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The functional purification of P-glycoprotein is dependent on maintenance of a lipid–protein interface

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Abstract

P-Glycoprotein (P-gp) is a 180-kDa membrane-bound transporter which can confer the multi-drug resistance phenotype on tumor cells. We have examined the factors required to preserve activity of P-gp during its purification. The starting material for purification was plasma membranes from Chinese hamster ovary (CH^rB30) cells, overexpressing P-glycoprotein. These membranes displayed drug stimulated ATPase activity ($V_m = 897 \pm 55$ nmol min^{-1} mg^{-1}; $K_m = 1.8 \pm 0.4$ mM) and high affinity binding of [^{3}H]vinblastine ($K_d = 36 \pm 5$ nM; $B_m = 161 \pm 11$ pmol/mg). Several non-ionic detergents which readily solubilized P-glycoprotein significantly inhibited ATPase activity and drug binding at concentrations well below their respective CMC values. This inactivation was prevented by excess crude lipid mixtures, with the greatest protection afforded against dodecyl-maltoside. Furthermore, the significantly reduced binding affinity and capacity of solubilized P-gp was partly reversed by the addition of lipids. A combination of anion-exchange and hydroxyapatite chromatography were used to purify P-gp with high yield to greater than 90%. The purified, reconstituted P-gp displayed high ATPase activity ($V_m = 2137 \pm 309$; $K_m = 2.9 \pm 0.9$ mM) which was stimulated by verapamil ($EC_{50} = 3.8 \pm 0.6$ μM) and inhibited by orthovanadate (3.1 ± 0.8 μM). Pure P-gp also displayed high affinity vinblastine binding ($K_d = 64 \pm 9$ nM) with a capacity of 2320 ± 192 pmol/mg. This purification scheme yields the highest P-gp activity reported to date, and indicates a dependence of function on maintaining a lipid–protein interface. © 1997 Elsevier Science B.V.

Keywords: Multi-drug resistance; P-glycoprotein; Drug binding; Purification; Lipid-protein interface

1. Introduction

The development of a multiple-drug resistance (MDR) phenotype frequently results in the failure of chemotherapeutic treatment of neoplastic disorders. An important factor in MDR is the 180-kDa membrane-bound P-glycoprotein (P-gp) [1,2]. P-gp is thought to confer drug resistance by maintaining low

Abbreviations: MDR, multiple drug resistance; P-gp, P-glycoprotein; LUVs, large unilamellar vesicles; PEG-6000, polyethylene glycol 6000; CMC, critical micellar concentration; PC, phosphatidylcholine; PA, phosphatidic acid

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cellular levels of chemotherapeutic drugs in tumor cells. The protein is also expressed in many normal tissues [3] and belongs to a large group of eukaryotic and prokaryotic membrane proteins known as the ABC superfamily [4]. P-gp is post-translationally modified by phosphorylation [5] in the highly charged 'linker' region and glycosylation in the first extracellular loop [6]. However, it appears that neither phosphorylation, [7,8] nor glycosylation [6] affects the ability of P-gp to confer multi-drug resistance.

A large number of unrelated compounds specifically bind or label P-gp [9] in its native environment. In addition, membranes containing P-gp display high levels of ATP hydrolytic activity which is altered by several P-gp substrates [10,11]. The precise mechanism by which ATP hydrolysis and substrate binding are coupled to facilitate transport remains unclear. The conventional model in which P-gp contains a distinct binding site(s) with a localised conformational change leading to substrate reorientation across the bilayer, as demonstrated for several other membrane transporters may be envisioned [12]. However, to account for the broad substrate specificity of P-gp, some novel transport models have been proposed [13–15]. For example, the hydrophobic vacuum model proposes that a non-selective hydrophobic surface on P-gp is used to accept the wide range of substrates whose only consistent link is their hydrophobicity. However, the majority of pharmacological [16–18], mutational [19] and photo labeling data [20] support a conventional transport model in which multiple specific drug acceptor sites accessible from the cytoplasm are found on P-gp.

In order to address the mechanisms of P-gp-mediated drug transport, including turnover number, multiplicity of binding sites, coupling of ATP hydrolysis to transport and to obtain structural data, the purification of functional P-gp is required. Several purification procedures have been developed for P-gp, resulting in protein ranging from 20 to 90% purity. Among the methods used are: (1) immunoaffinity chromatography [21]; (2) selective detergent extraction [22]; (3) ion-exchange chromatography [23]; (4) affinity chromatography [24] and various combinations of these [25,26]. However, in several of these procedures the specific P-gp activity did not increase above levels in the starting material, reflecting significant levels of inactivation. It has been proposed that interaction of P-gp with several chromatographic matrices is a major cause of inactivation [26,27].

The first step in purification of an integral membrane protein involves extraction from its lipid environment, usually by detergents. P-gp appears to be influenced by its membrane environment. In particular, (1) expression of P-gp tends to 'destabilize' the plasma membrane [28,29], (2) modification of lipid composition or biophysical properties alters P-gp activity [30,31], and (3) the ATPase activity of soluble P-gp is affected by several phospholipids [26]. Therefore the delipidation of proteins which can occur during solubilization and chromatographic separation [32] may have significant consequences for the functional integrity of P-gp.

In the present study, we have investigated the solubilization and chromatographic conditions that retain maximal drug binding and ATP hydrolyzing activity of P-gp. It was concluded that disruption of the lipid–protein interface is a major factor responsible for inactivation of P-gp during purification. The resulting purification scheme generated P-gp with significantly increased ATPase activity and drug binding capacity compared with the starting material.

2. Materials and methods

2.1. Materials

[^{3}H]Vinblastine sulfate (13.5 Ci/mmol), and [^{3}H]phosphatidyl choline (62 Ci/mmol) were purchased from Amersham (Amersham, UK). Hydroxyapatite (CHT-II) and anion-exchange (Q) Econo-Pac prepacked chromatography columns (1 ml) and the Detergent Compatible protein assay kit were from Bio-Rad (Hemel Hempstead, UK). Bovine serum albumin (> 95% pure), γ-globulin (> 95% pure), ascorbate, verapamil hydrochloride, orthovanadate and molybdate were from Sigma (Poole, UK). GF/F glass fiber, 0.1 and 0.2 μm nitrocellulose filters and crude soya bean lipids (asolectin) were obtained from BDH (Poole, UK). The Rapid-Ag silver stain kit was purchased from ICN chemicals (Thame, UK), and bovine spinal cord lipid extract from Lipid Products (Surrey, UK). The detergents dodecyl-β-D-maltopyranoside, octyl-β-D-glucoside and zwittergent 3–12 were purchased from Calbiochem (Nottingham, UK).

2.2. Plasma membrane preparations

CH^rB30 cells were grown as described previously in media supplemented with 30 μg/ml colchicine [1]. Preparation of plasma membranes from CH^rB30 cells was based on previously published methods [33]. Briefly, CH^rB30 cells (1×10^9) were suspended to a density of 2.5×10^7 cells/ml and subjected to two rounds of N_2 cavitation (20 min) at 1000 p.s.i. and 4°C. Following the final centrifugation, the membranes were resuspended at a protein concentration of 5–7 μg/μl and stored at −80°C for up to 6 months.

2.3. P-gp purification procedure

Frozen plasma membranes were rapidly thawed and collected by centrifugation at 150 000 × *g* for 40 min. The pellet was resuspended at a protein concentration of 5 mg/ml in low ionic strength buffer A (18% (v/v) glycerol, 10 mM PIPES, pH 6.9, 1 mM EDTA, 0.02% NaN_3), containing 0.2% (w/v) sonicated bovine spinal cord lipids and 0.4% (w/v) dodecyl-maltoside. This mixture was incubated at 4°C for 30 min and subsequently centrifuged at 100 000 × *g* for 20 min. The supernatant containing solubilized P-gp was injected onto a 1-ml Q-column previously equilibrated with buffer A containing 0.02% (w/v) dodecyl-maltoside. The column was washed with 4-vols. of buffer A and then subjected to a linear gradient of NaCl to reach 1 M over 20 min (2 ml/min flow rate). P-gp eluted in the void volume under the above conditions. The appropriate fractions were concentrated using 100 kDa molecular weight cut-off Centricon 100 (Amicon) filters centrifuged at 500 × *g* until a protein concentration of 0.25–0.35 μg/μl was achieved. This concentrated post-anion-exchange protein solution was then loaded onto an hydroxy-apatite column (1 ml) previously equilibrated with buffer C (15 mM NaP_i, pH 6.6, 0.02% (w/v) dodecyl-maltoside). Proteins bound to the matrix were eluted with a linear gradient to 500 mM NaP_i in buffer C over 36 min (0.8 ml/min flow rate). Samples containing P-gp were detected using SDS–PAGE and concentrated to 0.3–0.4 μg/μl using Centricon 100 filters by centrifugation at 500 × *g*.

2.4. Reconstitution procedures

P-gp was reconstituted into preformed unilamellar vesicles (LUVs) made by extrusion of multi-lamellar vesicles through 200 nm nitrocellulose filters as described previously [34]. Electron microscopic evidence has confirmed the unilamellar nature of these liposomes (data not shown). To determine conditions at which the liposomes were completely solubilized or saturated with detergent [35], the LUVs were progressively solubilized with octyl-glucoside. Briefly, LUVs were resuspended at concentrations of 2.5, 5, 10, 12.5, 15, 20 and 25 mM in a total volume of 500 μl dialysis buffer D (150 mM NaCl, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine). Increasing concentrations of octyl-glucoside were then added until light scattering at 750 nm by the LUVs was relieved, reflecting a micellar suspension of lipid and detergent. An equilibration period of 2–4 min (allowing equilibration of the signal) was used at each concentration of octyl-glucoside.

P-gp reconstitution into preformed or solubilized vesicles was achieved by slow dialysis or gel filtration to remove detergent. In each case, a saturating concentration of detergent was added to a 20-mM suspension of LUVs and incubated at room temperature for 10 min. Protein suspension was added to the detergent/lipid mixture to a final protein concentration of 0.1–0.15 μg/μl and incubated at 4°C for 40 min. In the case of slow dialysis, the detergent/lipid/protein mixture was placed in a 6- to 8-kDa cut-off dialysis bag and dialysed against buffer D for 72 h at 4°C, with buffer changes every 12 h. Removal of detergent by gel filtration was achieved with a 1 × 60 cm Sephadex G-50 column equilibrated previously with dialysis buffer D. Prior to the initial use of each new column, the non-specific binding sites were coated by flushing the column with 100 mg asolectin. The protein/lipid/detergent mixtures were loaded onto the column and cloudy fractions of the void volume pooled. These proteoliposomes were collected by centrifugation at 150 000 × *g* for 2 h at 4°C. The pellet was resuspended in buffer D at a protein concentration of 0.4–0.5 μg/μl.

The efficiency of reconstitution was followed by inclusion of [^{3}H]phosphatidyl-choline (1 μCi) in the LUVs. Typically, 15–20 μg reconstituted protein was mixed 1:1 with buffer E (100 mM K_2SO_4, 20

mM PIPES, pH 6.9) containing 60% (w/v) sucrose and 0.05% Triton X-100 to render the liposomes leaky. Buffers containing 20, 10, 5 and 0% (w/v) sucrose were layered over the liposomes in 30% sucrose and the gradients centrifuged at $150\,000 \times g$ for 4 h at 4°C. The location of lipid was determined by using 10% of each sucrose fraction for liquid scintillation counting. A further 20–30% of each fraction was used to assay for protein by SDS–PAGE following precipitation with trichloracetic acid.

2.5. ATP hydrolytic activity of P-glycoprotein

The ATPase activity of P-gp in CH^{r}B30 membranes (1–2 μg protein) or reconstituted into liposomes (0.4–0.6 μg protein) was measured by the method of Chifflet et al. [36]. The incubation period used was 25 min at 37°C and the effects of vanadate and verapamil (5×10^{-8} to 5×10^{-5} M) on ATP hydrolysis were assessed. Vanadate stock solutions were prepared as previously described [37]. ATPase activity was expressed as nmol inorganic phosphate liberated per mg of protein and plotted as a function of added drug concentration. The potency of each compound to alter ATPase activity of P-gp was expressed as an EC_{50} concentration (dose required to give 50% of maximal effect). The EC_{50} concentrations were determined from non-linear regression using the general dose–response equation [38] below:

$$A_o = \left\{(a-b)/\left(1+(X/c)^d\right)\right\} + b \quad (1)$$

where A_o = % basal ATPase activity; a = initial ATPase activity; b = final ATPase activity; $c = EC_{50}$ concentration; d = slope factor; and X = concentration of drug.

2.6. Equilibrium binding of [^{3}H]vinblastine to P-glycoprotein

Binding of [^{3}H]vinblastine to P-glycoprotein was assessed by a rapid filtration assay based on published methods [16]. CH^{r}B30 membranes (20 μg), or reconstituted protein (1–4 μg), were incubated with [^{3}H]vinblastine in a 200-μl volume of binding buffer F (0.05 M Tris HCl, pH 7.4) at 20°C for 120 min. Following incubation, 3 ml ice-cold buffer G (0.02 M Tris HCl, pH 7.4, 20 mM $MgCl_2$) was added and the samples filtered through GF/F and 0.2-μm nitrocellulose filters (presoaked with 0.1% BSA in buffer F) to retain bound drug and subsequently washed twice with 3 ml ice-cold buffer G. Radioactivity associated with the filters was determined by liquid scintillation counting. Filtration of proteoliposomes with GF/F and 0.2-μm nitrocellulose filters retained greater than 80% of CH^{r}B30 membranes, but less than 30% of proteoliposomes. However, greater than 85% of proteoliposomes were retained with a GF/F and 0.1-μm nitrocellulose filter combination.

Saturation isotherms were generated with 0.1–100 nM [^{3}H]vinblastine in the absence or presence of 3 μM vinblastine, the latter indicating the level of non-specific binding. Data were expressed as pmol of [^{3}H]vinblastine bound per mg of protein. The equation below was fitted to saturation isotherms by non-linear regression:

$$B_o = (B_{max} {}^* F)/(K_d + F) \quad (2)$$

where, B_o = amount of [^{3}H]vinblastine bound; B_{max} = maximum density of binding sites; K_d = dissociation constant; F = concentration of free [^{3}H]vinblastine.

Displacement assays were performed with a single concentration of [^{3}H]vinblastine (25–30 nM) in the presence of competing compound at concentrations indicated in the figure legends. Non-specific binding of [^{3}H]vinblastine to CH^{r}B30 membranes was determined in the presence of 3 μM vinblastine. The results for each compound were expressed as a fraction of the total specific binding of [^{3}H]vinblastine in the absence of competing agent. The potency of each compound to displace [^{3}H]vinblastine binding was expressed as an EC_{50} concentration determined by non-linear regression of Eq. (1) to displacement curves. In this case the parameters obtained were; A_o = fraction total [^{3}H]vinblastine bound, a = maximal binding, b = minimum binding, $c = EC_{50}$ concentration, d = slope factor and X = concentration of competing compound.

In order to use the rapid filtration technique to measure binding of [^{3}H]vinblastine to solubilized P-gp, the drug–protein complex was precipitated as described [39]. Briefly, following the appropriate incubation period, the samples were cooled to 4°C in an ice-bath. BSA (0.1 mg), γ-globulin (0.1 mg) and 3

ml of PEG-6000 10% (w/v) in binding buffer F were added to the samples and left for 40 min at 4°C prior to filtration as described above. The washing buffer G contained 5% (w/v) PEG-6000.

2.7. Routine procedures

SDS-gel electrophoresis [40] and immunoblotting [41] were performed according to published methods. Precipitation of dilute protein solutions by trichloroacetic acid was based on published methods [42] and protein concentration determined with a detergent compatible kit according to the suppliers instructions (BioRad, Hemel Hempstead).

3. Results

3.1. Characterization of P-glycoprotein in CH^{r}B30 membranes

The starting material for purification of P-gp was plasma membranes obtained from CH^{r}B30 cells. The activity of P-glycoprotein in its native environment served as a reference for retention of activity during purification as assessed by ATP hydrolysis and equilibrium binding of [^{3}H]vinblastine. The results for CH^{r}B30 membranes are summarized in Fig. 1.

Equilibrium techniques were used to determine the affinity and capacity of vinblastine binding to CH^{r}B30 membranes. The dissociation constant (K_d) of 36.3 ± 4.7 nM ($n = 3$, independent preparations) obtained from saturation isotherms indicates that [^{3}H]vinblastine bound with high affinity. The density of sites for [^{3}H]vinblastine binding to CH^{r}B30 membranes was 161 ± 11 pmol/mg protein. If 1 mol of vinblastine binds to 1 mol of P-gp ($Mr = 180$ kDa) then this B_{max} translates into 2.9% of total membrane protein, which is borne out by silver staining (Fig. 3). Non-specific binding to membranes and filters accounted for 10–15% of total binding and all binding assays were performed in hypotonic buffer to minimize possible accumulation of drug in the intravesicular space. Membranes from the parental cell line AuxB1, which does not express P-gp, displayed negligible specific binding of the radioligand (data not shown).

ATPase assays were performed in the presence of EGTA, NaN_3 and in the absence of Na^+ or K^+ in order to minimize contributions from P-, F- and V-type ATPases present in membranes. The basal ATP hydrolytic activity ($V_{max} = 897 \pm 55$ nmol P_i min^{-1} mg^{-1} $K_m = 1.84 \pm 0.37$ mM) obtained from CH^{r}B30 membranes ($n = 9$) was insensitive to 2 mM ouabain. Verapamil stimulated the basal activity (2 mM ATP) by a factor of 1.59 ± 0.05 ($n = 13$) with high potency ($EC_{50} = 0.60 \pm 0.21$ μM). In contrast, sodium orthovanadate decreased the basal ATPase activity of P-gp by $91 \pm 8\%$ ($n = 9$) with an EC_{50} concentration of 1.8 ± 0.6 μM.

3.2. Effects of non-ionic detergents on P-glycoprotein activity

Prior to their use for solubilization of P-gp, dodecyl-maltoside, octyl-glucoside and zwittergent 3–12 were examined for their effects on function. In particular, the ability of these detergents to inhibit ATPase activity of, and displace [^{3}H]vinblastine binding to P-gp is summarized in Table 1. Complete dose–response curves were performed in the presence or absence of a crude bovine spinal cord phospholipid mixture. On a molar basis, dodecyl-maltoside was the most potent inhibitor of the ATPase activity and drug binding by P-gp with EC_{50} values of 59 ± 10 and 26 ± 7 μM, respectively. The critical micelle concentration (CMC) of this detergent is 200 μM and therefore the EC_{50} values were equivalent to 13–30% of the CMC. However, in the presence of excess lipid, the EC_{50} value for inhibition of basal ATPase activity of P-gp was increased to 368 ± 56 μM. Similarly, the EC_{50} value for displacement of the equilibrium binding of [^{3}H]vinblastine to P-gp was raised to 259 ± 43 μM following addition of lipids. Therefore, a crude mixture of lipids was able to protect the ATPase activity (6-fold) and drug binding (10-fold) by P-gp from the deleterious effects of dodecyl-maltoside. In both cases, the EC_{50} values were raised above the CMC.

The ATPase activity of P-gp was also sensitive to the detergent octyl-glucoside with EC_{50} values of 6.4 ± 3.0 mM (40% of CMC) in the absence, and 18.8 ± 1.7 mM in the presence of spinal cord lipids. The magnitude of protection afforded by the lipids (3-fold) was not as large as observed for dodecyl-maltoside. Binding of [^{3}H]vinblastine to P-gp was

also perturbed by octyl-glucoside with an EC_{50} value (1.2 ± 0.1 mM) equivalent to only 8% of the CMC. A two-fold protection against the effects of octyl-glucoside by excess lipids did not raise the EC_{50} value (2.4 ± 0.8 mM) above the CMC for the detergent.

The zwitterionic detergent, zwittergent 3–12, was also a potent inhibitor of ATPase activity ($EC_{50} = 132 \pm 39$ μM; 7% of CMC) and drug binding ($EC_{50} = 262 \pm 73$ μM; 13% of CMC) associated with P-gp. The inhibition of ATPase activity was partly reversed

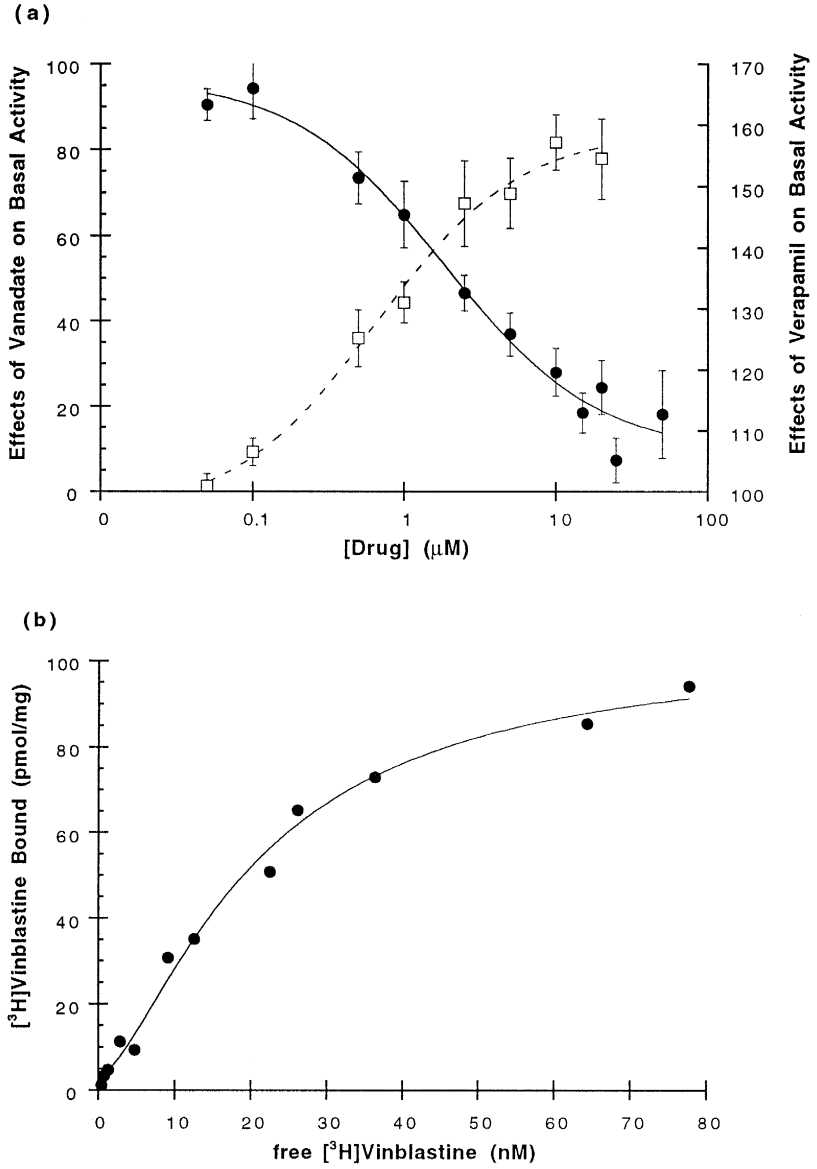

Fig. 1. Characterization of ATPase activity and [^{3}H]vinblastine binding by P-gp in CH^{r}B30 membranes. a: the effects of verapamil (□) and orthovanadate (●) on ATPase activity of membranes (1 μg) in the presence of 2 mM MgATP. Data (mean ± S.E.M.) are expressed as a percentage of ATPase activity in the absence of drug. b: saturation isotherm for [^{3}H]vinblastine binding to CH^{r}B30 membranes. [^{3}H]Vinblastine (1–80 nM) was incubated with 20 μg membrane at 23°C for 2 h before bound and free ligand were separated by rapid filtration. Non-specific binding was defined as the amount of ligand bound in the presence of 3 μM excess cold vinblastine and was linear as a function of [^{3}H]vinblastine concentration. The data shown were obtained from a single representative experiment and parameters (±asymptotic S.E.) obtained from curve fitting as described in Section 2 (Materials and methods) were: $K_d = 21 \pm 2$ nM and $B_{max} = 103 \pm 4$ pmol/mg.

Table 1
The ability of bovine spinal cord lipids to protect against the effects of the non-ionic detergents (1 nM–0.1 M) dodecyl-maltoside, octyl-glucoside and zwittergent 3–12 on the ATPase activity and [^{3}H]vinblastine binding properties of P-gp in CH^{r}B30 plasma membranes

	Dodecyl-maltoside		Octyl-glucoside		Zwittergent 3–12	
	ATPase (μM)	Binding (μM)	ATPase (mM)	Binding (mM)	ATPase (μM)	Binding (μM)
Control	59 ± 10	26 ± 7	6.4 ± 3.0	1.21 ± 0.06	132 ± 39	262 ± 73
0.2% lipid	368 ± 56	259 ± 43	18.8 ± 1.7	2.44 ± 0.75	444 ± 98	269 ± 77
Fold protection	6.2	10.1	2.9	2.0	3.4	1.0
P-value	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01	n.s.

Results shown are the mean EC_{50} values (± S.E.M.) obtained from 3–5 plasma membrane preparations.

by the addition of spinal cord lipid ($EC_{50} = 444 \pm 98$ μM; 3.4-fold increase). However, lipids did not protect P-gp against the displacement of [^{3}H]vinblastine binding ($EC_{50} = 269 \pm 77$ μM) caused by zwittergent 3–12.

In summary, all three detergents inhibited ATPase activity and drug binding by P-gp to varying degrees. However, the potencies did not vary significantly when expressed as a percentage of CMC. Furthermore, the two activities measured displayed different sensitivities to detergent in the absence or presence of added lipid. Solubilization of integral membrane proteins usually requires detergent concentrations significantly greater than the CMC. Of the detergents tested only dodecyl-maltoside-induced inactivation of P-gp occurred at values above the CMC in the presence of excess lipid.

3.3. Binding of [^{3}H]vinblastine to detergent solubilized P-glycoprotein

In the previous section, the ability of lipids to protect against P-gp inactivation by detergent was assessed. To assess whether the inactivation was reversible, P-gp was solubilized by detergent in the absence of added lipids and saturation isotherms performed to examine [^{3}H]vinblastine binding. P-gp bound [^{3}H]vinblastine with a $K_d = 130 \pm 9$ nM ($n = 3$), a significant reduction (3.6-fold) in affinity from CH^{r}B30 membranes (Fig. 4). Furthermore, the binding capacity of P-gp was reduced approximately 6-fold from CH^{r}B30 membranes to 25 ± 9 pmol/mg protein.

To examine whether the inhibition of drug binding capacity of soluble P-gp by detergent could be reversed, crude spinal cord lipids were added to the solubilized protein. Fig. 2 shows that binding of [^{3}H]vinblastine to solubilized P-gp was increased by 3.5 ± 0.4-fold with an EC_{50} value of 0.33 ± 0.12% (w/v). Maximal recovery of activity was obtained at approximately 8 mg/ml (0.8% w/v) lipid. P-gp was solubilized with 4 mg/ml dodecyl-maltoside which is equivalent to a lipid/detergent ratio of 2, roughly the R_{sol} value for dodecyl-maltoside (see Section 3.6). The R_{sol} value is the ratio of lipid and detergent

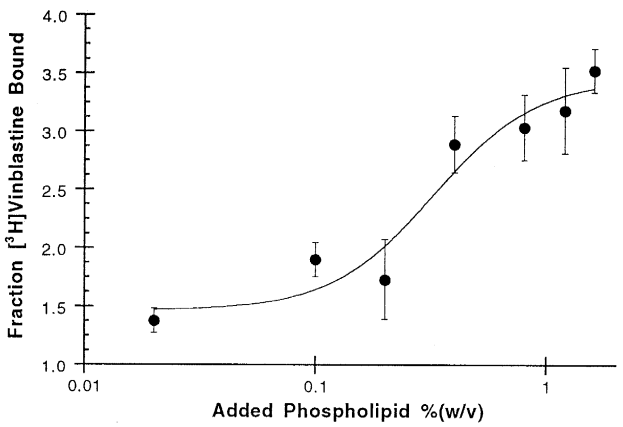

Fig. 2. Reversibility of the detergent-impaired [^{3}H]vinblastine binding to solubilized P-gp. P-gp from CH^{r}B30 membranes was solubilized with 0.4% (w/v) dodecyl-maltoside. Specific binding of 25–30 nM [^{3}H]vinblastine to soluble protein (10 μg) was determined using a rapid filtration assay. Non-specific binding in the presence of 3 μM cold vinblastine was subtracted from total binding. Sonicated bovine spinal cord lipids were added at the concentrations indicated and the results expressed as a fraction of binding in the absence of added lipid. All data points are the mean ± S.E.M. from three independent preparations.

at which lipids are in the micellar form. Under these conditions, lipids will saturate the protein/detergent micelles and maximally protect activity. At higher lipid/detergent ratios, added lipids will begin to form liposomes.

Therefore, besides preventing inactivation of P-gp in the presence of dodecyl-maltoside, the addition of excess lipids may convert some P-gp from a conformation which cannot bind [^{3}H]vinblastine, to a conformation with higher binding capacity.

3.4. Purification of P-glycoprotein from CH^{r}B30 membranes

P-Glycoprotein was considered to be fully solubilized by the detergent dodecyl-maltoside since centrifugation at $100\,000 \times g$ did not pellet the protein. A concentration of 0.4% (w/v) dodecyl-maltoside solubilized greater than 90% of P-gp and approximately 50% of total membrane protein (see Fig. 3 and Table 2) thereby resulting in a two-fold enrichment. A crude mixture of sonicated bovine spinal cord lipids was included during solubilization to preserve drug binding and ATPase activity of P-gp (see above). The use of anion exchange as a first chromatographic step necessitated the use of a low ionic strength buffer for the solubilization step. Efficiency of solubilization was unaffected in the pH range 6.0–7.5. However, profound effects on chromatography were observed. At pH 7.4, all the P-gp bound to the anion-exchange resin, eluting at 180–200 mM NaCl. In contrast, at pH 6.9, P-gp eluted quantitatively in the void volume. The chromatographic resolution of P-gp was similar at either pH (data not shown). An approximately 4-fold purification of P-gp, based on protein yield, was achieved using the anion-exchange step. Fractions containing P-gp were concentrated into smaller volumes (< 1 ml) to enable efficient loading on an hydroxy-apatite column (1 ml bed volume). It was necessary to use 100-kDa cut-off membranes to prevent concentration of dodecyl-maltoside which has a micelle molecular weight of 50-kDa. Low recovery of P-gp was observed from hydroxy-apatite columns at pH values over 7.0, due to irreversible binding to the matrix. Increased recovery of protein was achieved by lowering the pH to 6.6. P-gp eluted from the matrix at phosphate concentrations between 200 and 250 mM and, based on protein yield, this chromatographic step resulted in a 5- to 6-fold purification. Approximately 100 μg of P-gp was obtained from 5 mg of CH^{r}B30 membranes, a 45-fold purification. Fig. 2 illustrates the

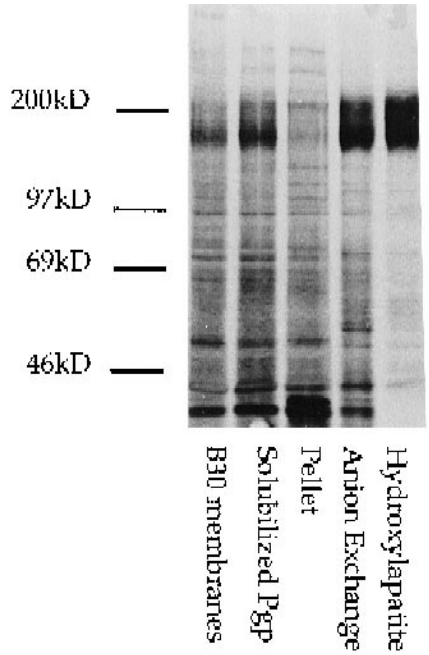

Fig. 3. SDS–PAGE analysis of protein at each stage of P-gp purification. Proteins were separated by electrophoresis on 7.5% SDS–PAGE gels and detected by silver staining. Lanes (from left to right): CH^{r}B30 membranes; membrane proteins solubilized by 0.4% (w/v) dodecyl-maltoside; insoluble material; proteins obtained in void volume of the anion-exchange column; and purified P-gp obtained from the hydroxy-apatite column. Each lane contained 2 μg protein.

Table 2
Constants obtained from solubilization of LUVs composed of asolectin, PC/PA (95:5) and bovine spinal cord (BSC) lipids by octyl-glucoside

Lipid composition	Saturation		Solubilization	
	R_{sat}	D_w	R_{sol}	D_w
Asolectin	1.1	8	1.5	20
PC/PA (95:5)	1.1	18	4.4	22
BSC	1.1	18	3.3	22

Constants were obtained by linear regression of the equation $[OG] = D_w + R[Lipid]$ to plots of [OG] required for saturation or solubilization of LUVs vs. amount of lipid. D_w is the monomeric concentration (mM) and R is the ratio of detergent to lipid at saturation or solubilization.

relative purity of P-gp at each stage of the purification and the final product was estimated to be of 90% purity.

3.5. Activity of soluble P-glycoprotein during purification

The [^{3}H]vinblastine binding capacity of soluble P-gp was used to assess its functional state at each stage of purification (Fig. 4). The decrease in [^{3}H]vinblastine binding to P-gp following solubilization in dodecyl-maltoside, outlined above, was also observed for the detergents octyl-glucoside (B_{max} = 8.9 ± 1.4 pmol/mg) and zwittergent 3–12 (B_{max} = 17.2 ± 7.7 pmol/mg). Therefore, it appears that the impaired drug binding capacity of solubilized P-gp is not confined to any one detergent. Rather it is a consequence of removing the protein from its native lipid environment.

Subsequent anion-exchange chromatography under conditions where P-gp was bound by the matrix (pH 7.4) further inactivated the protein. Despite the 4-fold purification during this step, the binding capacity of soluble P-gp was actually reduced by 4-fold to 5.8 ±

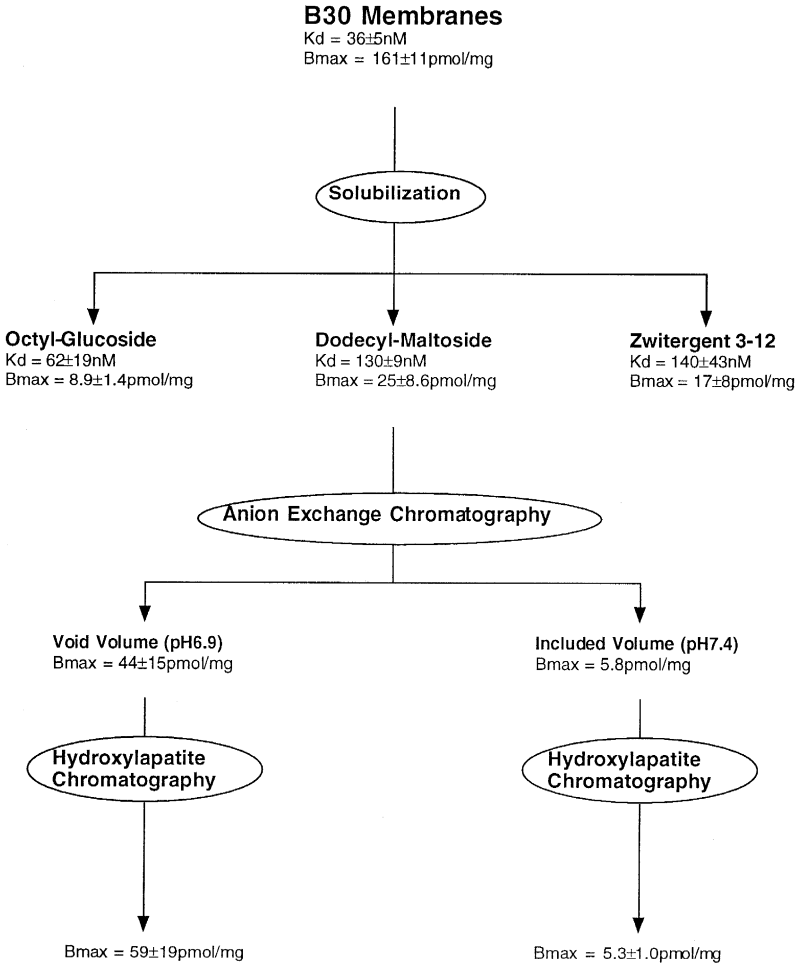

Fig. 4. Scheme outlining the ability of P-gp to bind [^{3}H]vinblastine during purification. Binding capacity (B_{max}) of P-gp was determined from saturation isotherms (1–150 nM [^{3}H]vinblastine) obtained by a rapid filtration assay as described in Section 2: Materials and methods. Initially, CH^{r}B30 membranes were solubilized by 1.25% (w/v) octyl-glucoside, 0.4% (w/v) dodecyl-maltoside or 1.0% (w/v) zwittergent and binding assays performed on soluble protein (10 μg). Protein solubilized by dodecyl-maltoside was subjected to anion-exchange chromatography at either pH 6.9 or 7.4 and binding assays performed on 5 μg pooled P-gp-containing fractions. Saturation isotherms were also obtained for P-gp-containing fractions (1–2 μg protein) following hydroxy-apatite chromatography. Binding capacity was obtained from the mean of 2–3 independent determinations using non-linear regression analysis.

0.4 pmol/mg. However, if anion-exchange chromatography was performed at pH 6.9, the binding capacity of P-gp increased to 44 ± 15 pmol/mg. An increase in binding capacity ($B_{max} = 59 \pm 19$ pmol/mg), although not in proportion to the increase in purity (5-fold), was also observed following hydroxyl-apatite chromatography. These results demonstrate that allowing P-gp to interact with the anion-exchange matrix caused a significant reduction in the ability of the protein to bind substrate. Thus, the purification scheme resulting in the most functional form of P-gp employed anion-exchange chromatography at pH 6.9. There was no advantage gained by solubilizing P-gp in either octyl-glucoside or zwittergent 3–12. This is in agreement with data outlined in the section above describing effects of detergents on P-gp activity in native membranes.

3.6. Liposome solubilization by octylglucoside and reconstitution of P-gp

P-gp was reconstituted into liposomes composed of asolectin lipids using the detergent octylglucoside. Octylglucoside was chosen since the low CMC of dodecyl-maltoside (0.2 mM) prevented easy removal by dialysis techniques.

To investigate the interaction between unilamellar liposomes and octyl-glucoside, solubilization profiles were performed. The solubilization of unilamellar asolectin liposomes (5–25 mM) was examined by following the change in light scattering caused by intact liposomes (Fig. 5a). The initial point of inflection occurred at the maximal concentrations of octylglucoside tolerated by the liposome and was defined as $[OG]_{sat}$. At higher concentrations of octylglucoside solubilization progressed to the point where light scattering was negligible, reflecting mixed micelles of lipid–detergent [43]. This was defined as the solubilizing ($[OG]_{sol}$) concentration of octylglucoside. Values of $[OG]_{sat}$ and $[OG]_{sol}$ were plotted as a function of phospholipid concentration and linear relationships obtained of the form $[OG] = D_w + R[Lipid]$ (Fig. 5b). D_w corresponds to the monomeric concentration of detergent and R is the ratio of detergent to lipid in the liposomes. Solubilization of asolectin liposomes by octylglucoside was described by the relationship $[OG]_{sol} = 19.9 + 1.54[Lipid]$ ($r = 0.961$, $n = 15$) and saturation by $[OG]_{sat} = 7.61 + 1.17[Lipid]$ ($r = 0.949$, $n = 13$). The concentrations of octyl-glucoside required to saturate or completely solubilize a given concentration of asolectin liposomes may be estimated from these relationships.

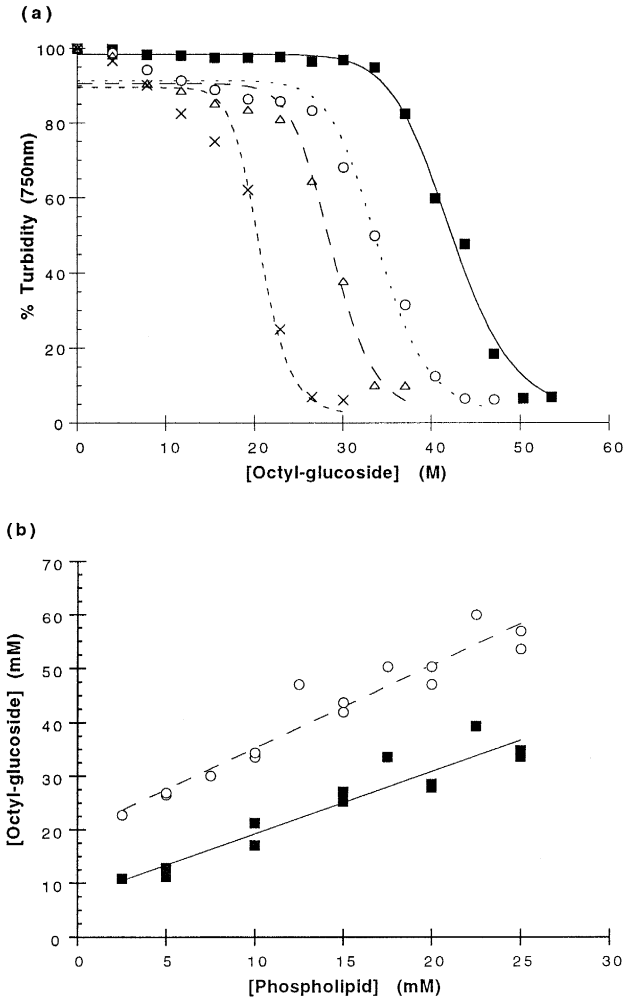

Fig. 5. Solubilization of unilamellar liposomes composed of asolectin by the detergent octyl-glucoside. a: representative curves of liposome (5 (×), 10 (△), 15 (○) and 22.5 mM (■)) solubilization by increasing concentrations of octyl-glucoside as evidenced by light scattering at 750 nm in a 500 μl reaction volume. b: concentrations of octyl-glucoside resulting in saturation ($[OG]_{sat}$, ■) and solubilization ($[OG]_{sol}$, ○) of liposomes were plotted as a function of lipid concentration. The linear relationships obtained were defined by the expressions $[OG]_{sat} = 7.6 + 1.2[Lipid]$ ($r = 0.949$, $n = 13$) and $[OG]_{sol} = 19.9 + 1.5[Lipid]$ ($r = 0.961$, $n = 15$). These data were obtained at 8 different lipid concentrations from several independent experiments.

The relatively low level of R_{sol} for octylglucoside solubilization compared with previous reports using model lipid systems prompted further investigation. Table 2 shows the values of R_{sat}, R_{sol} and the respective D_w obtained for solubilization of asolectin, bovine spinal cord lipids and liposomes composed of egg phosphatidylcholine and phosphatidic acid (95:5) by octyl-glucoside. Interestingly, significantly higher octylglucoside concentrations were required to solubilize liposomes composed of crude spinal cord or defined lipid species. The lower R_{sol} value of asolectin lipids appears peculiar to this complex lipid mixture rather than a general octylglucoside–lipid phenomenon.

In order to reduce the exposure of P-gp to detergents, reconstitution was performed using liposomes saturated with [35], rather than completely solubilized by octylglucoside as in conventional methods. Pure P-gp had been concentrated to approximately 0.4 mg/ml and liposome suspensions made at 15 mg/ml. Therefore, to reconstitute at a lipid/protein ratio of 100, the protein solution was diluted roughly 3-fold corresponding to a dodecyl-maltoside concentration in the final protein/lipid/detergent mixture of 0.007% (w/v). Since the CMC for dodecyl-maltoside is 0.01% (w/v), following dilution the micelle/monomer equilibrium of the protein/lipid/detergent mixture was shifted towards the monomeric form of dodecyl-maltoside. The monomeric dodecyl-maltoside was then removed by dialysis.

P-gp reconstitution using Sephadex chromatography was used initially since there is a significantly shorter contact time between detergent and protein compared with conventional dialysis. Lipid recoveries greater than 80% were typically achieved, although, 40–50% of the protein was lost during chromatography. Consequently, for reconstitution of the post hydroxy-apatite fraction, slow dialysis was the preferred method due to the relatively smaller amounts of protein available.

The efficiency of P-gp reconstitution was assessed by migration profiles of liposomes and protein through sucrose gradients during centrifugation (Fig. 6). There were two major lipid bands: the first floating upwards through the gradient to 0% sucrose; however, this did not contain protein; the second and major band spanned the interface between 5 and 10% sucrose.

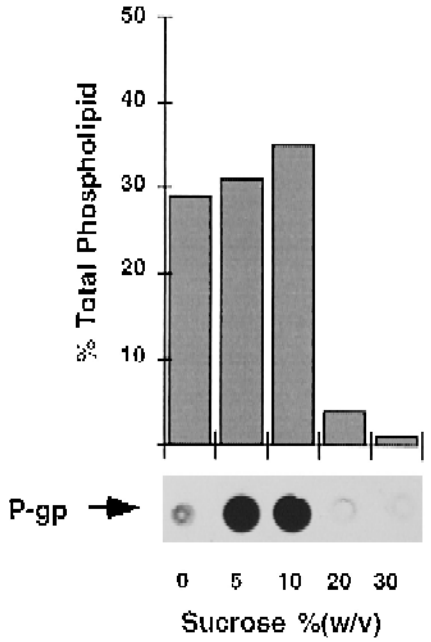

Fig. 6. Efficiency of P-gp reconstitution into preformed unilamellar liposomes saturated with octyl-glucoside. Following dialysis, the lipid/protein mixtures were centrifuged at $150\,000 \times g$ through a discontinuous sucrose gradient as described in Section 2: Materials and methods. Localization of lipids was assessed by migration of tracer amounts of [^{3}H]phosphatidyl-choline through the gradient. The amount of lipid in each sucrose fraction were expressed as a percentage of total phospholipid. The inset shows localization of purified P-gp in the sucrose gradient fractions. Protein was determined by immuno dot-blotting using the monoclonal antibody C219.

This band contains greater than 90% of the P-gp and is evidence of reconstitution. Confirmation of reconstitution of P-gp into these liposomes is two-fold. Firstly, electron microscopic images demonstrate insertion of P-gp into liposomes [44]. Secondly, if pure P-gp was centrifuged through sucrose gradients in the absence of lipid, the protein did not migrate out of the 30% sucrose fraction (data not shown). Using a vesicle radius of 100 nm and an approximate phospholipid surface area of 50 nm^2 [45] the estimated number of P-gp molecules per liposome was 1–1.5. Similar results were obtained under reconstitution conditions employing solubilizing concentrations of octyl-glucoside (data not shown). In the less pure post-solubilization and anion-exchange stages of purification, greater than 90% of the P-gp was also associated with the liposomes.

3.7. Activity of purified P-glycoprotein reconstituted into asolectin liposomes

Protein obtained at each stage of purification was reconstituted into asolectin liposomes at lipid/protein ratios of 70–100 by weight, equivalent to a molar lipid/protein ratio of 14 000–20 000. The ATPase and [^{3}H]vinblastine binding activities of reconstituted P-gp are shown in Table 3.

ATPase activity of reconstituted protein was performed at several concentrations of ATP in order to determine the maximal rate of hydrolysis and the affinity for ATP. The K_d for ATP hydrolysis by P-gp in CH^{r}B30 membranes (1.8 ± 0.4 mM) was not significantly altered during purification (Table 3) reflecting an unchanged affinity for the nucleotide. The V_{max} for ATP hydrolysis by P-gp increased from 897 ± 55 nmol min^{-1} mg^{-1} in CH^{r}B30 membranes to a value of 2137 ± 309 nmol min^{-1} mg^{-1} ($n = 4$) for the purified, reconstituted protein. This represents the highest reported rate of ATP hydrolysis by purified P-gp to date. However, the proportional increase in specific ATPase activity for each fraction did not quantitatively match the degree of purification. Solubilization of P-gp from CH^{r}B30 membranes with dodecyl-maltoside, despite a two-fold enrichment in P-gp, did not increase the V_{max}. The initial solubilization step contains the highest detergent concentration (0.4% w/v) during the procedure and therefore some loss in activity is not surprising. In contrast, hydroxyl-apatite chromatography (5-fold purification) during which detergent concentration was reduced to 0.02% (w/v) resulted in a 2-fold increase in V_{max}. The lack of quantitative agreement between purification and specific activity was due to a combination of: (a) the presence of contaminating ATPases in the less pure earlier fractions; and (b) partial inactivation of P-gp during the purification.

As reported earlier, the ATPase activity of P-gp in its native membrane is sensitive to verapamil and orthovanadate. Full dose–response relationships for ATPase activity (2 mM ATP) in the presence of either verapamil or orthovanadate were carried out at each stage of purification. The concentrations at which half-maximal effect occurred were estimated by non-linear regression and are shown in Table 3. Verapamil, retained the ability to stimulate the ATPase activity of P-gp (range 1.3- to 1.8-fold) at each stage of purification. The degree of stimulation by verapamil did not significantly differ between any stage of purification and the native membranes, reflecting correct coupling of drug–protein interaction and ATPase activity. However, the EC_{50} value of 4.86 ± 1.16 μM obtained for purified reconstituted protein was higher than in CH^{r}B30 membranes. Similarly, higher concentrations of verapamil were required to cause 50% stimulation of the ATPase activity of P-gp reconstituted from the solubilized (1.4 ± 0.5 μM),

Table 3
ATPase activity and vinblastine binding by P-gp during the course of purification

	CHrB30 membranes	Solubilized protein	Anion exchange	Hydroxy-apatite
Protein				
Amount (μg)	5000	2380	580	107
Purification	1.0	2.1	8.2	45
ATPase activity				
K_m (ATP)	1.82 ± 0.37	2.19 ± 0.47	2.19 ± 0.26	2.79 ± 0.51
V_{max} (ATP (nmol min^{-1} mg^{-1})	897 ± 55	926 ± 30	1129 ± 179	2137 ± 309
EC_{50} (verapamil) (μM)	0.61 ± 0.25	1.25 ± 0.62	4.29 ± 1.45	2.91 ± 0.96
EC_{50} (vanadate) (μM)	1.52 ± 0.55	1.95 ± 0.85	2.92 ± 1.47	3.13 ± 0.77
[^{3}H]Vinblastine binding				
K_d (nM)	36 ± 5	62 ± 12	57 ± 13	64 ± 9
B_{max} (pmol/mg)	161 ± 11	404 ± 81	807 ± 117	2320 ± 192

ATPase activity and [^{3}H]vinblastine binding were determined for CH^{r}B30 membranes and P-gp at various stages of purification. P-gp was reconstituted into asolectin LUVs at lipid/protein ratios of 100 (w/w). All values are the mean ± S.E.M. obtained from 3–5 independent preparations.

and post anion-exchange fractions (5.6 ± 0.7 μM). The ATPase activity of P-gp was inhibited at each stage of purification by sodium orthovanadate with complete inactivation at concentrations of 100–150 μM. The EC_{50} values for inhibition of ATP hydrolysis by vanadate were not significantly different between CH^rB30 membranes (1.5 ± 0.6 μM, $n = 5$) and purified reconstituted P-gp (3.1 ± 0.8 μM, $n = 5$).

To date, there has been no quantitative assessment of substrate binding to purified P-gp. We examined the protein–ligand interaction using equilibrium binding techniques to measure the affinity and capacity of the [^{3}H]vinblastine–P-gp interaction. Saturation isotherms were carried out for [^{3}H]vinblastine binding to reconstituted protein each stage of purification. Following solubilization in dodecyl-maltoside and reconstitution into asolectin liposomes, P-gp bound 404 $\pm$ 81 pmol/mg ($n = 4$) with a $K_d = 62 \pm 12$ nM. The affinity was not significantly different from that in native membranes ($K_d = 36 \pm 5$ nM, $B_{max} = 161 \pm 11$ pmol/mg) and the 2.5-fold increase in capacity quantitatively agreed with the degree of purification. In contrast, soluble P-gp bound 16-fold less [^{3}H]vinblastine with a $K_d = 130 \pm 9$ nM (Fig. 4). This suggests that P-gp has a strict requirement for the presence of phospholipids to bind substrate with high affinity. The specific binding capacity of P-gp increased as the protein was further purified. High affinity binding of [^{3}H]vinblastine ($K_d = 64 \pm 9$ nM) was retained by purified reconstituted P-gp. The binding capacity of pure P-gp (2320 pmol/mg) was 15-fold higher than observed in CH^rB30 membranes. Based on the B_{max} observed in CH^rB30 membranes, the theoretical maximum binding capacity of purified P-gp is estimated at 7245 pmol/mg protein. Assuming that 1 mol of [^{3}H]vinblastine binds to 1 mol of P-gp, and accepting that P-gp has been purified to homogeneity, 35–40% of P-gp retains this biological activity.

Reconstitution with a large excess of lipids such as employed in the present manuscript makes it difficult to assay transport of typical P-gp substrates such as vinblastine due to their inherent hydrophobicity and therefore avid partitioning into the lipid environment. Successful reconstitution at significantly lower lipid/protein ratios will allow accurate measurement of drug transport by P-gp.

4. Discussion

To date, protocols for purification of P-gp have resulted in protein with a poor specific activity compared with starting material and in some cases low yield. In the present study we have quantitatively assessed the ATPase activity and drug binding capacity of P-gp throughout its purification in order to determine conditions under which maximal function was retained. P-gp was shown to be most susceptible to inactivation under conditions that perturb the lipid–protein interface. The purified, reconstituted protein displayed the highest ATPase activity reported thus far and high affinity binding of vinblastine.

The basal P-gp ATPase activity of the starting material for purification, CH^rB30 membranes, displayed similar pharmacological characteristics to the well-characterised CH^rC5 cell line [22]. Furthermore, the high affinity binding of vinblastine to hamster P-gp obtained using equilibrium techniques in the present study was similar to that reported for human P-gp [18]. Drug binding to P-gp was assessed using equilibrium techniques which, unlike labeling with photo-affinity compounds, permit quantitation of the affinity and capacity of binding. Furthermore, the use of a photo affinity ligand, such as azidopine, to examine drug binding to P-gp is limited by: (1) low affinity compared with vinca alkaloids; (2) the large number of orientations the molecule may assume; (3) the reactive azide may not reside in the pharmacophoric region [46]; and (4) only binding displacement data is possible.

The key first step in purification of integral membrane proteins is extraction from the membrane environment whilst retaining function. Extraction from the membrane involves the use of ampiphilic compounds, usually detergents. CHAPS [22], octylglucoside [23], zwittergent 3–12 [25] and dodecylmaltoside (this study) have previously been used to solubilize P-gp. One would expect that ampiphiles such as these detergents would perturb the lipid–protein interface [47]. Indeed, it has been demonstrated that membrane protein function is inactivated under conditions where even a small fraction of annular lipids are displaced [48,49]. This was evident in the present study since all the non-ionic detergents examined were able to inactivate P-gp at concentrations

well below their respective CMC values. The inactivation of P-gp function by detergents was both preventable and partly reversible if an excess of phospholipids was present. This 'protective' effect may be explained by either: (1) partitioning of detergent between added lipids and P-gp containing membranes; or (2) direct protection of protein activity, perhaps by increasing lipid content at the annulus. Irrespective of the precise mechanism, not all the detergents behaved similarly. For example, greater functional protection was observed for dodecyl-maltoside compared with the related detergent octylglucoside. It has previously been reported that detergents with longer alkyl chain length, such as on dodecyl-maltoside, may protect the hydrophobic portion of integral membrane proteins to a greater extent than shorter 8-carbon alkyl chain detergents like octylglucoside [48,50]. However, despite having a 12-carbon alkyl chain, zwittergent 3–12 remained a potent inactivating agent even in the presence of excess phospholipids in the present study. This apparent inconsistency may be explained by the inability of detergents, such as zwittergent 3–12, dodecyl-dimethyldiamine oxide and the Tween series, to adequately segregate hydrophobic α-helices from the aqueous environment due to their bulky head groups [50]. In the present study, the conditions which preserve both substrate binding and ATPase activity of P-gp to the greatest degree involve the use of dodecyl-maltoside and an excess of crude lipids.

Following extraction from its native environment, solubilized P-gp displayed a markedly reduced affinity and capacity to bind vinblastine. This impaired substrate binding was independent of the detergent used. There is a large body of evidence indicating that agents which perturb plasma membrane biophysical properties are able to modify the activity of P-gp [30,31,51]. The results obtained from the present study suggest that the mechanism by which membrane-active agents affect P-gp involves disruption of a protein–phospholipid interface which transforms P-gp into a conformation that binds vinblastine with low affinity. Binding of solubilized P-gp to an anion-exchange matrix also led to decreased affinity and substrate binding capacity of P-gp. It has previously been shown that the ATPase activity of P-gp was damaged following binding to other chromatographic matrices [27]. Phospholipids may also bind to anion-exchange media, and therefore the possibility of disrupting lipid–protein interactions exists [32]; a property which has been used to generate delipidated protein [48]. Such delipidation of P-gp would impair its function in a manner analogous to the use of high detergent concentrations.

To date, P-gp has been reconstituted following removal of detergent from protein–detergent and detergent–lipid micelles by dialysis, dilution or gel filtration chromatography [22,24,25]. Reconstitution of proteins from mixed micelles has several drawbacks including: (1) the use of high detergent concentrations to generate phospholipid micelles; (2) no control of the size of proteoliposomes formed following detergent removal; and (3) symmetric orientation of proteins into liposomes. Several, integral membrane proteins (e.g., bacteriorhodopsin) have been reconstituted into preformed liposomes mildly disrupted by detergents or fusogenic agents [35,52]. We have demonstrated for the first time that P-gp is efficiently reconstituted into preformed unilamellar liposomes containing only saturating concentrations of octylglucoside. Higher ATPase activity and drug-binding capacity were obtained than following reconstitution into mixed micelles of solubilized phospholipid, presumably due to the lower detergent/protein ratios [44]. Lipids used during solubilization and for reconstitution comprised either crude bovine spinal cord extract or asolectin (30–35% PC) in order to retain any specific lipid requirements for P-gp. It has previously been shown that addition of various lipids to solubilized P-gp has a variety of specific effects on ATPase activity [26] and lipid composition modified the basal and drug-stimulated characteristics of reconstituted P-gp ATPase activity [24].

Reconstitution of soluble P-gp into phospholipid bilayers at various stages of purity resulted in the restoration of high affinity substrate binding by the protein. In contrast to most of the previous purification procedures for P-gp, the final product displayed specific activity significantly greater than that of the starting material. Approximately 40% of the total vinblastine binding capacity of P-gp was recovered following purification. From the present study, it also appears that the ATPase activity of P-gp (approximately 20% retained) is more sensitive to inactivation than is drug binding. Binding of substrate has a specific requirement for a lipid–protein interface

which concurs with reports that substrate interacts directly with P-gp through the lipid environment [14]. Furthermore, the ATPase activity of P-gp was sensitive to perturbations of the protein–lipid interface. ATP hydrolysis occurs in the soluble nucleotide binding domains which one would expect to be relatively insensitive to membrane perturbation. Transmembrane helices have in the past been thought to play a passive role, merely maintaining hydrophobic interaction with membrane lipids. However, it is clear that the amphipathic nature of α-helices in integral membrane proteins modulate anchoring, helix–helix interactions, ligand binding and an aqueous pore in the case of ion channels [53]. The transmembrane helices of P-gp may also be involved in generating transport by correct coupling of substrate binding and ATP hydrolysis.

In summary, we have developed an improved purification procedure for P-gp generating high protein yield, a specific activity significantly increased over levels in the starting material, whilst retaining similar pharmacological characteristics. The generation of this purification scheme demonstrated that disruption of the lipid–protein interface led to both impaired ATPase activity in conjunction with reduced affinity and capacity for drug binding.

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