel to liquid-crystalline phase transition (Figure 3). The main phase transition was broadened significantly, even at low protein concentrations, and a noticeable alteration in the DSC scan was observed at the highest lipid:protein ratio of 63:1 (w/w). For lipid:protein ratios in the range 63:1 to 20:1 (w/w), the transition was broadened in a fairly symmetrical manner, and there was no evidence of multiple components of distinct peak width in the DSC scan. The onset of melting gradually declined from

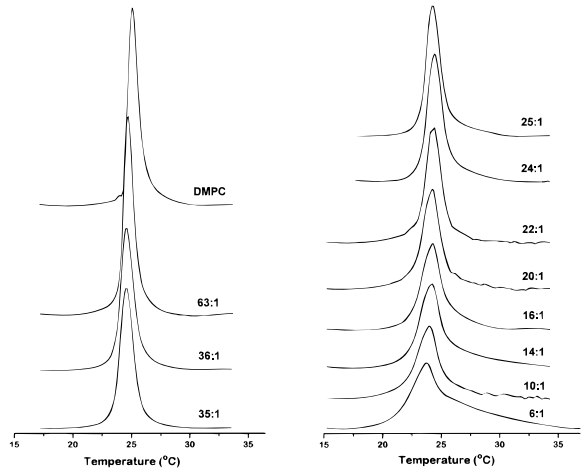

FIGURE 3: Dependence of the DSC scan shape on Pgp mole fraction for proteoliposomes of DMPC containing reconstituted Pgp at lipid:protein ratios varying between 63:1 and 6:1 w/w (as indicated on each trace). DMPC liposomes containing no Pgp were prepared using the same gel filtration technique as for reconstitution of Pgp (DMPC trace). Reconstituted proteoliposomes were resuspended in sample buffer at a DMPC concentration of ~0.5 mg/mL, prewarmed above the phase transition temperature of DMPC, cooled to 4 °C, and thermally analyzed at a scan rate of 1.5 °C/min. Duplicate scans of the same sample were essentially superimposable.

about 23 °C in pure DMPC liposomes to 21 °C at 20:1 DMPC:Pgp, and the completion of melting remained at about 28 °C. At lower lipid:protein ratios of 16:1 (w/w) and below, a second very broad shoulder centered at a slightly higher temperature appeared in the DSC traces. Onset of DMPC melting was reduced to about 19 °C at 6:1 (w/w) DMPC:Pgp, and completion of melting was delayed to much higher temperatures, about 33 °C. Thus, at low Pgp contents, the DMPC melting peak remained fairly symmetrical, but as Pgp levels increased it became skewed to the high-temperature end of the phase transition.

Unilamellar DMPC liposomes prepared by detergent removal exhibited a pretransition at 15.0 °C, which was very similar to the pretransition temperature of 15.1 °C for multilamellar DMPC liposomes (not shown in Figure 3). The pretransition was not observed even in the sample of lowest Pgp content at lipid:protein 63:1 (w/w). Other research groups have noted that the pretransition of DMPC is highly sensitive to the incorporation of protein and peptides into the bilayer (Zhang et al., 1992; Naderi et al., 1995).

Effects of Pgp on the Gel to Liquid-Crystalline Phase Transition of DMPC. Inclusion of increasing quantities of Pgp in the proteoliposomes progressively lowered the phase transition temperature, T_m, in a protein concentration-dependent fashion (Figure 4). The decrease in T_m appeared to occur in two stages. The first phase, which took place from 63:1 to 16:1 DMPC:Pgp (w/w), gave rise to a drop of ~0.75 °C, which was steep at first, and plateaued out at a lipid:protein ratio of about 25:1. In the second phase, there was a steeper drop in T_m with decreasing lipid:protein ratio, which resulted in a further 1.35 °C decline in T_m.

Pgp incorporation caused a marked effect on the width of the main phase transition, as observed by a large increase in the peak width, $\Delta T_{1/2}$. As shown in Figure 5, the width of the DMPC phase transition rose in a monotonic fashion with increasing mole ratio of Pgp in the bilayer. The peak was

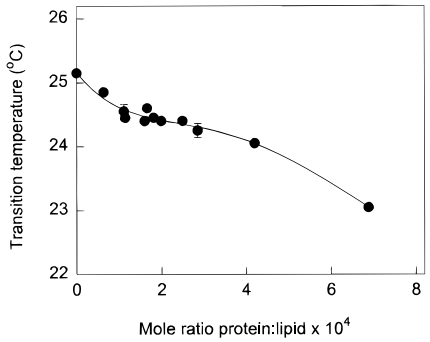

FIGURE 4: Effect of increasing mole fractions of Pgp on the gel to liquid-crystalline phase transition temperature, T_m, of DMPC bilayers. Data are presented as the means for two consecutive scans of the same proteoliposome sample.

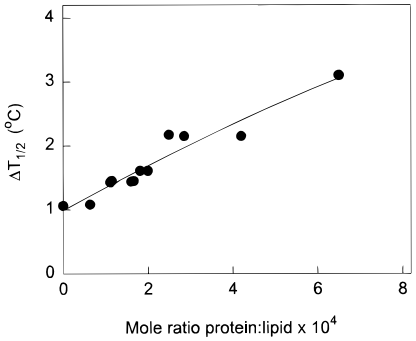

FIGURE 5: Effect of increasing mole fractions of Pgp on the width of the main gel to liquid-crystalline phase transition, $\Delta T_{1/2}$, of DMPC bilayers. Data are presented as the means for two consecutive scans of the same proteoliposome sample.

over 3-fold broader at 6:1 lipid:protein (w/w) compared to pure DMPC.

The cooperative unit, CU, is a parameter that describes the degree of intermolecular cooperation between phospholipid molecules undergoing the gel to liquid-crystalline phase transition, *i.e.*, the number of phospholipid molecules undergoing melting simultaneously. The value of the CU may be obtained from the van't Hoff enthalpy, which is, in turn, calculated from T_m and $\Delta T_{1/2}$ (see Materials and Methods). The value of the CU is very sensitive to impurities in the lipid bilayer, and other attributes of the sample. However, comparison of the CU for phospholipid bilayers containing different amounts of reconstituted protein is useful in evaluating the effects of protein on the cooperativity of the phase transition. In the present experiments, the value of the CU for multilamellar DMPC liposomes, prepared by vortexing, was found to be 157. Liposomes of DMPC alone, prepared by detergent removal, displayed an almost identical CU of 154, which suggests that no traces of residual detergent remain in the liposomes. Figure 6 indicates that the value of the CU drops dramatically on incorporation of Pgp into the bilayer. The decrease in the CU is dependent on the concentration of added protein, and approaches a value of 40 at the lowest lipid:protein ratio of 6:1 (w/w) DMPC:Pgp. Thus, Pgp markedly disrupts the cooperativity of the DMPC phase transition.

Effects of Pgp on the Thermodynamic Characteristics of DMPC Bilayers. The areas under the peaks in the DSC scans were normalized for the amount of phospholipid analyzed, and a value for the phase transition enthalpy, ΔH, was calculated for each reconstituted sample. The data were

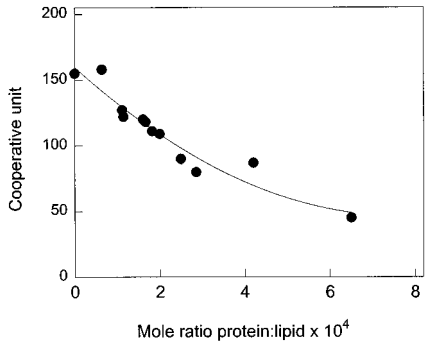

FIGURE 6: Variation of the cooperative unit for the main gel to liquid-crystalline phase transition of DMPC with increasing mole fraction of Pgp in the bilayers. Data are presented as the means for two consecutive scans of the same proteoliposome sample.

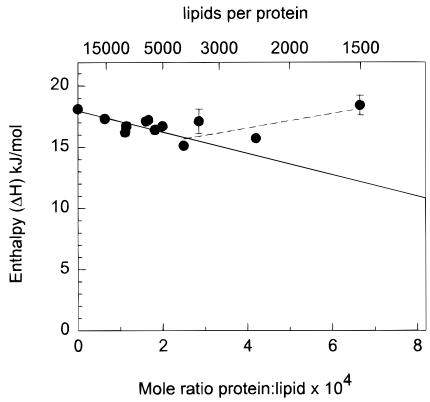

FIGURE 7: Effect of increasing mole fractions of Pgp on the total enthalpy change per mole of phospholipid, ΔH, for the main gel to liquid-crystalline phase transition of DMPC. Each data point represents an individual reconstituted sample, prepared independently using several different Pgp preparations. The values for the enthalpy of melting of DMPC were generated from the DSC scans shown in Figure 3, as outlined under Materials and Methods. Each data point shows the mean value of ΔH for two consecutive scans of the same proteoliposome sample. The number of phospholipids per protein is indicated on the top scale.

fitted to the equation (Van Zoelen et al., 1978):

$$\Delta H = \Delta H_o[1 - m(\mathrm{P/L})]$$

where ΔH_o and ΔH are the transition enthalpies in the absence and presence of protein, respectively, P/L is the protein:lipid mole ratio, and m is the number of phospholipids prevented from participating in the gel to liquid-crystalline phase transition by one molecule of protein. A plot of ΔH *vs* P/L (Figure 7) shows that reconstitution of increasing amounts of Pgp into the DMPC bilayer decreased the enthalpy of the transition about 17%, in a linear fashion, in the lipid:protein ratio range 63:1 to 16:1 (w/w). Extrapolation of this line to the *x*-axis indicated the value of m to be 485. Thus, assuming that interaction of phospholipids with Pgp causes their complete removal from the phase transition, almost 500 DMPC molecules appear to be perturbed by a single molecule of Pgp. At lower lipid:protein ratios, from 16:1 to 6:1 (w/w), the slope of the plot changed direction, and the transition enthalpy increased to close to the value for pure DMPC (Figure 7). A rise in ΔH with higher protein content has been observed previously for the human erythrocyte anion exchanger (Chicken & Sharom, 1984), the Na^+,-Mg^{2+}-ATPase from *Acholeplasma laidlawii* (McElhaney, 1986), and the human erythrocyte hexose transporter (Naderi et al., 1995). This behavior has been interpreted as arising from either protein aggregation within the bilayer at high protein:lipid ratios, which leads to a reduction in the number of phospholipids perturbed by proteins, or a change in the conformation of hydrophilic regions of the protein, such that they differ in their interactions with the bilayer.

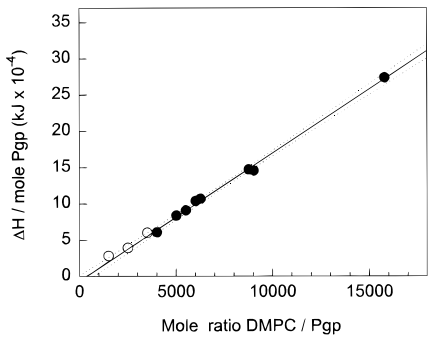

FIGURE 8: Dependence of the DMPC phase transition enthalpy per mole of Pgp on the lipid:protein mole ratio. Each data point represents an individual reconstituted sample, prepared independently using several different Pgp preparations. The values for the enthalpy of melting of DMPC were generated from the DSC scans shown in Figure 3, as outlined under Materials and Methods. Each data point shows the mean value of ΔH for two consecutive scans of the same proteoliposome sample, and error bars are contained within the symbols. The data points indicated by the open circles are those found on the section of positive slope in Figure 7 (the dotted line), and they were not included in the linear regression analysis of the data. The 95% confidence interval for the data points included in the linear regression analysis (closed circles) is indicated by the dotted lines.

Figure 8 shows how the phase transition enthalpy per mole of incorporated Pgp changes with increasing mole ratio of lipid in the host bilayer. The interaction of Pgp with DMPC caused a linear decline in the value of ΔH. Extrapolation to zero enthalpy (the intercept on the *x*-axis) of the line for samples with lipid:protein ratios in the range 63:1 to 16:1 (w/w) (*i.e.*, the downward linear section of Figure 7) led to another estimate of the number of lipids removed from the phase transition. Figure 8 indicated that around 375 ± 197 (mean ± SEM) DMPC molecules are prevented from participating in the phase transition by each molecule of Pgp. The average transition enthalpy of those phospholipids that do undergo melting in the presence of Pgp can be obtained from the slope of this plot (Schiffer et al., 1993). A ΔH value of 17.6 kJ/mol was derived from Figure 8, which compares with ΔH values of 17.4 and 18.1 kJ/mol for multilamellar DMPC liposomes made by vortexing, and unilamellar liposomes prepared by detergent removal, respectively. These results suggest that the thermodynamic properties of the lipids undergoing melting in the presence of Pgp are essentially unchanged by the protein.

It should be noted that the value of ΔH obtained from Figure 8 should be considered a rough estimate, since the equation used to describe lipid−protein interactions (see earlier) assumes that only two types of lipid molecules exist: either unperturbed "free" lipids in the bilayer, or fully perturbed lipids in an annular ring around the reconstituted integral protein. This "two-state" model of lipid behavior in reconstituted lipid−protein systems is clearly an oversimplification. In reality, we would also expect lipid molecules located further away from the integral protein, in the second or third solvation layers, to demonstrate significant broadening of their phase transition, and a reduced value of

ΔH. Nevertheless, useful information can be obtained using this method of analysis, especially if the goal is to make comparisons between different integral proteins.

DISCUSSION

The present study is the first to examine the lipid−protein interactions of a member of the ABC superfamily of transporters. It also represents the first report of the reconstitution of purified Pgp into defined bilayers of a single phospholipid species. Unlike the preparations described by other researchers (Urbatsch et al., 1994; Shapiro & Ling, 1994), the purified Pgp used in this work contains no added exogenous lipids, which makes it ideal for direct reconstitution into a defined phospholipid such as DMPC. The only additional lipids present in the reconstituted preparations are the endogenous phospholipids (53−55 per molecule of Pgp, consisting mainly of PE and PS), which copurify with Pgp during detergent extraction and affinity chromatography (Sharom et al., 1995a). We have previously shown that removal of these tightly-bound lipids leads to complete inactivation of the ATPase activity of Pgp (Doige et al., 1993).

The current work focused on examination of the interactions of Pgp with PC, since this phospholipid, together with its sphingolipid counterpart sphingomyelin, makes up the major lipid in the plasma membrane of MDR cells in which Pgp is expressed. We previously showed that egg PC, which is a highly fluid PC mixture, was highly effective at restoring ATPase activity following delipidation of Pgp (Doige et al., 1993), and also increased the catalytic activity of pure Pgp (Sharom et al., 1995a). DMPC was also effective at restoring ATPase activity after delipidation, but had no effect on Pgp ATPase activity, and was unable to protect Pgp from thermal inactivation. Previous studies in our laboratory have also revealed that Pgp excludes phospholipids with choline headgroups (PC and sphingomyelin) from its immediate "boundary" layer (Sharom et al., 1995a). Pgp therefore appears to have distinct preferences for different types of membrane lipids immediately surrounding it, and in the bulk lipid bilayer into which it is inserted.

The size distribution of the reconstituted DMPC:Pgp proteoliposomes used in this study was characterized by QELS and detergent permeabilization. A bimodal population of vesicles was observed at 50:1 (w/w) DMPC:Pgp, similar to that previously observed with proteoliposomes of 1:1 (w/w) egg PC−DPPE containing Pgp at a 45:1 (w/w) lipid:protein ratio (Sharom et al., 1993). At 6:1 (w/w) DMPC:Pgp, a single relatively uniform population of vesicles was observed. CHAPS permeabilization experiments indicated that at a high lipid:protein ratio, Pgp was reconstituted with ∼40% of the molecules facing inward, and ∼60% facing outward, with their ATP binding domains in the vesicle lumen. We previously noted that ∼55% of the Pgp reconstituted into egg PC−DPPE vesicles faced inward (Sharom et al., 1993). At a low DMPC:Pgp ratio, the situation was reversed, and ∼75% of the transporters faced inward. This change in the symmetry of Pgp reconstitution at a low lipid:protein ratio may result from steric crowding inside the vesicle lumen when the Pgp content is increased. The two ATP binding domains are relatively large, and might be expected to favor a location on the outer surface of the vesicle when the lipid:protein ratio is low. Taken together, our results suggest that the proteoliposomes remain relatively homogeneous and largely unilamellar over a range of DMPC:Pgp ratios from 50:1 to 6:1 (w/w).

The interaction of Pgp with DMPC led to a downward shift in the temperature of the main phase transition, and a marked broadening of the DSC peak, resulting from a large reduction in the cooperativity of melting. For lipid:protein ratios in the range 63:1 to 16:1 (w/w), the presence of Pgp decreased the transition enthalpy in a linear fashion (Figure 7), which can be attributed to the withdrawal of phospholipids from the phase transition by interaction with the protein. The slope of the plot in Figure 8 indicates that the lipid which still undergoes the phase transition basically melts with the same enthalpy as pure DMPC, despite the presence of Pgp. Using two different methods of analysis (Figures 7 and 8), each molecule of Pgp appears to remove between 375 and 485 DMPC molecules from the phase transition at the low to medium Pgp densities likely to be found in a typical MDR cell plasma membrane. This is many more lipids than would be expected purely on the basis of interactions with 12 membrane-spanning helices. Using a hexagonal packing model, it was estimated that 12−18 phospholipids are present in the boundary layer around a single membrane-spanning α-helix (Bradrick et al., 1989). Another study of a membrane-spanning peptide indicated that about 18 phospholipids are needed to form a single solvation layer around 1 α-helix (Morrow et al., 1985). On this basis, 12 isolated α-helices would be expected to interact with a maximum of between 144 and 216 phospholipids. Membrane-spanning helices of polytopic integral proteins pack into bundles via helix−helix interactions, thus substantially reducing the number of lipids associated with each protein. We would, therefore, expect that a single molecule of Pgp would interact with even fewer lipid molecules than the maximum numbers alluded to above. Clearly, Pgp affects many more membrane lipids than would be expected based on these types of considerations.

One explanation for the large number of perturbed lipids may be that, like the Ca^{2+}-ATPase from sarcoplasmic reticulum (Lentz et al., 1983), the membrane-spanning segments of Pgp partially perturb a second shell of phospholipids. It is also possible that the additional phospholipids which appear to be affected by Pgp are in fact interacting with the two nucleotide binding domains of the protein. Although these regions are usually pictured as being separately folded soluble domains, evidence is accumulating that they may interact more closely with the membrane bilayer than has been previously envisaged. The ATPase activity of Pgp is absolutely dependent on the presence of membrane lipids, and their removal leads to inactivation, which is reversible by re-addition of phospholipids (Doige et al., 1993). Both constitutive and drug-stimulated ATPase activity appear to be modified by the presence of specific phospholipids (Urbatsch & Senior, 1995; Sharom et al., 1995b). In addition, the kinetic parameters for ATP hydrolysis can be modulated by the phase state of the bilayer (Sharom, 1997). Either lipid stabilization of the membrane-spanning regions of Pgp is necessary for ATPase activity at the nucleotide binding domains, or, alternatively, these domains themselves interact with the membrane bilayer, so that their integrity depends on the presence of phospholipids. Finally, when the isolated nucleotide binding domains were expressed separately, they were observed to associate with

the membrane fraction of the cell, which led to the suggestion that they might normally interact with the membrane (Sharma & Rose, 1995). We previously noted that human erythrocyte band 3, a 110 kDa anion exchange transporter which is also believed to possess at least 12 membrane-spanning segments, appeared to perturb about 685 phospholipids when analyzed by DSC (Chicken & Sharom, 1984). In this case, it was also postulated that the hydrophilic domains of the transporter interacted with the bilayer surface, and contributed to the perturbation of membrane phospholipids.

The estimates of the number of DMPC molecules perturbed by Pgp discussed above assume that these phospholipids are completely withdrawn from the phase transition by their interaction with Pgp. However, if they have a reduced, but nonzero, transition enthalpy, it is conceivable that an even larger number of lipid molecules may be perturbed by interaction with Pgp. Partial chain melting has been proposed for perturbed lipids in some lipid−protein systems [see, for example, Zhang et al. (1992) and Heyn et al. (1981)].

At lipid:protein ratios lower than 16:1 (w/w), the slope of the plot of ΔH vs protein mole ratio is changed, and the value of ΔH increases. Such biphasic plots have been reported previously for both the hexose transporter and the anion exchanger from human erythrocytes (Naderi et al., 1995; Chicken & Sharom, 1984), as well as the Na^+,Mg^{2+}-ATPase from *Acholeplasma laidlawii* (McElhaney, 1986). One explanation for this behavior is that it arises from protein aggregation within the bilayer at high protein:lipid ratios (Naderi et al., 1995; Chicken & Sharom, 1984). Protein oligomers interact with many fewer phospholipids than a monomer, and phospholipids would thus be released back into the bulk lipid, where they can undergo the phase transition once more. For example, bacteriorhodopsin exhibits this type of behavior. At low temperatures, a protein lattice forms, for which protein−protein interactions are predominant, and only 10 phospholipids are perturbed per protein. After dispersion as monomers, each bacteriorhodopsin molecule perturbs 60 phospholipids (Heyn et al., 1981). Pgp has been reported to exist as dimers (Boscoboinik et al., 1990; Loo & Clarke, 1996) and/or oligomers (Poruchynsky & Ling, 1994) in the plasma membrane of MDR cells.

An alternative explanation for the biphasic ΔH plot involves a change in the conformation of Pgp at high protein density in the bilayer. If extramembranous regions of the protein (*e.g.*, the ATP binding domains, the glycosylated extracellular loop) are involved in perturbing phospholipids, then one can postulate that crowding in the bilayer plane might cause these regions to undergo a conformational change, such that the altered conformation has greatly reduced interactions with bilayer phospholipids. Such a change in conformation has been reported for human erythrocyte glycophorin; the highly perturbing and less perturbing conformations affect 300 and 100 phospholipids, respectively (Rüppel et al., 1982).

At high Pgp:lipid mole ratios, we observed that the transition enthalpy of the DMPC bilayer increased until it reached the value observed for pure DMPC. However, this lipid does not behave like pure DMPC, as indicated by the shape of the DSC scan for the sample with lipid:protein ratio 6:1 (w/w). Although it melts with a transition enthalpy typical of DMPC, the cooperativity of the melting transition is markedly lowered, resulting in a very broad peak for the phase transition. At high Pgp mole ratios, a large fraction of the lipid in the bilayer appears to exist in this perturbed state. The possible effect of this highly perturbed lipid on the physical behavior of chemotherapeutic drugs within the lipid bilayer, and the transport function of Pgp, remains to be determined.

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