Projection Structure of P-glycoprotein by Electron Microscopy

EVIDENCE FOR A CLOSED CONFORMATION OF THE NUCLEOTIDE BINDING DOMAINS*

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The structure of P-glycoprotein (Pgp) from mouse has been studied by electron microscopy and image analysis. Two-dimensional crystals of Pgp in a lipid bilayer were generated by reconstituting pure, detergent-solubilized protein containing a C-terminal six-histidine tag using the lipid monolayer technique. The crystals belong to plane group P1 with a = b = 104 ± 2 Å and γ = 90 ± 4°. The projection structure of Pgp calculated at a resolution of 22 Å shows two closely interacting protein domains that can be interpreted as the N- and C-terminal halves of the protein. The projection structure of Pgp is consistent with the recently published x-ray structure of MsbA, a lipid A flippase from Escherichia coli with high sequence homology to Pgp but only when the two MsbA subunits are rotated to bring their nucleotide binding domains together.

P-glycoprotein (Pgp,1 also called multidrug resistance protein) is found in the plasma membrane of higher eukaryotes where it is responsible for ATP hydrolysis-driven export of hydrophobic molecules (1–3). In animals, Pgp plays an important role in excretion of and protection from environmental toxins; when expressed in the plasma membrane of cancer cells, it can lead to failure of chemotherapy by preventing the hydrophobic chemotherapeutic drugs from reaching their targets inside cells (4). Pgp is a member of the superfamily of ABC binding cassette (ABC) transporter proteins (1, 5). ABC transporters typically consist of four domains, two nucleotide binding domains (NBDs) located in the cytoplasm and two transmembrane domains (TMDs) responsible for binding and transport of substrates and/or drugs. Pgp contains in the order of 1280 amino acid residues, and the arrangement of the domains in the primary sequence is: TMD1-NBD1-TMD2-NBD2. The N- and C-terminal halves of Pgp show strong sequence homology to each other and in bacterial ABC transporters, the functional domains are often composed of pairs of identical separate polypeptides.

There is firm evidence that the two nucleotide binding sites function in a strongly cooperative mechanism to bind and hydrolyze ATP in an alternating mode, effectively coupling drug transport to the collapse of the transition state in one of the NBDs (6–9). High resolution x-ray crystal structures exist for the nucleotide binding domains of a variety of ABC transporters, including HisP (10), MalK (11), Rad50 (12), MJ1267 (13), MJ0796 (14), and TAP1 (15). However, no atomic resolution structural information is available for Pgp. Three-dimensional models of Pgp obtained from electron microscopic images of detergent-solubilized single enzyme molecules (16) and two-dimensional crystals of delipidated Pgp (17) indicated that Pgp is a more or less globular molecule with a central chamber with access from the cytoplasmic and the extracellular side as well as an opening to the hydrophobic interior of the lipid bilayer. Such a structure would support the “vacuum cleaner” mechanistic model of Pgp, which predicts that drugs to be exported are initially bound from the interior leaflet of the lipid bilayer rather than from the aqueous cytosol (18). Refined structural pictures for two full-length ABC transporters from Escherichia coli have recently been reported for the lipid A flippase, MsbA, (19) and for the vitamin B12 importer, BtuCD (20). MsbA shares more than 30% of its primary sequence with Pgp, and the similarity is spread over both the transmembrane and nucleotide binding domains. The 4.5-Å x-ray structure of MsbA shows the identical halves of the protein arranged in a V-shaped structure, with the periplasmic ends of the TMDs interacting and the cytoplasmic nucleotide binding domains as far as 50 Å apart. The x-ray model of MsbA shows that its TMDs contain six bilayer-spanning α-helices, and this membrane topology is also predicted for Pgp. The arrangement of the functional domains is quite different in the 3.2-Å resolution model of BtuCD that shows the two nucleotide binding subunits (BtuD) in close contact with each other, giving the protein a much more compact appearance. However, BtuCD cannot be considered a close homolog to Pgp. The very limited sequence similarity between the two proteins is concentrated around the functional regions in the NBDs with no significant similarity in the membrane domains. Indeed, the TMDs of this transporter (BtuC) contain a total of 10 membrane-bound α-helices.

None of these crystallographic studies, however, shows the protein in its native environment, the lipid bilayer. Here we report the projection structure of mouse Pgp at 22 Å resolution, obtained from electron microscopic analysis of two-dimensional crystals of the protein reconstituted in a lipid bilayer by the lipid monolayer technique. The structure of Pgp shows two closely interacting domains that can be interpreted as the N- and C-terminal halves of the transporter. Comparison of the projection structure of Pgp with the x-ray structures of MsbA and BtuCD indicates that in lipid-bound Pgp the nucleotide binding domains are in close contact. The implications of this structure of Pgp with respect to its proposed mechanism are discussed.
Materials—Pgp (mouse MDR3) containing a C-terminal His<sub>6</sub> tag and three mutations (Asn to Gln) at potential glycosylation positions 83, 87, and 89, respectively, was expressed in yeast Pichia pastoris as described (21–22). All lipids including DOGS-NiNTA were from Avanti Polar Lipids, Inc. N-Dodecyl-β-maltoside (DM) was from Anatrace Inc. Amphilph A8–75 was synthesized as described (23).

Native Gel Electrophoresis—Pgp was bound to Ni-NTA-agarose (Qiagen) and washed on the column with 50 mM Tris/HCl, pH 7.4, 10% glycerol, 100 mM NaCl, 0.02% amphiphil A8–75 to exchange the amphiphil with Pgp. Pgp was then eluted in the same buffer containing 200 mM imidazole. For native gels, 1% agarose in 10 mM Tris, 100 mM glycine, 1 mM dithiothreitol, adjusted to pH 7, with acetic acid was used.

Two-dimensional Crystallization—The verapamil-stimulated ATPase activity of Pgp in the presence of a 2-fold excess (w/w) of E. coli lipids and 8 mM dithiothreitol was tested for each preparation before crystallization and was ~4–5 units/mg when measured in an ATP regenerating assay (24). For two-dimensional crystallization, a Teflon block was prepared as described in Levy et al. (25). A variety of crystallization conditions were tested with the following giving the most consistent results. 0.5 μl of a 0.1 mg/ml lipid solution in hexane containing NiNTA-DOGS/EggPC in a ratio of 1:4 was spread on 60 μl of 50 mM Tris/HCl, 100 mM NaCl, pH 8, and allowed to incubate for 12 h at 4 °C.

Pgp, which had been incubated for 12 h at 4 °C at a protein concentration of 1 mg/ml in the same buffer in the presence of 0.2% DM, with or without additional lipid (EggPC), was injected into the lipid monolayer and into a second addition of BioBeads after 6 h). Crystals were transferred to 37 °C, and detergent was removed by polystyrene Teflon block was then transferred to 37 °C, and about 1 BioBeads SM2 (Bio-Rad) were added through the side channel. Incubation was continued for another 24 h at 37 °C (with a fresh addition of BioBeads after 6 h). Crystals were transferred to carbon-coated copper grids by touching the surface of the drops and negatively stained with 0.5–1% uranyl acetate. Analysis of the protein in the crystallization solution by SDS-PAGE revealed that no significant degradation of the protein takes place during the time periods of the incubation (not shown).

Electron Microscopy—Grids were examined in a Philips CM300 transmission electron microscope operating at 100 kV. Electron micrographs were recorded on a 128 × 128 pixel camera (Gatan, Inc.) or on Kodak SO163 film that was developed in full-strength Kodak D19 developer at room temperature for 12 min. Images were recorded at an electron optical magnification of ×35,000 or 47,000 with defocus settings of ~384 and ~576 nm, placing the first zero of the contrast transfer function between 1/18 and 1/24 Å<sup>-1</sup>. Electron micrographs (×35,000) were digitized on an Operetta ColorGentherm in the presence of 0.1% dodecyl maltoside. Previous SDS-gel electrophoresis has shown that the resultant preparation is highly purified (22). Pgp prepared in this way is soluble in aqueous buffer and highly monodisperse. Fig. 1 shows a native gel electrophoresis and electron microscopy of Pgp. As can be seen from Fig. 1A, Pgp migrates as a single sharp band in the gel, indicating the homogeneous nature of the preparation with respect to its oligomeric state. Fig. 1B shows a sample of Pgp negatively stained with uranyl acetate. Again, the preparation appears highly homogenous with an average size of the particles of about 80–100 Å, consistent with a monomeric form of Pgp.

Two-dimensional Crystallization of Pgp—For two-dimensional crystallization of Pgp, we used the lipid monolayer technique modified for the crystallization of membrane proteins (25, 30). Briefly, detergent-solubilized Pgp containing a His<sub>6</sub> tag at the C terminus is covered with a monolayer consisting of a mix of Egg-PC and NiNTA- derivatized lipid (DOGS-NiNTA). After sufficient time to allow the protein to bind to the monolayer (1 h at room temperature), the Teflon block was transferred to 37 °C, and detergent was removed by polystyrene beads over a time period of 24 h. During the detergent removal, Pgp was reconstituted into a lipid bilayer, which formed underneath the lipid monolayer. The advantage of this method versus the conventional reconstitution method is that the protein exhibits a uniform orientation in the lipid bilayer, thus facilitating the subsequent structure analysis. Two-dimensional crystals of Pgp were observed under a variety of conditions; the condition giving the most reproducible results is described under “Experimental Procedures.” Fig. 2 shows a collection of images of two-dimensional crystals of Pgp generated this way. Crystals that formed directly on the solvent-exposed side of the lipid monolayer as a single layer (these were more often observed at lower temperatures) were generally limited in size and often distorted due to breakage of the
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**Fig. 2.** Two-dimensional crystals of Pgp. A, portion of the monolayer covered by an array of Pgp molecules. The monolayer, which is overlapping a hole in the carbon support film, is distorted from the transfer to the EM grid and/or the exposure to the electron beam. B, small crystalline patch of Pgp supported by continuous carbon film. An area of about 0.18 × 0.18 μm² is covered by an array of ~25 × 25 Pgp molecules. C, small vesicle containing small crystalline areas. The edge of the vesicle shows a dense packing of the cytoplasmic nucleotide binding domains (see the small area surrounded by the circle enlarged in the inset) indicating a uniform orientation of the molecules in the bilayer. D, enlarged view of a larger crystalline patch from the upper box in the large vesicle shown in the inset. The box is 0.27 × 0.27 μm². E, computed power spectrum (truncated) from the area in the lower box shown in the inset of panel D. The power spectrum indicates two overlapping crystalline layers. F, computed power spectrum (truncated) of the area shown in panel D. The appearance of the power spectrum is consistent with a single crystalline layer. The weak (3, 3) reflection (see small circle) occurs at a spatial frequency of 1/24 Å. Bar in panel E is 0.01 Å⁻¹. The crystals shown were from experiments in which Pgp was added to the crystallization drop without additional lipid. The crystals in panels A and B were generated at 4 °C, whereas the crystals in panels C and D were grown at 37 °C.

monolayer during the transfer to the EM grid. Fig. 2, A and B shows two examples of crystalline arrays formed this way. Panel A shows a small area of lipid monolayer covered by an array of Pgp molecules. The crystalline area is overlapping a hole in the “holey” carbon film used to pick up the monolayer. Panel B shows a slightly larger patch supported by continuous carbon film.

Better quality crystals were observed in vesicles or tubes formed from the lipids contained in the monolayer and/or the lipids added together with the protein after incubation at 37 °C. Fig. 2C shows a small vesicle containing densely packed Pgp molecules. At the edge of the vesicle, little globular protrusions, ~50 Å wide and 50 Å high, can be seen (see inset, Fig. 2C). The size of these protrusions is consistent with the expected size of the two cytoplasmic nucleotide binding domains of Pgp, which would suggest an inside out orientation of the protein. More commonly, two-dimensional crystals were observed in tubular vesicles 1–2 μm in diameter and up to 20 μm in length. Both single- and double-layered two-dimensional crystals were observed. Fig. 2D shows a crystalline patch from a larger vesicle (see inset) with its computed power spectrum shown in Fig. 2F. The power spectrum indicates that this area of the vesicle contains a single crystalline layer. Fig. 2E shows the power spectrum of the lower box in the inset in Fig. 2D. The power spectrum shows two overlapping patterns that are rotated by ~45° with respect to each other, indicating the presence of two crystalline layers. The weak 3,3 reflection close to the first zero of the contrast transfer function in the power spectrum calculated from the image shown in Fig. 2D (Fig. 2F, circle) occurs at a spatial frequency of 1/24 Å⁻¹, indicating that the crystals contain information to a resolution of at least 24 Å. The clearly visible 3,2 reflection occurs at 1/27 Å⁻¹.

The exact mechanism by which the vesicular two-dimensional crystals of Pgp are formed is not fully understood. Lipid tubes and vesicles shaped similar to the ones containing the Pgp two-dimensional crystals were also observed in the absence of added Pgp, detergent and/or lipid, indicating that they are formed from the lipids contained in the monolayer. However, such lipid structures formed in the absence of protein did not show any crystalline arrays. Formation of two-dimensional crystals was observed with or without addition of extra lipid (EggPC) to the crystallization drop. With no extra lipid added, the lipid required to reconstitute the monolayer-bound Pgp upon detergent removal probably comes from some lipid solu-
bilized from the monolayer by the detergent (the amount of lipid added to cover the crystallization drop is about 1.5 times the amount needed to cover the surface of the drop with a single layer) and from residual lipid contained in the Pgp preparations. What seems crucial in the formation of the Pgp two-dimensional crystals is the initial oriented binding of the protein to the lipid head groups of the intact monolayer. No crystals could be obtained when spreading the monolayer lipids on the drop, which already contained Pgp and detergent, in agreement with the original report (25) describing the lipid monolayer technique for membrane proteins. Especially for 37 °C incubations, we always observed some evaporation of the buffer during crystallization, resulting in a slight decrease of the surface area of the crystallization drop. This reduction in surface area might then lead to some folding of the monolayer, with or without Pgp bound, which in turn might lead to the formation of the observed bilayer structures. Consistent with this idea is that the observed tubes and vesicles seem always attached to the residual monolayer on the surface of the drops and that we have not observed any vesicular structures by electron microscopy of the remaining crystallization solution after transfer of the monolayer to the EM grid. Another possibility is that the vesicular structures are formed during transfer of the monolayer to the EM grid. However, formation of closed lipid structures such as shown in Fig. 2, C and D is a slow process, which would suggest that this is a less likely explanation.

Image Analysis by Correlation Averaging—To further analyze the two-dimensional crystals, we used correlation averaging as described under “Experimental Procedures.” Correlation averaging is the method of choice for small to medium-sized two-dimensional crystals with limited long range order. Fig 3A shows the result of correlation averaging with a 256 × 256 pixel image excised from a 1024 × 1024 crystalline patch as a starting reference. In the first round, the 64 highest peaks in the cross-correlation function were used to extract 64 images from the crystalline patch, resulting in a slight decrease of the surface area of the crystallization drop. This reduction in surface area might then lead to some folding of the monolayer, with or without Pgp bound, which in turn might lead to the formation of the observed bilayer structures. Consistent with this idea is that the observed tubes and vesicles seem always attached to the residual monolayer on the surface of the drops and that we have not observed any vesicular structures by electron microscopy of the remaining crystallization solution after transfer of the monolayer to the EM grid. Another possibility is that the vesicular structures are formed during transfer of the monolayer to the EM grid. However, formation of closed lipid structures such as shown in Fig. 2, C and D is a slow process, which would suggest that this is a less likely explanation.

Fig. 3. Correlation averaging of Pgp two-dimensional crystals. A, correlation averaging with a 256 × 256 pixel area. The appearance of the individual Pgp molecules indicates a uniform orientation of the protein in the bilayer. The number of averaged images is 256. B, computed power spectrum of the image shown in panel A. Fourth order reflections are now clearly visible. The reciprocal lattice vectors are 0.0094 Å⁻¹. C, contour plot showing the unit cell of the crystal. The average was calculated from 1000 60 × 60 pixel images excised from the area outlined in Fig. 2C, lower box. The length of the edges of the square is 104 Å. Positive contours (protein) are black, and negative ones (stain) are white. One Pgp monomer is outlined by the dashed oval. D, estimate of the resolution of the average shown in panel C. At y = 0.003 (3/n with n = 1000), both the S- (triangles) and I-image (diamonds) indicate that the final average contains information to a resolution of 22 Å.
visible in the power spectrum (up to the sixth order) probably do not contribute much to the signal content in the average, but they indicate the high degree of order in the crystalline lattice of Pgp. To get a statistically more significant picture of the unit cell, correlation averaging was performed starting with a 128 × 128 pixel image that was later reduced to 60 × 60 pixels, corresponding to an area of 9 unit cells. Smaller areas (down to 6 unit cells) were tried but did not result in better averages (data not shown). Fig. 3C shows the final average, which was calculated from 1000 images excised from a 1024 × 1024-μm crystalline patch (0.53 × 0.53 μm²). All crystalline patches analyzed that showed defraction to the second order lead to very similar images with minor variances in the projection caused by differential stain distribution and/or small tilts (not shown). The unit cell contains two Pgp monomers and has dimensions a = b = 104 ± 2 Å and γ = 90 ± 4°. The size of the projection of the individual Pgp molecules is ~68 × 45 Å when using positive contours to define the boundary of the molecule (one molecule is surrounded by the dashed line in Fig. 3C). The two-domain structure of Pgp is now clearly visible, with both domains related by a pseudo 2-fold symmetry axis through the middle of the molecule where there is less density. The two domains visible in the projection can be readily interpreted as the N- and C-terminal halves of the Pgp molecule, which display 38% sequence identity (31), suggesting that they possess a very similar tertiary fold. Both orientations of the molecules show the same weak handedness, again indicating the uniform inside out orientation of the protein induced by the initial binding to the lipid monolayer. The resolution in the final average was estimated by calculating the S- and I-images as implemented in IMAGIC 5 (Fig. 3D). Both algorithms indicate that the final average contains information to a resolution of 22 Å.

Structural features of Chinese hamster Pgp from electron microscopy of single molecules and two-dimensional crystals of delipidated protein grown at the air-liquid interface were previously reported (16, 17). Although the top view projection obtained from stained detergent-solubilized single Pgp molecules is similar in overall shape (with a longer and shorter dimension), its size is somewhat larger (100 Å in the long dimension; Ref. 16) than the mouse Pgp projection reported here. The size of the monomers in the two-dimensional crystals of hamster Pgp (60–70 Å; Ref. 17) is more consistent with the long dimension of lipid-bound mouse Pgp reported in this study. However, the shape of the hamster protein in the two-dimensional crystals is more ring-like with a central pore (17). The larger size of the hamster Pgp single molecule projection is probably caused by a layer of stain-excluding detergent and/or a more open conformation of the NBDs (see below). The different appearance of the projections of hamster Pgp in the two-dimensional crystals could be due to the fact that these crystals were grown in the absence of lipid, which might result in different staining compared with lipid-bound Pgp. The influence of the method of specimen embedding on the appearance of the projection maps is also evident from the images obtained from unstained two-dimensional crystals of hamster Pgp, which look somewhat different compared with the images of the same crystals in negative stain (17). The differences in the projections of nucleotide-free hamster (17) and mouse Pgp (this study) might also be caused by a slightly different orientation...
of the protein molecules in the two-dimensional crystals. For elongated molecules such as Pgp, even slight variations in the viewing direction can lead to significant differences in the appearance of the projections.

Comparison of the Projection Structure of Pgp with the Crystal Structures of E. coli MsbA and BtuCD—Recently, the first detailed structures of intact ABC transporters, the E. coli lipid A flippase, MsbA, and the vitamin B12 importer, BtuCD, were reported from x-ray crystallographic data at 4.5 and 3.2 Å resolution, respectively (19–20). Both proteins show some sequence homology to Pgp within the nucleotide binding domains, but in the case of the transmembrane domains, only MsbA is similar. It is therefore likely that the structure of Pgp will be more similar to the structure of MsbA. The functional MsbA molecule consists of two identical subunits, each containing a transmembrane and a nucleotide binding domain, whereas BtuCD contains four subunits, comprising two identical NBDs (BtuD) and TMDs (BtuC). The backbone representations of the two x-ray structures is shown in Fig. 4, A and G (side view) and B and H (top view). It is salient that the two nucleotide domains are well separated in the MsbA structure (left panel), whereas they are in close contact in BtuCD (right panel). To be able to compare the x-ray models with our projection structure of Pgp, the Protein Data Bank coordinate files for MsbA (1JSQ) and BtuCD (1L7V) were imported into IMAGIC 5, low pass filtered to a resolution of 16 Å, and projected along a direction perpendicular to the lipid bilayer. The resulting projections are shown in Fig. 4, C and I. Fig. 4J shows the projection of Pgp outlined by the dashed oval in Fig. 3C, displayed at the same scale as the x-ray structure projections. As can be seen, the dimensions of the projection of MsbA are roughly 115 × 45 Å. The dimensions of the Pgp projection (Fig. 4J) are −68 × 45Å (see above) and therefore not compatible with the dimensions of the MsbA projection (Fig. 4C).

There is strong evidence from Pgp mechanism studies that the nucleotide binding domains interact cooperatively in at least one step in the catalytic cycle (see references in the Introduction). There is also structural evidence for potential close proximity of the nucleotide binding domains. For example, conserved cysteine residues in the Walker A sequence of human Pgp are able to form a disulfide bond connecting both NBDs (32–33). The crystal structure of the ABC transporter analog, Rad50, shows clear evidence of contact of the NBDs (12), and the ArsA ATP-driven arsenic exporter from E. coli evinces both functional and structural interaction of its two NBDs (34). We therefore modified the MsbA coordinate file to bring the nucleotide binding domains into contact (Fig. 4, D and E). This involved a 30° rotation of the MsbA subunits around an axis parallel to the lipid bilayer leaving enough space for residues 344–418, which were not resolved in the MsbA structure (19). Residues 344–418 occur within the MsbA NBDs and include the Walker A consensus sequence. Thus the projected structures in Fig. 4, C and F are also missing densities corresponding to these regions. As can be seen from Fig. 4F, the resulting projection structure is now much more similar to the projection structure of Pgp shown in Fig. 4J, including the weak handedness. The similarity in the dimensions of the projections of Pgp and the manually closed MsbA suggests that Pgp crystallized in the lipid bilayer in a “closed” conformation (as compared with the open MsbA structure) even in the absence of nucleotides. This is not unreasonable, because the intra-NBD disulfide cross-link in the human Pgp can occur in the absence of added nucleotides (33). At this level of resolution, the projection structure of Pgp is also very similar to the projection of the BtuCD transporter (Fig. 4, G–I) despite the lack of sequence similarity between the two proteins in the membrane domain (which, of course, does not exclude the possibility that they possess a similar transmembrane architecture).

As mentioned above, naturally occurring cysteines in the P-loops of the human Pgp have been shown to cross-link via a disulfide bond. It is possible that the closed conformation seen here in mouse Pgp is stabilized by a similar disulfide bond because the cysteines responsible for the cross-link in human Pgp are conserved in the mouse protein. In BtuCD, the equivalent positions in the P-loops are alamines, and in the x-ray model, these alamines are more than 30 Å apart. The relatively small area of buried surface between the two NBDs as seen in the BtuCD structure (20) also suggests that the NBDs did not form a very tight interface. However, it can be anticipated that during the catalytic cycle large conformational changes are taking place that might transform Pgp from the open conformation as seen in the MsbA structure via a closed conformation as seen in BtuCD to a closed conformation in which the P-loops come as close as 4–6 Å to enable the disulfide cross-link observed in the mammalian Pgp. Major substrate-dependent structural changes have been seen in delipidated hamster Pgp crystallized at the air/liquid interface (17), but the projections obtained in that study were hard to interpret in terms of the two-domain structure of Pgp, possibly because of a different viewing angle (see above).

In summary, we present a projection structure of Pgp, which although at a modest 22 Å resolution, is the first detailed structural information for Pgp obtained with the protein embedded in its native environment, the lipid bilayer. The molecule appears in a closed conformation with the nucleotide binding domains in contact in contrast to the open conformation of the protein as seen in the x-ray structure of MsbA. Previous mutagenesis studies have indicated the occurrence of both open and closed conformations of Pgp in the catalytic cycle (35). Speculatively, these may correlate with the conformations of MsbA and Pgp discussed here.

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