Nucleotide-induced Structural Changes in P-glycoprotein Observed by Electron Microscopy

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P-glycoprotein (Pgp)3 is an ATP hydrolysis driven multidrug efflux pump, which, when overexpressed in the plasma membrane of certain cancers, can lead to the failure of chemotherapy. Previously, we have presented a projection structure of nucleotide-free mouse Pgp from electron microscopic images of lipid monolayer-generated two-dimensional crystals (Lee, J. Y., Urbatsch, I. L., Senior, A. E., and Wilkens, S. (2002) J. Biol. Chem. 277, 40125–40131). Here we have analyzed the structure of cysteine-free human Pgp from two-dimensional crystals that were generated with the same lipid-monolayer technique in the absence and presence of various nucleotides. The images show that human Pgp has a similar structure to the mouse protein. Furthermore, the analysis of projection structures obtained under different nucleotide conditions suggests that Pgp can exist in at least two major conformations, one of which shows a central cavity between the N- and C-terminal halves of the molecule and another in which the two halves have moved sideways, thereby closing the central cavity. Intermediate conformations were observed for some nucleotide/verapamil combinations. A low-resolution, three-dimensional model of human Pgp was calculated from tilted specimen crystallized in the presence of the non-hydrolyzable nucleotide analog, adenosine 5’-O-(thiotriphosphate). The structural analysis presented here adds to the emerging picture that multidrug ABC transporters function by switching between two major conformations in a nucleotide-dependent manner.

P-glycoprotein (Pgp)3 is an ATP hydrolysis-driven drug efflux pump found in the plasma membrane of mammalian tissues involved in detoxification of a wide variety of hydrophobic organic molecules (1, 2). Pgp expression in cancer cells can result in multidrug resistance and subsequent failure of cancer chemotherapy by preventing the mostly hydrophobic anticancer drugs from reaching their targets in the cytoplasm or nucleus. Thus, circumvention of Pgp activity could lead to improved cancer treatments. Pgp, a member of the ATP binding cassette (ABC) family of membrane transporters, is expressed as an ~1280 amino acid containing single polypeptide chain that is folded in two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) in the order N-TMD-NBD-TMD-NBD-C. Each TMD is predicted to contain six membrane-spanning α helices and there is evidence that the two TMDs together constitute the drug transport pore (3). Each NBD contains nucleotide binding motifs including a Walker A (P-loop) and Walker B sequence and the ABC signature motif (LSGGQ). Both NBDs of Pgp can bind and hydrolyze ATP. There is ample evidence that for efficient ATP hydrolysis to occur, the two NBDs have to interact by forming a sandwich dimer so that the ABC signature motif (LSGGQ) of one NBD comes in contact with the P-loop of the other NBD to form the nucleotide-binding pocket (4, 5). A model for the catalytic mechanism of cooperative ATP hydrolysis has been proposed in which the two NBDs hydrolyze MgATP in an alternating fashion with each net hydrolysis step being coupled to drug translocation across the lipid bilayer (6).

The mechanism, however, by which ATP hydrolysis taking place on the NBDs is coupled to drug transport across the TMDs is much less well understood. This lack of understanding is largely due to the fact that there is no atomic resolution structural information available for Pgp or any other related mammalian multidrug transporter. Low-resolution topology information for Pgp from human, mouse, and Chinese hamster ovary has been reported from cysteine-mediated chemical cross-linking (7), fluorescence resonance energy transfer experiments (8), and electron microscopy (9, 10). These studies point to a pseudo dimeric front-to-front arrangement of the N- and C-terminal halves of the molecule, much like as is seen in recent crystallographic structures of bacterial members of the ABC transporter family including BtuCD (11), HI1470/1 (12),

V_i, inorganic orthovanadate; TEM, transmission electron microscopy; CCD, charge coupled device; CFTR, cystic fibrosis transmembrane conductance regulator; ATPγS, adenosine 5’-O-(thiotriphosphate); AMPPNP, 5’-adenylyl-β,γ-imidodiphosphate.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S5.

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3 The abbreviations used are: Pgp, P-glycoprotein; ABC, ATP binding cassette; NBD, nucleotide-binding domain; TMD, transmembrane domain;
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Sav1866 (13), and ModBC (14). However, whereas BtuCD and HI1470/1 show two loosely interacting TMD/NBD pairs, in Sav1866 and ModBC, the TMDs and NBDs are seen arranged in an intertwined fashion, indicating that the arrangement of the functional domains of ABC proteins depends on the nature of the substrate they transport. Sav1866 is currently the closest homolog of Pgp for which high-resolution structural information is available. It shows considerable sequence similarity to Pgp for the NBDs (around 50% identity), however, the homology in the TMDs is much less prominent (around 15% identity). In the nucleotide-bound Sav1866 structure, the TMDs are seen in what appears to represent an outward facing conformation, leading the authors of that study to propose that the TMDs alternate between an outward and an inward facing conformation (15) similar to what has been proposed for secondary transporters from the major facilitator superfamily (16). However, whereas the energy consuming conformational switch in members of the major facilitator superfamily is driven by syn- or antiport along a transmembrane chemical gradient, in ABC transporters, binding of MgATP and/or subsequent hydrolysis in the NBDs is tightly coupled to structural changes in the TMDs that are thought to be associated with drug efflux. Interestingly, the first ADP-containing structure of Sav1866 (13) did not change much when ADP was replaced by AMPPNP (17), suggesting that the ATP- and ADP-bound conformations are very similar at the current level of resolution. The authors further suggested that major structural changes occur upon ATP binding and NBD dimerization (17). Indeed, in other ATPases, such as mitochondrial F-ATPase, the biggest structural change is seen between the nucleotide-free and nucleotide-bound (ADP or ATP) states (18). On the other hand, efficient ATP hydrolysis in detergent-solubilized, delipidated mouse and human Pgp requires the addition of lipid, suggesting that some of the structural changes occurring during the catalytic cycle are dependent on a proper membrane environment (19). We therefore decided to investigate the structure of Pgp and the conformational changes the drug transporter is undergoing during the catalytic cycle by electron microscopy of lipidi bilayer reconstituted protein.

We have recently reported the first electron microscopic images of lipid bilayer bound Pgp (mouse Mdr3) obtained from two-dimensional crystals of the protein (9). The projection structure showed two protein densities, which we interpreted as the N- and C-terminal halves of the molecule arranged around a pseudo 2-fold symmetry axis. These crystals were obtained in the absence of nucleotide. In the present study, we have analyzed two-dimensional crystals of lipid bilayer bound human Pgp (MDR1) incubated with various nucleotides with or without vanadate. Furthermore, we have calculated a three-dimensional structural model of the transporter incubated with the non-hydrolysable ATP analog, ATPγS in the presence of the drug verapamil. Overall, the data are consistent with Pgp existing in two major conformations, a nucleotide-free and a nucleotide-bound form, interconversion of which involves a sliding motion of the two halves of the transporter.

EXPERIMENTAL PROCEDURES

Materials

Egg phosphatidylcholine, Escherichia coli total lipids, and 1,2-dioleoyl-sn-glycero-3-[[N-(5-amino-1-carboxypentyl)imidodiacetoyl]succinyl] (nickel salt) (Ni-NTA-DOGS) were obtained from Avanti Polar Lipids, Inc. n-Dodecyl-β-d-maltoside was obtained from Anatrace Inc. Bio-Beads SM-2 were obtained from Bio-Rad. ATP, ADP, AMPPNP, ATPγS, and other biochemical reagents were obtained from Sigma. Electron microscopy supplies were from Ted Pella.

Purification of Cysteine-free Human P-glycoprotein

Cysteine-free (Cys-free) human Pgp (MDR1) that had all seven natural cysteines replaced by alanines and that contained a C-terminal deca-histidine tag (His10) was used throughout this study (20). The Cys-free Pgp was expressed in the yeast Pichia pastoris and purified to homogeneity as described (19, 21). Purity and ATPase activity of the preparations used for two-dimensional crystallization were confirmed by SDS-PAGE and ATPase assays, respectively. The ATPase activity was stimulated by verapamil (~10-fold) to a maximum of ~0.4 ± 0.2 μmol/min/mg in the presence of a 2-fold excess (w/w) of E. coli lipids. This activity is about 40% of our previous reported value likely due to a lipid/protein ratio of 2:1 rather than 100:1 used in Ref. 21.

Two-dimensional Crystallization and Transmission Electron Microscopy

Two-dimensional crystallization was performed by the lipid-monolayer technique as described previously (9) with the following modifications: Pgp was premixed with E. coli lipids at a protein-lipid ratio of 2:1 or 4:1 (w/w) at 4 °C for 6–12 h. After injecting the protein/lipid mixture underneath the lipid monolayer, 1 unit of phospholipase A2 (PLA2) was added during protein binding to the lipid monolayer at 4 °C for 6–12 h. The Teflon block was transferred to room temperature (instead of 37 °C), and then 10 Bio-Beads were added into the crystallization solution followed by a second addition of 10 Bio-Beads after 6 h. After incubating the drop for 24 h, 5 mM nucleotides and 9 mM MgCl₂ were added and allowed to bind to the pre-formed Pgp two-dimensional crystals between 10 min and 48 h. The monolayers with the attached two-dimensional crystals were transferred to EM grids by touching the surface of the drops with the carbon side of holey- or plain carbon grids and stained with 0.5 or 1% uranyl acetate. Grids were then examined in a Philips CM300- or FEI Tecnai 12 transmission electron microscope operating at 100 or 120 kV, respectively. Electron micrographs were recorded in low-dose mode with a 1024 × 1024- (CM300) or a 2048 × 2048 (Tecnai12) pixel CCD camera (both from Gatan, Inc.). Images were recorded at an electron optical magnification of ×30,000 or 42,000 at a defocus setting of between −400 and −700 nm, placing the first zero of the contrast transfer function at around 0.05–0.04 Å⁻¹. The tilt series was recorded on the 2048 × 2048 pixel camera at a magnification of ×30,000 and at tilt angles between −40 and +40° in 5° increments. The pixel size on the specimen level was calibrated using two-dimensional crystals of bacteriorhodopsin.
**Image Analysis of Pgp Two-dimensional Crystals**

**Correlation Averaging**—Two-dimensional crystal images were analyzed by correlation averaging as described (9). Briefly, crystalline patches, which showed a power spectrum consistent with a single crystalline layer, were band-pass filtered to remove low (<0.01 Å⁻¹) and high (>0.1 Å⁻¹) spatial frequencies. The contrast transfer function was corrected by inverting the phases between the first and second zero as implemented in IMAGIC 5. Correlation averaging was started with a reference 256 × 256-pixel image excised from the 2048 × 2048-pixel CCD images or 128 × 128-pixel images from the 1024 × 1024-pixel CCD image. Correlation averaging starting from the first reference was iterated 2–4 times using an average of the best sub-images from the previous round until no further improvement was seen in the averages of the crystal patches. At this point, the motif was centered and the size of the reference was reduced to cover 16 (and later 9) unit cells, and correlation averaging was continued until stable results were obtained. Occasionally, crystalline patches were observed that produced two similar but rotated power spectra, indicating the presence of double layered crystals. In cases where the two layers were rotated sufficiently, the two layers could be processed individually, resulting in projection maps with opposite handedness. Depending on the quality of the starting image (crystal size, crystalline order), final averages were calculated from 1000 to 2000 images. To estimate the resolution in the final averages, the I- and S-image methods as implemented in IMAGIC 5 were used (22, 23). Prior to calculating the resolution, the extracted images were treated with a circular mask (radius 0.7) with a soft Gaussian fall-off of 5 pixels to avoid high frequency correlations from the edges of the images and the mask.

**Crystallographic Approach**—Electron micrographs were first examined by using fast Fourier transform (FFT) as implemented in XIMDISP to identify well ordered areas (24). Images were corrected for lattice distortions, effects of the contrast transfer function, and astigmatism using the MRC Image Processing Package (MRC package) (25). One pass of unbending was performed with a reference area of 10% of the total pixel area, and the initial phases and amplitudes for each image were obtained by MMBOX. Two images were merged and the phase origins were refined without assuming any symmetry (plane group p1), using ORIGTILT. Averages of amplitudes and phases were calculated by AVRGAMPHS, and FOMSTAT was used to calculate the phase errors for the different resolution bins (26). An inverse temperature factor $B = -3500$ Å⁻² was found sufficient to compensate for the resolution-dependent attenuation in the amplitudes (27, 28). The final projection density map was contoured to $\sigma = 0.222$ the standard deviation of the density histogram by using CCP4 (29).

**Three-dimensional Image Reconstruction**

For calculating a first three-dimensional model of lipid bilayer bound human Pgp, a well ordered crystal incubated with 5 mM MgATP·$\gamma$S and 150 μM verapamil was used. The individual images of the tilt series were first examined by fast Fourier transform, and the +40° image was discarded due to insufficient information in the power spectrum. Lattice distortion, contrast transfer function effects, and the astigmatism were then corrected as described above. Following one round of unbending, images of two independent untilted crystals were merged in plane group p2. After adding the data from the tilt series (14 images), lattice lines were fitted with LATLINE as implemented in the MRC package. Weighted amplitudes and phases were then used for the three-dimensional reconstruction, and the three-dimensional density map was plotted to 1 σ above standard deviation of the density map by using CCP4. An inverse temperature factor $B = -2000$ Å⁻² was imposed to compensate for the resolution dependent attenuation in the amplitudes.

**RESULTS**

**Two-dimensional Crystallization of Cys-free Human Pgp**—Previously, we have employed the lipid monolayer technique to generate two-dimensional crystals of lipid bilayer-bound mouse P-glycoprotein (Mdr3/Abcb1), crystallized in the absence of nucleotides (9). The two-dimensional crystals were analyzed by transmission electron microscopy and projection images obtained by correlation averaging showed individual Pgp molecules with closely interacting N- and C-terminal nucleotide binding domains. In the current study we expanded our analysis to human Pgp and to the analysis of structural changes Pgp is undergoing in response to binding of nucleotides. We decided to conduct the analysis with Cys-free Pgp to avoid cysteine oxidation and inactivation due to the formation of intra- and/or intermolecular disulfide bonds (21). By optimizing the crystallization conditions (see “Experimental Procedures”), we were able to obtain crystals more reproducibly and with improved long-range order. During crystallization, we observed that Pgp molecules were reconstituted into small proteoliposomes, which over time grew into larger, often tubular vesicles below the lipid monolayer. As shown in Fig. 1A, we routinely obtained large vesicular (or tubular) two-dimensional crystals with a size ranging from 0.5 to 2 μm wide and 1 to 10 μm long. Fig. 1B shows part of the edge from the longer tube in Fig. 1A, revealing uniformly packed and oriented protein molecules (circled area and inset). Suchperiodical appearance is typical for collapsed negatively stained two-dimensional crystal vesicles. Fig. 1C shows the power spectrum of a representative two-dimensional crystalline area, with reflections clearly visible to the third order, e.g. the 2,3 reflection, indicating structural information to a resolution of better than 28 Å.

**Image Analysis by Correlation Averaging and Crystallographic Methods**—Electron micrographs of two-dimensional crystals were further analyzed by correlation averaging as described under “Experimental Procedures.” Fig. 1D shows a signal-improved image obtained by averaging 1000 raw images excised from the crystalline area shown in Fig. 1B. This image shows 4 unit cells with a regular grid-like arrangement of Pgp molecules (white density) and interfaces (black density). The calculated power spectrum in Fig. 1E reveals reflections up to the fifth order (black arrow). The resolution in the average image shown in Fig. 1D was estimated by S- and I-image methods as implemented in IMAGIC 5, and both algorithms indicate that the final average contains structural information to ~20 Å resolution (data not shown). A comparison between the image
In order to monitor some of the structural rearrangements Pgp is assumed to undergo as part of the catalytic cycle, various nucleotides in the presence or absence of vanadate were added to preformed two-dimensional crystals. We used the non-hydrolyzable ATP analogs MgAMPPNP and MgATPγS as well as MgADP to produce pre- and post-hydrolysis forms of Pgp two-dimensional crystals. In addition, orthovanadate was included in conjunction with MgATP or MgADP to produce the vanadate-trapped state (30). To grow crystals of nucleotide-bound Pgp, we initially tried to co-crystallize protein and nucleotide, but this approach did not result in ordered arrays, possibly due to heterogeneity in nucleotide occupancy as a consequence from the relatively modest affinity of Pgp for nucleotides (31, 32). Crystallization was therefore started in the absence of nucleotide and once crystals were formed, nucleotide was added for various amounts of time before preparation of the crystals for electron microscopy (supplemental Fig. S1). Electron microscopic images of the resulting Pgp two-dimensional crystals were analyzed by correlation averaging, and the results are summarized in Fig. 3. As can be seen, the overall appearance of the crystal lattice of the nucleotide-bound protein is very similar to the nucleotide-free protein (Fig. 3A) in that they contain two molecules per unit cell in a square P1 lattice with a pseudo 2-fold axis in the center of each molecule. However, close inspection of the averages revealed subtle but reproducible differences between nucleotide-free and nucleotide-containing Pgp.

The projection image of nucleotide-free Pgp (Fig. 3A) has an overall rectangular appearance with a large stain-filled cavity in the center. Compared with the projection of nucleotide-free Pgp (Fig. 3A), all the nucleotide-bound forms in Fig. 3, B–F, appear to have less density at the interface of the two halves of the molecule. In addition, all nucleotide-bound forms show a more or less distinct left-handed kink in the two peripheral high-density domains (white arrowheads). This is most apparent in the AMPPNP- and ATPγS-bound forms marked with solid ovals in B and C, but also in all of the vanadate-trapped nucleotide-bound Pgp molecules in D and E (solid and dashed ovals). The two-dimensional crystal analyzed in Fig. 3C was incubated with both MgATPγS and vanadate (a projection of

FIGURE 1. Image analysis of Pgp two-dimensional crystals by correlation averaging. A, two-dimensional crystals of Pgp were formed in the shape of elongated tubular vesicles with dimensions of 0.5–1 μm wide and up to 1–10 μm long (scale bar, 2 μm). B, a uniform orientation of the Pgp molecules in the lipid bilayers was observed at the edge of the two-dimensional crystal (see white arrowheads in inset). The scale bar is 60 nm. C, the power spectrum computed from the area outlined in B, black square, revealed reflections to the third order (white circle), indicating that the image contains structural information to at least 28-Å resolution. D, average of 1000 128 × 128 pixel images extracted from the crystal shown in B by correlation averaging (scale bar, 8.0 nm). E, calculated power spectrum (truncated) of an average similar to the one shown in D. Reflections up to 5,3 are now clearly visible (black arrow).
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Interestingly, two populations of molecules with slightly different shapes can be distinguished in the AMPPNP- and ATPγS-bound Pgp forms: whereas one form appears more compact and slimmer in the middle (Fig. 3, B and C, solid ovals), the other population retains a small low density area in the center and the left-handed kink is less pronounced (Fig. 3, B and C, dashed ovals; see also supplemental Fig. S2). Such two population appearance was observed consistently with incubation periods from 1 to 24 h (data not shown). Of all the arrays of nucleotide-bound Pgp analyzed, the ADP-bound form (Fig. 3F) most closely resembles projections of nucleotide-free Pgp (Fig. 3A) with respect to two features: the unit cell parameters \((a = b = 102 \pm 2 \, \AA, \gamma = 90 \pm 1^\circ)\) are similar to the ones of nucleotide-free Pgp and both molecules in the asymmetric unit are similar to each other. However, a distinct left-handed kink and a smaller low density center do distinguish the ADP-bound from the nucleotide-free form. Because of the relatively short incubation time with MgADP of 10 min, this population of Pgp molecules may represent an intermediate form as the nucleotide-free form changes to one with loosely bound nucleotide. Finally, we added verapamil (150 \(\mu\)M) to the crystallization trays with some nucleotide/vanadate combinations; however, the resultant images did not differ significantly from the images obtained in its absence, indicating that drug binding may not produce a large enough structural change to be detectable at the current resolution (data not shown).

Topography of Nucleotide-free and Nucleotide-bound Pgp—To facilitate comparison of the various Pgp projection structures, we excised single molecules from the projection structures shown in Fig. 3 and outlined the two high-density domains from each projection by closed white lines (Fig. 4). In case of the projections of nucleotide-free protein, each molecule has a near rectangular shape (Fig. 4, A1 and A2) and a shallow, spindle-shaped cavity at the center (black arrowhead) of each projection separates two “boot-shaped” domains (outlined in white). In the projections of AMPPNP-, ATPγS/Vi, ATPγVγ, or ADP/Vγ-bound protein, the central low density area of one population is almost sealed by two “triangle-like” domains (Fig. 4, B1 and B2) and a narrow spindle-shaped cavity in the middle (somewhat similar to nucleotide-free Pgp), but the two high density domains are shifted toward each other and extend into a left-handed kink with a wider “shaft” in the boot-shaped domain. As mentioned above, the projections of ADP-bound Pgp (Fig. 4, F1 and F2) were most similar to nucleotide-free protein (Fig. 4, A1 and A2), albeit with a somewhat smaller central cavity (black arrow).

All in all, the projection structures summarized in Fig. 4 seem to indicate that Pgp can exist in a fully open (nucleotide-free) conformation, which is characterized by the rectangular outline and the central cavity, and a fully engaged (ATPγVγ, or ADP/Vγ, bound) conformation, in which the two halves of Pgp have moved sideways, thereby closing the central cavity. Intermediate conformations with a partially closed central cavity or partially engaged NBDs can be observed for some of the mole-
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The symmetry search of the two-dimensional crystals indicated that the projections were compatible with plane group symmetries p2, or p221 (supplemental Table S1). However, the crystals were processed with p2 symmetry to improve the statistical significance of the final three-dimensional map (image statistics are given in supplemental Table S2). Imposing no symmetry (p1 plane group) produced a projection that resembled the projection calculated with imposed p2 symmetry, albeit at a lower signal-to-noise ratio (data not shown). Analysis of the images of the untitled crystal with imposed symmetry (supplemental Fig. S2) revealed the presence of two populations of molecules in the unit cell, again consistent with the projection image shown in Fig. 3C (and the projections shown in supplemental Fig. S2).

Three-dimensional Image Analysis of ATPγS-bound Two-dimensional Crystals—To calculate a first three-dimensional model of lipid-bilayer-bound Pgp, a tilt series of a well ordered Pgp two-dimensional crystals incubated with ATPγS and the drug verapamil were recorded from −40° to +40° in 5° increments (the +40° was later discarded due to poor signal in the power spectrum). The image analysis was performed with programs implemented in the MRC Image Processing Package. The symmetry search of the two-dimensional crystals indicated that the projections were compatible with plane group symmetries p2, or p221 (supplemental Table S1). However, the higher symmetry plane group was ruled out during three-di-

### TABLE 1

Summary of three-dimensional data analysis

| Data                                                   | Value |
|--------------------------------------------------------|-------|
| Number of images                                       | 14    |
| Unit cell parameters                                    | a = 102 Å; b = 100 Å; γ = 86° |
| Two-dimensional plane group                            | p2    |
| Range of tilt angles                                   | −40° to +40° |
| Total number of measurements                           | 600   |
| Total number of fitted unique reflections              | 259   |
| Overall phase error                                    | 45.7° |
| Overall weighted phase error                           | 20.0° |
| Range of resolution                                    | 74.5–14.6 Å |
| Effective resolution cutoffs                           | 22.3 Å (in plane); 91.8 Å (vertical) |

* The +40° image was not included in the final analysis.
* Included reflections with IQ ≤ 8 to 14.6 Å.
* See supplemental Fig. S3.

mensional processing and consequently, the crystals were processed with p2 symmetry to improve the statistical significance of the final three-dimensional map (image statistics are given in supplemental Table S2). Imposing no symmetry (p1 plane group) produced a projection that resembled the projection calculated with imposed p2 symmetry, albeit at a lower signal-to-noise ratio (data not shown). Analysis of the images of the untitled crystal with imposed symmetry (supplemental Fig. S2) revealed the presence of two populations of molecules in the unit cell, again consistent with the projection image shown in Fig. 3C (and the projections shown in supplemental Fig. S2).

For the three-dimensional reconstruction, tilt geometry for the more highly tilted crystals was calculated by EMTILT, whereas nominal tilt angles were used for the projections recorded at low tilts. The statistics of the three-dimensional data set are summarized in Table 1. The resolution in the final model was estimated to ~22 and 92 Å in the planes parallel and perpendicular to the crystal plane, respectively (supplemental Fig. S3). From the top view perpendicular to the crystal plane (Fig. 5A), the three-dimensional density map shows the two populations of Pgp molecules designated as “p” molecule (dashed oval) and “q” molecule (solid oval). The p molecule appears rather smooth with shallow protrusions and has dimensions of ~70 × 50 Å. The q molecule on the other hand appears more compact with dimensions of ~65 × 50 Å, and more of the aqueous stain seemed to have penetrated the molecule producing overall more distinct features. This is true for both the top (Fig. 5A) and bottom (Fig. 5B) views. Inspection of the molecules from the side indicates that they have a height of ~125 Å (Fig. 5, C and D). In both molecules, four short protrusions with a length of ~15 Å are seen on the bottom (Fig. 5C) and top (Fig. 5D), respectively (short arrows), but at the current resolution it is not clear whether these features are genuine structural details or a result of the limited resolution in the direction perpendicular to the plane of the crystal. Consecutive rotations by 45° (middle and right panels) reveal stain accessible cavities at the top of the p and q molecules and in the bottom half of the q molecule (black arrowheads). Whereas the cavities near the top of the molecules are barely seen, the cavity in the bottom half of the q molecule is deeper and more obvious likely because it is more accessible to the aqueous staining solution (see also Fig. 6, below). In the q molecule this cavity reaches ~50 Å up into the three-dimensional volume where it is surrounded by long distinct lobes (marked by asterisks). The middle domain of the q molecule appears more compact and narrows into a slimmer “neck” of about 40 Å in length. In contrast, the p molecule appears relatively smooth over its entire length,
with a number of short protrusions sealing the top, and four distinct protrusions surrounding the wide, but sealed bottom.

Comparison of the Pgp Three-dimensional Model with the Crystal Structure of Sav1866—Because the q molecule seemed better resolved compared with the p molecule, we have focused on this three-dimensional map of Pgp for comparison with the three-dimensional crystal structure of the MgADP bound form of Staphylococcus aureus Sav1866 (Fig. 6, fitting of the p molecule is shown in supplemental Fig. S5) (Protein Data Bank (PDB) code 2hyd). Shown are surface representations of Pgp (Fig. 6, A and C) and Sav1866 (Fig. 6B). It is obvious that the three-dimensional model of Pgp is quite similar to Sav1866 in terms of overall dimensions and appearance, consistent with the relatively high sequence conservation between the two transporters. However, due to the limited resolution of the Pgp three-dimensional volume, it is not immediately obvious which orientation of Pgp matches Sav1866 (Fig. 6B), e.g. fitting the crystal structure of Sav1866 into the three-dimensional electron density of Pgp in the two possible orientations as shown in Fig. 6, A and C, resulted in virtually identical cross-correlation coefficients of 75.8 and 74.4, respectively. However, when the molecules are sliced along the long axis of the molecules, it appears that both Sav1866 (Fig. 6E) and Pgp (Fig. 6F) contain a large cavity (black arrow), which in the case of Sav1866 is formed by the two transmembrane domains. In contrast, the orientation of Pgp in Fig. 6A shows this cavity at the approximate location of the Sav1866 NBDs, inconsistent with the tight interaction of the NBDs in the crystal structure. Thus, Sav1866 and the three-dimensional volume of Pgp in the orientation shown in Fig. 6C were further compared by generating ~25-Å thick cross-sections as indicated by the black lines. As can be seen in Fig. 6G, the Sav1866 x-ray structure is filling out the Pgp three-dimensional model reasonably well for slices G1, G2, G4, and G5, including the cavity formed by the Sav1866 TMDs (see arrows). On the other hand, slice 3 (and to a lesser degree slice 4) does not seem to generate a good fit as the volume occupied by Pgp appears more rectangular in this slice compared with more compact Sav1866. It should be kept in mind, however, that the two-dimensional crystal-derived three-dimensional volume of Pgp corresponds to stain excluding regions contributed by both the protein and lipid components, and that protein/lipid packing forces may have altered the overall appearance of lipid-embedded Pgp versus detergent-solubilized Sav1866. Another reason for the mismatch between Pgp and Sav1866 in slices 3 and 4 might be due to dehydration-induced specimen flattening commonly observed with negative staining. Nevertheless, the comparison of the x-ray crystal structure of Sav1866 and the EM derived model of Pgp, despite the limited resolution of the Pgp three-dimensional volume, does reveal the presence of similar features in the two structural models, suggesting that detergent extraction as in the case of Sav1866 does not result in major structural alterations compared with the lipid bilayer bound transporter.

DISCUSSION
In an attempt to gain a better understanding of the conformational changes Pgp undergoes during ATP hydrolysis-driven drug translocation, we generated two-dimensional crystals of the nucleotide-free and nucleotide-bound protein for structural analysis by transmission electron microscopy. Previously, we provided the first projection structure of lipid bilayer-bound mouse Pgp (9), which revealed two closely interacting nucleotide-binding domains, even in the absence of nucleotide. In the current analysis, we continued to use the lipid bilayer-bound protein, because this is the physiological form of Pgp, and we utilized the human protein because of its optimal clinical relevance.

We chose to work with the human variant of Pgp in which all naturally occurring cysteines were replaced by alanines, to exclude the possibility of artificial structural changes due to disulfide bond formation during crystallization. Of particular concern were the naturally occurring cysteines located in the P-loops of the two NBDs, which are able to form a disulfide bond in the absence of nucleotide (21). The fact that the pro-
jection structures of Cys-free human MDR1 presented in the current analysis look virtually identical to the previously reported (9) projections obtained for Cys-containing mouse Mdr3 indicated that the molecules in the two-dimensional crystals of mouse protein were probably not oxidized, and that the two Pgps are not only catalytically (21) but also structurally analogous.

To obtain structural information for nucleotide-bound human Cys-free Pgp, we initially tried to crystallize the protein in the presence of nucleotide, but without success. A possible explanation for this behavior is that the affinity of membrane-bound Pgp for nucleotide is not high enough for generating a sufficiently homogeneous population of molecules for crystallization. Another explanation might be that the membrane domain of nucleotide-bound Pgp is too flexible to allow crystallization under these conditions as crystal contacts of membrane proteins are mostly mediated by contacts between membrane-embedded domains in two-dimensional lipid-protein crystals.

To overcome this problem, Pgp two-dimensional crystals were first generated in the absence of nucleotide, and then nucleotide was added to the crystallization mixture before preparation of the two-dimensional crystal for electron microscopy. Overall, the crystalline lattice of the nucleotide-free protein appeared not to be disrupted by binding of nucleotide and drug substrate (verapamil). The appearance of individual molecules, however, did change in a nucleotide-dependent manner. In general, whereas the nucleotide-free molecules consistently showed a large cavity between the N- and C-halves of the transporter, the very same cavity was occluded by an apparent lateral motion of the two half-molecules, consistent with previ-
ous cysteine-mediated cross-linking studies, where a cysteine in each P-loop of the N- and C-terminal NBD was able to form a disulfide bond in a nucleotide-dependent manner (21).

As noted earlier, the only available x-ray crystal structure (PDB codes 2onj and 2hyd) of a bacterial ABC transporter with significant sequence similarity to Pgp did not change much when bound ADP was replaced by the non-hydrolyzable ATP analog, AMPPNP (17). This finding is overall consistent with the EM projections of lipid-bound Pgp as shown in Fig. 3 in that there are also no significant differences between the two nucleotide conditions. In fact, our EM analysis predicts that the nucleotide-free structure will be distinctly different from the nucleotide-bound ones. A very similar situation can be observed in other energy transducing ATP-binding proteins. For example, in the crystal structure of the mitochondrial F-ATPase (1bmf), the difference between nucleotide-free (βp) and nucleotide-bound β subunits is much greater than structural differences between β subunits containing different nucleotides (βp and βT) (18). Unfortunately as of now no high-resolution structure is available for a nucleotide-free ABC transporter with homology to Pgp (such as Sav1866). However, the nucleotide-free structure of ModBC shows the NBDs in quite a different conformation compared with ADP-bound Sav1866 (see below), consistent with the idea that the NBDs are undergoing large structural changes between nucleotide-free and nucleotide-bound forms (32).

The results presented here appear to be in contrast to electron microscopic images obtained for Pgp from Chinese hamster ovary cells. In that study, using two-dimensional crystals of delipidated, detergent-solubilized protein, generated at the buffer-air interface, a very different projection structure was observed for AMPPNP-bound Pgp compared with ADP-bound and nucleotide-free protein (33). We have no explanation for this difference at this point, but it cannot be ruled out that differential staining and/or a different orientation of the Pgp molecules in the two-dimensional crystals might be responsible for the apparent discrepancy between lipid-bound and delipidated Pgp. Rosenberg et al. (34) also obtained two-dimensional crystals of AMPPNP-bound CFTR (ABCC7) by the same technique. Interestingly, in their study, Rosenberg et al. (34) obtained two crystal forms of AMPPNP-bound CFTR. When comparing these to the projections of Pgp reported by the same group (33), one crystal form of AMPPNP-bound CFTR appears to resemble AMPPNP-bound Pgp, whereas the other crystal form of AMPPNP-bound CFTR is more similar to nucleotide-free Pgp. More data are needed to reconcile the differences observed between crystals of lipid-bound Pgp on one hand and delipidated Pgp and CFTR on the other.

In the current study, we also obtained a first low-resolution three-dimensional model of lipid bilayer-bound Pgp, incubated with the non-hydrolyzable analog ATPγS and the drug verapamil. However, due to the limited size of the two-dimensional crystals, we were only able to record images with tilt angles up to 40° to calculate a low-resolution three-dimensional density map of membrane-bound Pgp (Fig. 5). The overall dimensions and appearance of the Pgp three-dimensional model are consistent with the molecular dimensions and domain organization of the related bacterial multidrug transporter, Sav1866, which was crystallized with MgADP in both catalytic sites (Fig. 6). This structure of Sav1866 was referred to as an “outward” facing conformation, with tightly interacting NBDs and a cavity formed by the two TMDs on the periplasmic side of the transporter. Orienting the three-dimensional volume of Pgp as shown in Fig. 6C and taking a cross-section along the long axis of the molecule (Fig. 6E) reveals a large cavity surrounded by two slightly outward tilted density lobes, such as seen in the crystal structure of Sav1866 (Fig. 6E). This would suggest that the three-dimensional model of ATPγS-bound Pgp shows the molecule in an outward facing conformation, and thus that binding of nucleotide (here ATPγS) alone is able to generate this conformation without the need for nucleotide hydrolysis. On the other hand, an “inward” facing conformation was recently observed in the crystal structure of yet another bacterial ABC transporter, the metal cluster importer ModBC (2onk). This structure was obtained from nucleotide-free protein and it shows the NBDs in an “open” conformation interacting mainly via two α-helices at the C-terminal ends of the NBDs. The TMDs in the ModBC structure open up to form a large cavity, in this case, on the cytoplasmic side of the transporter. A central cavity can be observed in the projection structures of Pgp and that cavity is most distinguished in the nucleotide-free protein (Fig. 3A), suggesting that in nucleotide-free Pgp the NBDs open up to present a drug binding site in the inner leaflet (cytoplasmic side) of the plasma membrane. This observation, together with the location of the cavity in the three-dimensional model, is overall consistent with the alternating access model put forth based on the crystal structures of Sav1866 and ModBC (15, 35, 36).

As shown in Fig. 3, B and C, incubation with non-hydrolyzable nucleotide analogs leads to two sets of projections with slightly different appearance. This is also apparent in the three-dimensional reconstruction, which shows two species, designated as p and q molecules. At this point we have no explanation for this observation. It is possible that the molecules have a different (up and down) orientation in the crystal analyzed, and that the molecule with the NBD oriented toward the inside of the liposome was not exposed to nucleotide..

Biochemical and structural studies have shown that ATP induces sandwich dimer formation between isolated NBDs from ABC transporters (37, 38). In E. coli MalK, binding of two ATP molecules induced a tight association between the NBDs (39), and ATP hydrolysis and/or product release converted the NBDs back to the resting state. NMR experiments as well as computer simulation have shown that the closely packed NBD dimer is dependent on nucleotide binding, and that slight shifts of the NBDs against each other may support an induced-fit mechanism (40, 41). Biochemical studies with Pgp have supported the idea that NBD dimerization of the NBDs occurs during the catalytic cycle to produce an “occluded nucleotide conformation” (6, 42).
Nucleotide-induced Structural Changes in Pgp

With the foregoing evidence in mind, together with the structural analyses of human nucleotide-bound and nucleotide-free Pgp presented in this study, we propose a working model for domain-domain interactions coupled to ATP hydrolysis in the NBDs of Pgp. In Fig. 7A, binding of MgATP to the two NBDs generates a tight sandwich dimer that was visualized here in the MgATP/δV\textsubscript{i} form seen in Fig. 4DI, representing a transition state. In the normal catalytic cycle, this transition state would collapse to a post-hydrolysis state containing MgADP, which was accessed in this study by adding MgADP plus Vi to MgATP/Vi (panel B, right). As shown in Fig. 7B, the transition state conformation can also be reached with MgADP following addition of Vi (panel B, right); however, in this case one NBD is likely occupied by MgADP, whereas the other NBD forms the occluded MgADP\textsubscript{V}, conformation (6, 48).

Although at low resolution, this study provides the first glimpse of structural changes that Pgp is undergoing during drug transport, and the overall three-dimensional structure of the lipid-bilayer bound transporter. One possible line of approach to disabling or circumventing the action of Pgp in cancer cells that has been suggested by studies on other ABC transporters is to develop small molecule inhibitors that would work by preventing dimerization of the NBDs, possibly by intercalating between them at points of interaction. The structural work presented here using human Pgp confirms that this approach might well be feasible. Much higher resolution structure will, however, be required to develop such compounds. To extend the resolution of the current study, analysis of larger, well ordered two-dimensional crystals, by cryo-electron microscopy, will be necessary. These studies are ongoing in our laboratories.

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FIGURE 7. Structural changes of Pgp during the catalytic cycle. Based on nucleotide-dependent changes in the appearance of the Pgp projections shown in Figs. 3 and 4, we propose the following mechanism. The molecule in the nucleotide-free resting state is a relatively symmetric, rather open form with a region of low density in the center (left, A, after binding of MgATP (or MgATP\textsubscript{δV})\textsubscript{5}, top middle) conformational changes occur possibly driven by partial dimerization of the two cytoplasmic NBDs. During these changes, the two high-density domains slide toward each other leading to a more compact sandwhich dimer might provide the energy for the power stroke. It has been suggested that tight association of the NBDs in the transmembrane domains of Pgp are interacting but the interaction might be confined to the C-terminal ends of the two NBDs, which are predicted to have similar secondary structure as the C-terminal ends of Sav1866 and to a lesser degree ModBC (not shown). Upon binding of MgATP to this “resting state,” both NBDs together form a rigid body, thus facilitating the alignment of ATP molecules between the P-loop of one NBD and the LSGGQ motif of the other (44). The transport cycle is then completed by the alternating ATP hydrolysis mechanism, which regenerates the resting state of the two NBD domains (6, 45).
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