P-glycoprotein (P-gp) is a member of the ATP binding cassette superfamily of active transporters and can confer multidrug resistance on cells and tumors by pumping chemotherapeutic drugs from the cytoplasm. P-gp was purified from CHB30 cells and retained the ability to bind substrates and hydrolyze ATP. Labeling of P-gp with lectin-gold particles suggested it is monomeric. An initial structure of purified P-gp was determined to 2.5 nm resolution by electron microscopy and single particle image analysis of both detergent-solubilized and lipid-reconstituted protein. The structure was further refined by three dimensional reconstructions from single particle images and by Fourier projection maps of small two-dimensional crystalline arrays (unit cell parameters: a, 14.2 nm; b, 18.5 nm; and γ, 91.6°). When viewed from above the membrane plane the protein is toroidal, with 6-fold symmetry and a diameter of about 10 nm. There is a large central pore of about 5 nm in diameter, which is closed at the inner (cytoplasmic) face of the membrane, forming an aqueous chamber within the membrane. An opening from this chamber to the lipid phase is present. The projection of the protein perpendicular to the membrane is roughly rectangular with a maximum depth of 8 nm and two 3-nm lobes exposed at the cytoplasmic face of the membrane, likely to correspond to the nucleotide binding domains. This study provides the first experimental insight into the three-dimensional architecture of any ATP binding cassette transporter.

The multidrug resistance P-glycoprotein (P-gp) is an active transporter located in the plasma membranes of many cells and tissues (1). P-gp has a relatively broad specificity for hydrophobic compounds. Although its natural substrate(s) is not known, when overexpressed P-gp can confer multidrug resistance on cells and tumors by pumping chemotherapeutic drugs from the cytoplasm. P-gp is a member of the ATP binding cassette (ABC) superfamily of transporters and channels (2). More than 100 ABC transporters have been identified in bacteria, yeasts, plants, and mammals, including the cystic fibrosis and adrenoleukodystrophy gene products, the pfmdr gene product associated with drug resistance of the malarial parasite, and the TAP (transporter associated with antigen presentation) peptide transporter, which is essential for antigen presentation. ABC transporters share a common domain organization and considerable amino acid sequence identity, implying a common architecture and evolutionary origin.

P-gp is a 170-kDa polypeptide that is glycosylated at a single site at the extracellular face of the membrane. From its primary sequence, P-gp is predicted to consist of four domains. Two hydrophobic transmembrane domains each consist of six membrane-spanning segments (putative α-helices) separated by hydrophilic loops (3, 4). These transmembrane domains are believed to form the pathway through which solute crosses the membrane and to play a major role in determining substrate specificity. The other two domains, the nucleotide binding domains (NBDs), are located at the cytoplasmic face of the membrane and couple ATP hydrolysis to the transport process (5, 6).

Although a considerable body of biochemical and genetic data has accrued for many ABC transporters, little is known about the three-dimensional organization of these proteins. Indeed, the absence of any structural data is the principal factor that limits our understanding of the molecular mechanisms of active transport. In this study we present an initial structure for P-gp, determined to 2.5 nm resolution. These data provide the first structural insights for any ABC transporter.

EXPERIMENTAL PROCEDURES

Cell Culture and Membrane Purification—Chinese hamster ovary CHB30 cells were grown and maintained in a medium containing 30 μg/ml colchicine as described previously (7). Plasma membranes from these cells were prepared according to the method of Lever (8) with minor modifications (7). Membranes were stored at a protein concentration of 5–7 μg/μl at −80 °C for up to 6 months without loss of P-gp activity.

Purification and Reconstitution of P-gp—Membranes from CHB30 cells were solubilized in the mild, nonionic detergent dodecyl maltoside and purified by anion exchange and hydroxyapatite chromatography as described in detail elsewhere.2 Fractions containing P-gp from the final hydroxyapatite chromatographic step were concentrated to approximately 0.2 μg/μl and stored at −20 °C.

Large unilamellar liposomes of asolectin lipids were generated at a lipid concentration of 15 mg/ml in dialysis buffer (10 mm Tris-HCl, pH 7.4, 150 mM NaCl) as described previously (10). The liposomes were solubilized with 40 mM octyl-β-D-glucoside and added to purified P-gp at a lipid-protein ratio of 10:1 (w/w) for 30 min at 4 °C. Detergent was subsequently removed, and P-gp was reconstituted into asolectin lipids by Sephadex G-50 gel filtration chromatography (1 × 60 cm) using 150 mm NaCl, 10 mm Tris-HCl, pH 7.4, elution buffer. The void volume fractions were collected, and proteoliposomes were concentrated by centrifugation at 150,000 × g for 4 h at 4 °C. The efficiency of reconstitution was assessed by the co-migration of P-gp with tracer [3H]phosphatidylcholine during sucrose density centrifugation (11).2

2 R. Callaghan, G. Berridge, D. R. Ferry, and C. F. Higgins, submitted for publication.
**ATPase Assay**—ATP hydrolysis by purified, reconstituted P-gp was determined essentially as described (12, 13). Each assay was carried out in a total volume of 150 μl of assay buffer (150 mM NH4Cl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% NaN3 containing 0.5 μg of protein and 2 mM MgATP for 25 min at 37 °C (during which hydrolysis rates were linear). The reaction was stopped by the addition of sample buffer (1 mM ammonium molybdate in 1 M HCl) and 150 μl of stabilizing (2% (w/v) each of Na metarsenite, Na citrate, and acetic acid) solutions and a 10-min incubation at 37 °C, the absorbance was measured at 750 nm. Basal ATPase activity was measured as nmol of Pi liberated per min per mg of protein. Assays carried out in the presence of verapamil or ouabain were expressed as the percent change from the donor-acceptor ATPase activity (mean ± S.E.) from at least five independent P-gp preparations. Background phosphate levels were measured by incubating samples in the absence of drug at 4 °C and were subtracted from all measurements.

[3H]Vinblastine Binding—The equilibrium binding of [3H]vinblastine to P-gp was determined using a rapid filtration assay based on published methods (14). Saturation isotherms were obtained by incubating 5–120 nM [3H]vinblastine with 20 pmol of membrane or 1 μg of reconstituted P-gp in a total volume of 200 μl at 25 °C for 2 h. Samples were filtered through a combination of Whatman GF/F fiber and 0.1-μm nitrocellulose filters, and radioactivity retained was determined by liquid scintillation counting. Nonspecific binding of [3H]vinblastine was determined by incubating to membranes and liposomes in the presence of 5 μM unlabeled vinblastine. Data were expressed as percent of [3H]vinblastine bound per mg of protein. The equation

\[
B = B_0 \left(1 - e^{-t/k_d}ight)
\]

was fitted to saturation isotherms by nonlinear regression analysis using Kaleidograph (Abelbeck Software). B, is the amount of [3H]vinblastine bound, B0, the density of binding sites, t, the apparent dissociation constant, and F, the concentration of free [3H]vinblastine.

**Photoaffinity Labeling of P-gp**—Photoaffinity labeling of P-gp (1–2 μg of protein) with the 1,4-dihydropyridine [3H]azidopropidine (45 nm) was determined by fluorography of SDS-polyacrylamide gels as described previously (15). Vinblastine (1–500 μM, as indicated) was used to inhibit [3H]azidopropidine labeling.

**Lectin-Gold Labeling**—Lentil lectin agglutinin from Lens culinaris (Sigma), specific for α-mannose residues, binds to glycosylated P-gp in the presence of divalent cations (16). Detergent-solubilized P-gp and gold-labeled lectin (5 nm) were mixed and incubated at a molar ratio of 1:1 at 4 °C overnight with constant agitation in buffer (10 mM Tris-Cl, pH 7.5, 25 mM MgSO4). Specimen preparation for electron microscopy was as described below.

**3D Reconstruction**—Copper grids (400 mesh/inch; Agar Scientific Ltd., Stansted, United Kingdom) were coated with a collodion/carbon film. Specimens were adsorbed to freshly glow-discharged grids (to render them hydrophilic) for 30 s, washed with filtered water for 30 s, and negatively stained with uranyl acetate (4% w/v in water; Electron Microscopy Laboratories Ltd., Reading, UK) or sodium phosphotungstate (17) for 1 min. Electron micrographs were recorded at calibrated magnification using Philips EM 300, Hitachi H-600, or Phillips CM 10 electron microscopes at 100 kiloelectron volts. Low dose conditions were achieved by focusing on an area of the carbon film adjacent to the area of interest to minimize irradiation damage to the specimen. The micrographs were digitized using a rotating drum microdensitometer (Joyce-Loebel) at 25-μm increments corresponding to pixel sizes in the range of 5–7 Å at the specimen level (depending on the primary magnification).

**Image Analysis Using SPIDER**—Single particle averaging was undertaken with the SPIDER software package on micro-VAX and Indigo-Silicon Graphics work stations (18, 19). The images were initially band-pass-filtered (20). Particles were interactively selected using the program WEB padded to 64 x 64 pixels, and the densities were normalized (21). Padded images were masked with a circular mask, and a Gaussian falloff filter was applied with a half-width of 1.0 pixel. Particles were rotationally aligned by an autocorrelation function-based method (20) and translationally aligned by cross-correlation using a reference particle (22). Initially, the first 20 particles were aligned with respect to a reference particle, and the best aligned particles were used to calculate a new reference, which was then used to align the rest of the particles. An average image was calculated by summing the aligned particles. The alignment was subjected to a refinement analogous to the random conical reconstruction method based on a χ2 minimization constraint (23). This method reduces the effects of missing angular range on the reconstruction. Phase residual assessment was used for the cross-reconstruction assessment, resulting in a resolution pass-filtered according to the resolution assessment. For viewing the results, a surface representation was used on a work station using WEB in the SPIDER package.

**Analysis of Small Two-dimensional Crystalline Arrays**—Crystallographic image processing of small, two-dimensional crystalline arrays of P-gp was carried out using the program CRISP and Trimerge (Calidris Bioimage Technologies). Images of micrographs (∼400,000 magnification) were digitized at 64 μm/pixel, giving 7.4 Å/pixel at the specimen level. Crystalline arrays were displayed, and the best ordered areas were judged by the sharpness and intensity of the first order reflections in the calculated Fourier transforms. The best areas were then used for lattice refinement, extraction of amplitudes and phases, and plane group assignment. Structure factors from 10 separate crystalline areas were gathered after phase refinement, and averaged phases and amplitudes were extracted for each spot. Since the plane group contained 1 asymmetric unit, the merging process required the operator to identify which of the two possible orientations of each crystalline area to apply. This decision was made by comparing the interimage phase residuals for the two possible orientations (rotated by 0 or 180° with respect to the first crystalline area used in the merging procedure); in each case the orientation with the lowest interimage phase residual was used. The final averaged structure factors were used to calculate an averaged projection map of the unit cell. The averaged interimage phase residual was 31° (a residual of 90° would be expected for completely random data).

**RESULTS**

**Purification and Reconstitution of Active P-gp**—Chinese hamster ovary CHB30 cells have been selected for high level drug resistance and overexpress P-gp to about 3% of total membrane protein. P-gp was solubilized from CHB30 membranes using the nonionic detergent deoxycholate and purified to >95% by ion exchange and hydroxyapatite chromatography (see "Experimental Procedures"). Fig. 1A shows a silver-stained SDS-polyacrylamide gel of the starting material (CHB30 membranes) and purified P-gp. Purified P-gp was reconstituted into liposomes of asolectin by gel filtration chromatography. The efficiency of reconstitution was assessed by...
co-migration of P-gp with tracer lipids during sucrose density gradient centrifugation. P-gp did not reconstitute efficiently at lipid:protein ratios of <10:1 (w/w). However, efficient reconstitution was obtained at higher lipid:protein ratios. The number of protein molecules reconstituted per unit area of liposome membrane observed by electron microscopy was as expected, given the relative amount of lipid and protein used (see below).

P-gp activity was assayed by measuring drug-stimulated ATPase activity and substrate binding (Fig. 1, B and C). The basal ATPase activity of purified, reconstituted P-gp (800 ± 48 nmol/min/mg; n = 4) was significantly greater than that measured for CH'B30 membranes (377 ± 129 nmol/min/mg; n = 9) and compared favorably with the activity reported for P-gp purified from CH'B30 membranes by immunofinity chromatography (29). ATPase activity was stimulated by verapamil (EC\textsubscript{50}, 1.9 ± 0.3 μM) and inhibited by orthovanadate (EC\textsubscript{50}, 2.4 ± 0.7 μM), with half-maximal concentrations indistinguishable from those determined for P-gp in native membranes (Fig. 1B),\textsuperscript{2} Thus, coupling between substrate binding and ATPase activity was unaltered following purification and reconstitution. Purified, reconstituted P-gp retained high affinity \textsuperscript{[3H]}vinblastine binding (K\textsubscript{d}, 46 ± 10 nM; n = 4), similar to that measured for P-gp in native membranes (37 ± 5 nM; n = 3) (Table I). The specific binding capacity of purified P-gp for \textsuperscript{[3H]}vinblastine (793 ± 108 pmol/mg) increased during purification (Table I). Azidopine, like other 1,4-dihydropyridines, is believed to bind at an allosteric site on P-gp (14). Concentrations of 1–5 μM vinblastine were required to displace 50% \textsuperscript{[3H]}azidopine photolabeling of purified P-gp (Fig. 1C), indistinguishable from the concentrations required to displace \textsuperscript{[3H]}azidopine binding from native membranes (15). Thus, the binding sites for azidopine and vinblastine remain coupled in a negative allosteric fashion in the purified P-gp. In conclusion, the activities of purified and reconstituted P-gp were not significantly different from those of P-gp in native membranes.

Electron Microscopy and Image Analysis of Single Particles of Reconstituted P-gp—Electron microscopy and single particle averaging (30) allow multiple images of a macromolecule in a characteristic orientation to be translationally and rotationally aligned using an iterative procedure of cross-correlation and autocorrelation and then averaged to produce a refined image (25). Single particle analysis was initially carried out on reconstituted P-gp because, compared with solubilized protein, the lipid bilayer confines all the particles in the absence of reconstituted P-gp. The area occupied by the particles was significantly different from those of P-gp in native membranes.

Proteoliposomes were negatively stained with uranyl acetate and examined by electron microscopy. The lipid vesicles were relatively uniform in size, between 50 and 200 nm in diameter (Fig. 2A). Small, uniform, 10-nm-diameter particles with a heavily stained central region were observed in these vesicles (Fig. 2A). The particles were also observed after staining with sodium phosphotungstate, pH 7.0 (data not shown). The contrast for reconstituted protein was low compared with detergent-solubilized protein (see below), as expected for a membrane-embedded protein protruding only a small distance from the bilayer. A montage of several typical particles at a higher magnification and with contrast maximized is shown in Fig. 2A. These particles were not observed in lipid vesicles in the absence of reconstituted P-gp. The area occupied by the particles at a 10:1 lipid:protein ratio, as a proportion of total membrane surface area, was 1:23, in good agreement with the expected value calculated from the partial specific volumes of lipid and protein and the greater “depth” of the protein (8 nm;
The molecules represented the same view. This is consistent with previous reports that, following reconstitution, >80% of P-gp is in the same orientation (16). Images of 25 individual particles with a high signal:noise ratio were aligned with respect to reference particles (18) and then averaged to produce an initial projection map of the protein (Fig. 3C). Further averaging with 135 particles generated the projection map shown in Fig. 3F. Since the molecules are fixed in the dimension parallel to the membrane plane (33), it is possible to use a relatively small number of molecules for the final average (25). The general structures of these two projections were very similar, although some detail was lost by averaging 135 particles due to increased variability in staining and imperfect rotational alignment of those particles with poorer signal:noise ratios. In this projection the protein is toroidal, with a ring of protein exhibiting hexagonal symmetry surrounding a 5-nm central pore. The intense staining of the pore with both uranyl acetate and sodium phosphotungstate implies it is aqueous. P-gp has a single glycosylation site at the extracellular face of the membrane, and lectin-gold labeling showed that this view corresponds to the face of the protein exposed extracellularly (see below).

**Electron Microscopy and Image Analysis of Single Particles of Detergent-solubilized P-gp—**To obtain projections of other orientations of P-gp, detergent-solubilized protein was studied. Fig. 2B shows that the detergent-solubilized protein consists of an essentially homogeneous collection of 10–12-nm-diameter particles. The sizes of the solubilized and reconstituted particles were similar, showing that their oligomeric state is the same. As expected, and in contrast to the reconstituted protein, solubilized P-gp showed more than one orientation with respect to the grid support film. However, the particles were not randomly orientated because of differential association of different faces of the protein with the electrostatically charged grid surface (21). Classification of 621 particles by correspondence analysis (21) identified three distinct projections, which represent distinct orientations of the protein with respect to the electron beam. A representative eigenvector plot is shown (for the unaligned data used in the three-dimensional reconstruction, see below). The three projection maps corresponding to these three orientations are shown in Fig. 3, A, D, and E. Each final projection map was generated from at least three rounds of iterative refinement. Similar projection maps were obtained from multiple independent preparations of P-gp.

One projection (Fig. 3D) closely resembled that of the reconstituted protein (Fig. 3C) and therefore represented the extracellular face of the protein. The detergent-solubilized protein had slightly larger dimensions (12 nm) in this projection than the reconstituted protein, probably reflecting an annulus of lipid or detergent molecules surrounding the protein (16, 34). The second projection (Fig. 3E) was also circular and 10–12 nm in diameter. However, this projection was distinct from the extracellular face-on views shown in Fig. 3, C, D, and F; there was no central pore, and two 3-nm lobes could be identified. These lobes are an appropriate size for the 200-amino acid nucleotide binding domains (5, 6). This projection was shown to correspond to a face-on view from the cytoplasmic face of the membrane by three-dimensional reconstruction (see below).

The third projection (Fig. 3A) was asymmetric in shape with a strongly stain-excluding, three-lobed structure across the center of the protein. This is indicative of a hydrophobic region normally embedded in the membrane and suggested that this projection was likely to be a side-on view of P-gp. Two 3-nm domains (Fig. 3A, arrows) extend from one side of this central structure. Studies on crystalline arrays of P-gp and three-dimensional reconstructions confirmed that this view repre-
sent a side-on view of the molecule and that the two 3-nm domains are on the intracellular face of the molecule (see below). In this side-on projection, P-gp is compact, about 8 nm in depth, which is about twice the depth of a lipid membrane bilayer (4 nm). The three-lobed appearance of the core of the protein in this side-on projection (Fig. 3A) is consistent with a side-on view of a protein with the hexagonal symmetry seen in the face-on projections (Figs. 3, C and D, and 4F).

Three-dimensional Reconstruction of Detergent-solubilized P-gp—Using single particle averaging it is possible to calculate a three-dimensional structure based on a random conical technique (26) and reference-free alignment algorithm (27). A specimen field containing particles in the preferred orientation was imaged with the specimen tilted to 45° and with the specimen in the untilted position. Particle projections appearing in the tilted image form a random conical projection set, since the particles assume random orientations with respect to an axis perpendicular to the specimen plane (27, 35–37). One of the major advantages of this method is that only one exposure of the specimen area is necessary, thus minimizing radiation damage to the molecule. A second exposure of the same specimen area is taken without tilt, but this is not used for the reconstruction but for measurement of the in-plane rotations and for the azimuthal placement of the corresponding tilted specimen projection on the cone. An iterative three-dimensional reconstruction method was then used to remove artifacts caused by missing angular information (27).

The micrographs shown in Fig. 2B represent a typical field of the tilted molecules used for three-dimensional reconstruction, except that the protein density of the sample used for this analysis was lower than that shown. After alignment, the untilted images were subjected to correspondence analysis and classification (21). The images fell into three major classes (designated I–III) by hierarchical ascendant classification, representing different preferred orientations of P-gp molecules on the grid. A dendrogram was calculated (Fig. 4A) showing the classes generated at a cutoff level of 0.1. The decision for cutting the dendrogram at this threshold was based on the demonstration of distinct orientations for each of the three major classes and a good within-group resolution (1/25 Å⁻¹).

The eigenvector plot (Fig. 4B) shows each image as a symbol representative of the cluster to which it belongs, using an alternative method of classification (the Diday method). The first two factors were identified as the most important for representing the image set; thus the main division of the data was shown in a display of factor 1 versus factor 2. The symbols (1–9 and A–Z) follow the ranking of the clusters, with cluster 1 being the most abundant. By averaging over images in clusters 1–3, the same motifs were again obtained, and these three clusters corresponded closely to classes I–III described above. The demonstration that two alternative classification methods (Diday versus hierarchical ascendant classification) generated similar averages implied that stable orientations were assumed by the particles and that the final partition in our classification scheme was largely correct.

As the P-gp molecules prepared on a single carbon layer could be classified into three distinct classes of views, three-dimensional reconstructions were performed, separately, on each of these classes. Fig. 5 shows a computer-generated montage of the three-dimensional structure of P-gp using surface rendering to give an impression of the overall shape of the molecule. This surface was calculated from the major class of
Fig. 4. Definition of particle classes for three-dimensional analysis. A, the linkage tree obtained from hierarchical ascendant classification using complete linkage as a merging criterion. The cutting level used was 0.1, which generated a suitable within group resolution of the major...
particle (class I). This three-dimensional model represents the whole molecule, because information from the opposite side of the protein is derived from the thinly distributed stain, visualized by tilting the specimen support film. Merging the random conical data sets obtained from each of the differently oriented classes of particle was not necessary to obtain an initial structure, because classes II and III contained significantly fewer particles than class I, giving a poorer resolution. Each particle class, however, yielded essentially the same three-dimensional structure. Similar results have been described by Carazo et al. (37) for the 50 S ribosomal subunit. The estimated resolution of this structure was 4.7 nm as determined by phase residual analysis of the three-dimensional volumes.

The initial image of the displayed molecule (Fig. 5A) corresponds to a view from the extracellular face of the membrane. The displayed molecule has been rotated around a horizontal axis, with the direction of rotation moving the top of the molecule in the panel away from the observer. The rotation sequence in the montage finishes after a 192° rotation. Small satellite features disconnected from the main body of the molecule are probably due to residual noise in the three-dimensional map and have been ignored in the discussion of the structure. The threshold level used for this surface-rendering display was chosen so that this peripheral noise was virtually eliminated. At this threshold, the overall dimensions of the three-dimensional representation correlated well with the dimensions of the molecule derived from contour maps of projections from untilted particles (Fig. 3).

FIG. 5. Three-dimensional reconstruction. Computer graphical representation of the three-dimensional reconstruction showing a shaded surface representation of the structure. Surface reliefs of the three-dimensional maps were generated using a density threshold of 0.14. A, view from the extracellular face of the membrane. Other views (B–D) were produced by rotating the P-gp molecule around the horizontal axis by angles of 108° (B), 132° (C), and 192° (D). Thus, D represents a view from the cytoplasmic or solvent-accessible side. Straight arrows, putative ATP binding domains; curved arrow, possible contact between the lipid phase and the aqueous core of P-gp. Bar, 3.7 nm.

FIG. 6. Structure of P-gp. Projections of the three-dimensional reconstruction of P-gp from Fig. 5 are shown enlarged and annotated. P, aqueous pore open at the extracellular face of the membrane. TMD, two thumbs, each of which probably corresponds to one of the two transmembrane domains. NBD, 3-nm lobes projecting from the structure at the cytoplasmic face of the membrane, probably corresponding to the two nucleotide binding domains. A, view perpendicular to the extracellular surface of the lipid bilayer, corresponding to projection of Fig. 5A. The NBDs seen in this reconstruction are below the plane of the membrane and so were not visualized in two-dimensional projections of solubilized P-gp (e.g. Fig. 3C). B, side view of P-gp, corresponding to projection of Fig. 5B. The approximate position of the lipid bilayer is indicated by the two horizontal dashed lines. Arrow, asymmetric opening providing access from the lipid phase to the aqueous core of the protein. Bar, 1.7 nm.

classes (1/25 Å⁻²), B, eigenvector plot of factor 1 versus factor 2 showing three major clusters (circled). C, averages of the three most populated classes determined by hierarchical ascendat classification. Class I particles were used for the three-dimensional reconstruction and represent a partial (slightly tilted) face-on view of the molecules. Bar, 5.3 nm.
would have been obscured by the lipid bilayer. Rotation of the molecule through approximately 90° shows that these two intracellular domains extend outward, giving the side view of the protein a truncated conical shape. They do not project far into the cytoplasm but are intimately associated with the rest of the molecule such that P-gp is relatively compact with a depth of about 7.5–8.0 nm in the plane of the membrane. Each intracellular domain appears to be more contiguous with one thumb than the other, enhancing the apparent double helical packing of the protein. The left domain is slightly larger than the right domain, with an additional mass extending downward. Although it is not possible to rule out, unambiguously, the possibility that this additional mass may be due to residual noise in the map, this seems unlikely, as a similar asymmetric appearance was also noted from the side view projection maps (Fig. 3, A and B). Thus, the two intracellular domains appear to exhibit structural differences within the molecule. It is likely that these intracellular domains correspond to the nucleotide binding domains (see “Discussion”).

A further major feature of P-gp is an elongated cavity formed between the left intracellular domain and the thumb lying closest to the observer in the side-view (Figs. 5C, curved arrow, and 6B, straight arrow). This opening does not have a symmetry-related equivalent on the other side of the molecule. This asymmetry is also seen as a “gap” in the toroidal representation of P-gp seen from the extracellular projection of the reconstituted protein (Fig. 3C). This opening is located within the region of protein predicted to be embedded in the lipid bilayer and could provide access between the lipid phase and the aqueous chamber within the protein core (see “Discussion”).

Analysis of Small Two-dimensionally Ordered Arrays of P-gp—Small, single layers of epitaxial two-dimensional crystals of P-gp formed spontaneously when the electron microscopic grid support film was incubated with concentrated (0.3 mg/ml) solutions of detergent-solubilized P-gp. These are shown in Fig. 2B (boxes). The arrays are small and superimposed by many randomly oriented (unordered) protein molecules that are distributed throughout the grid, making the arrays hard to visualize. Image processing of these arrays (Fig. 7) was therefore required to average the repeat units and to average out the randomly oriented protein. The detection of small crystalline arrays demonstrates the structural homogeneity of the protein prepared by this purification methodology. The crystal packing was similar to that observed for some three-dimensional mem-

brane protein crystals in which lattice contacts occur between hydrophilic extracellular and intracellular loop regions, presumably because the bulky annulus of disordered detergent molecules around the transmembrane regions prevents lattice contacts between hydrophobic regions (38). Fourier transforms of selected crystalline areas showed visible diffraction with a signal/noise ratio >5, sufficient to measure the lattice parameters and to apply image processing via Fourier filtering (i.e., extraction of structural information from the surrounding noise). Merging the data from 10 separate small crystalline areas significantly enhanced the signal/noise ratio of the resulting averaged projection map, giving structural data up to the fifth diffraction order (~3 nm resolution) (Fig. 7) with an average (amplitude-weighted) interimage phase residual of 31°. The unit cell of these crystals had a symmetry with the plane group containing 1 asymmetric unit, with a = 14.2 nm, b = 18.5 nm, and γ = 91.6°. These dimensions are slightly larger than those predicted by single-particle analysis. This could be due to the direction of packing in the lattice producing a longer b axis than expected. The projection map calculated from these arrays (Fig. 3B) showed a strong resemblance to one of the projections (the side-on projection) obtained by single-particle averaging (Fig. 3A). It should be noted that the map generated by single-particle alignment was at a slightly higher resolution (2.5 nm) than that generated by Fourier analysis. A poorly staining, three-lobed central structure was observed, consistent with a side-on view of a molecule with hexagonal symmetry in its face-on view (Fig. 3, C, D, and F). Two 2–3-nm-diameter lobes protrude from one side of the molecule (Fig. 3B, arrows), which correspond to the intracellular lobes (putative NBDs) obtained by three-dimensional reconstruction from single particle images (see above).

Lectin-Gold Labeling of P-gp—P-gp is glycosylated on a single extracellular hydrophilic loop (1). Lectin-gold labeling was used to evaluate the orientation and oligomeric state of P-gp (Fig. 8). It should be noted that this preparation was slightly less pure (75% compared with >95%) than those used for image analysis, possibly explaining why not all the molecules are labeled. Aggregation was also observed, probably due to the overnight incubation. The lectin-gold consistently labeled the face of the protein corresponding to the projection shown in Fig. 3, C, D, and F, showing that these projections correspond to the face of P-gp exposed at the extracellular face of the membrane (as glycosylation only occurs on an extracellular site). No more than one gold particle was ever associated with a nonclustered P-gp particle, even when the majority of particles were labeled, demonstrating that lectin-gold was not limiting. Thus, active P-gp appears to be monomeric. However, these data do not
formally exclude the possibility that P-gp is dimeric and that
dimerization sterically occludes one of the glycosylation sites
such that the dimer can only be labeled by a single lectin-gold particle.

**DISCUSSION**

The absence of structural data for any ABC transporter has
limited our understanding of the mechanisms of active transport.
In this study we have used electron microscopy to generate
an initial structure (to 2.5 nm resolution) of the multidrug
resistance P-glycoprotein. Three separate approaches were
used that gave consistent results and, together, enabled the
orientations of images with respect to the membrane to be
ascertained: two-dimensional projections from single particle
imaging of lipid-reconstituted protein; two-dimensional and
three-dimensional reconstructions from single particle image
analysis of detergent-solubilized P-gp; and Fourier transform
projection maps of small crystalline arrays of P-gp. These are
the first structural data for any ABC transporter.

P-gp was purified from membranes of cells selected for high
level drug resistance and retained activity following purification
as assessed by drug binding and drug-stimulated ATPase activity.
The P-gp particles observed by electron microscopy were of similar dimensions whether detergent-solubilized or
reconstituted into liposomes. Lectin-gold labeling indicated that
purified P-gp is monomeric. The volume of the structure
determined here is also consistent with a monomer (see below).
As purified P-gp retains activity, this suggests that P-gp can
function in the monomeric state. Other recent, biochemical
studies have also suggested P-gp can function as a monomer
(39), and another ABC protein, cystic fibrosis transmembrane
conductance regulator, also appears to be monomeric (40).
Whether P-gp is monomeric in native membranes or forms
oligomeric aggregates is not known. It has previously been
suggested that P-gp may be oligomeric, based on radiation
inactivation and cross-linking of P-gp overexpressed in native membranes (41–43).

From the data presented here, an initial structural model for
P-gp has been derived (Fig. 6), which is entirely consistent with
available biochemical and genetic data. In overall shape, P-gp
approximates a cylinder of about 10 nm in diameter with
a maximum height (in the plane of the membrane) of about 8 nm.
This compares with a depth of the lipid bilayer of about 4 nm,
suggesting that about one-half of the molecule is within the
membrane. Viewed from the extracellular surface of the mem-
brane (identified by reconstitution into lipids and by lectin-gold labeling), P-gp is toroidal with a large central pore of about 5
nm in diameter. The ring of protein surrounding the central
pore is roughly hexagonal, consisting of two thumbs, each with
three lobes (seen most clearly in Figs. 3, C and D, and 6A). The
domain appearance in side-on views of the membrane
region of P-gp (Fig. 3, A and B) is also consistent with an overall
6-fold symmetry. Biochemical data have shown that P-gp con-
sists of 12 membrane-spanning segments (3, 4), each predicted
to exist as an α-helix. These 12 membrane-spanning segments
are separated by six extracellular hydrophilic loops, one of which
is glycosylated. The internally repeated amino acid se-
quencies of the two transmembrane domains predict a 2-fold
pseudosymmetry. It is, therefore, reasonable to speculate that
the two thumbs represent the two transmembrane domains
and that the six lobes (three per thumb) reflect the six extra-
cellular loops connecting pairs of membrane-spanning seg-
ments (putative α-helices).

The aqueous pore open at the extracellular face of the mem-
brane is much larger in diameter (−5 nm) than is required for
the passage of known P-gp substrates. Such a large pore, if
open across the membrane, would destroy the permeability
barrier. However, the pore is closed at the cytoplasmic face of the
membrane. Thus, P-gp forms a large aqueous chamber
within the membrane, open to the extracellular milieu. In
addition, an opening to the pore-translocation pathway from
the lipid phase (44–46).

P-gp has two 3-nm lobes at the cytoplasmic face of the mem-
brane. The two NBDs of P-gp are the only substantial portion
of P-gp not embedded in the membrane and are primarily
cytoplasmic (4, 5). The 3-nm lobes are of an appropriate size for
the 200 amino acid NBDs. It is, therefore, likely that these
lobes correspond to the NBDs. Each lobe (putative NBD) is
more closely associated with one of the transmembrane thumbs
than with the other. The two lobes (putative NBDs) appear
asymmetrically organized within the P-gp molecule. It is
known that P-gp undergoes conformational changes in the
presence of ATP and substrate (47). The present structural
data were obtained in the absence of ATP; it will be interesting
to ascertain whether the conformation or asymmetry of these
lobes (putative NBDs) is altered on ATP binding and/or
hydrolysis.

The surface area occupied by P-gp in the membrane (the 12
predicted membrane-spanning α-helices per monomer) is
approximately 60 nm2. For comparison, cytochrome oxidase (22
membrane-spanning helices) occupies an area of about 54 nm2
(48), the bacterial photo reaction center (11 membrane-span-
ning helices) an area of 32 nm2 (49), and light-harvesting
complex 2 (18 helices) an area of about 49 nm2 (50). Thus, if
P-gp is monomeric, the transmembrane helices of P-gp must be
relatively loosely packed. The mass of a solid 10 × 8-nm cylin-
der (the overall dimensions of P-gp) can be calculated as about
540 kDa, assuming 0.7 cm3 g−1 as a partial specific volume for the
protein (51). Although protein-packing and volume calcula-
tions are notoriously variable, this is closer to the mass of a
dimer, rather than that of the 170-kDa P-gp monomer.

The structural data show that the protein is, indeed, loosely packed, and the central chamber and spaces between intracellular lobes (putative NBDs) occupy a significant propor-
tion of the volume of P-gp. When these factors are taken into
account, the structure is not inconsistent with a 170-kDa mon-
omer. Interestingly, hexameric annexin has approximately
the same overall dimensions as P-gp (a 10 × 7-nm cylinder with
a large central pore) and is of similar molecular mass, 210 kDa
(52).

The aqueous chamber within the membrane is closed at the
cytoplasmic face of the membrane. This closure is presumably
achieved by the two intracellular lobes (putative NBDs) and
the hydrophilic cytoplasmic loops that separate the 12 mem-
brane-spanning segments. This may provide an explanation for
the finding that the NBDs of certain ABC transporters are
accessible from the outside of the cell yet do not contain seg-
ments that would normally be expected to span the bilayer (53, 54); the transmembrane pore is sufficiently large to allow
agents to gain access to the NBDs at the intracellular face of
the membrane. Most kinetic models of transport predict the
alternate exposure of a substrate binding to the two sides of the
membrane. As the energy required to reorientate a binding site
from one face of the membrane to the other would be large, it
has generally been assumed that any conformational change
would be relatively localized within the membrane. The struc-
ture of P-gp suggests that this occurs at the inner face of the
membrane. This is consistent with data that show that the
substrate binding site of P-gp is at the cytoplasmic face of the
membrane: it is accessible from the inner face of the mem-

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brane; substrates can be cross-linked to regions of the protein predicted to be at the cytoplasmic face of the membrane (55); and mutations altering amino acids at the cytoplasmic face of the membrane can influence substrate selectivity (9, 56).

P-gp is a member of the ABC superfamily of transporters, and it is not unreasonable to suppose that the general architecture of other ABC transporters may be similar to that of P-gp. Some ABC transporters handle very large substrates (e.g., polypeptides or polysaccharides), whereas others handle rather small substrates (e.g., inorganic ions). A common architecture with a large pore could readily be adapted to accommodate different sized substrates with minor changes to the “gate” at the cytoplasmic face of the membrane. The availability of structural data for P-gp is necessary for directing future experiments aimed at understanding the molecular mechanisms by which P-gp and other ABC transporters operate.

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