Three-dimensional Reconstruction of the Saccharomyces cerevisiae Multidrug Resistance Protein Pdr5p*

Received for publication, December 2, 2002, and in revised form, January 27, 2003
Published, JBC Papers in Press, January 27, 2003, DOI 10.1074/jbc.M212198200

Antonio Ferreira-Pereira‡, Sergio Marco§, Annabelle Decottignies‡, Joseph Nader‡, André Goffeau,** and Jean-Louis Rigaud§

From the ‡Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmacia and Departamento de Microbiologia/Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, CEP 21949–900, Rio de Janeiro, Brazil, the §Institut Curie, Section Recherche, Unité Mixte de Recherche-CNRS 168 et Laboratoire de Recherche Correspondant-Commissariat à l’Energie 34V, 11 Rue Pierre et Marie Curie, 75231 Paris Cedex 05, France, and the ‡Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2–20, B-1348 Louvain-la-Neuve, Belgium

Pdr5p, the major multidrug exporter in Saccharomyces cerevisiae, is a member of the ATP-binding cassette (ABC) superfamily. Pdr5p shares similar mechanisms of substrate recognition and transport with the human MDR1-Pgp, despite an inverted topology of transmembrane and ATP-binding domains. The hexahistidine-tagged Pdr5p multidrug transporter was highly overexpressed in yeast strains where other ABC genes have been deleted. After solubilization and purification, the 160-kDa recombinant Pdr5p has been reconstituted into a lipid bilayer. Controlled detergent removal from Pdr5p-lipid-detergent micelles allowed the production of peculiar square-shaped particles coexisting with liposomes and proteoliposomes. These particles having 11 nm in side were well suited for single particle analysis by electron microscopy. From such analysis, a computed volume has been determined at 25-Å resolution, giving insight into the structural organization of Pdr5p. Comparison with the reported structures of different bacterial ABC transporters was consistent with a dimeric organization of Pdr5p in the square particles. Each monomer was composed of three subregions corresponding to a membrane region of about 50 Å in height that joins two well separated protruding stalks of about 40 Å in height, ending each one with a cytoplasmic nucleotide-binding domain (NBD) lobe of about 50–60 Å in diameter. The three-dimensional reconstruction of Pdr5p revealed a close arrangement and a structural asymmetric organization of the two NBDs that appeared oriented perpendicularly within a monomer. The existence of different angular positions of the NBDs, with respect to the stalks, suggest rotational movements during the catalytic cycle.

Drug resistance is a crucial clinical problem in the treatment of human cancers and infection of bacterial or fungal origin (1–5). The most important resistance mechanism, ubiquitous from bacteria to man, which leads to multidrug resistance (MDR) is the overexpression of membrane-associated transporters that extrude drugs out of the cell. The best characterized and the clinically most important MDR transporters are members of the ATP-binding cassette (ABC) superfamily such as the human P-glycoprotein (Pgp) and the MDR-associated proteins (6–9).

The identification of yeast genes sharing homology with the mammalian drug resistance genes provided interesting possibilities for genetic and molecular manipulations (10, 11). The yeast Saccharomyces cerevisiae genome project revealed the existence of 31 distinct genes encoding ABC proteins, several of which are implicated in multidrug resistance (5, 12, 13). In the yeast S. cerevisiae, a phenotype resembling the mammalian multidrug resistance phenotype and known as pleiotropic drug resistance (PDR), confers resistance to most currently available classes of clinically and agriculturally important fungicides and also to many antibiotics and herbicides (14–19). The first and, by now, the best characterized yeast PDR transporter is the PDR 5 gene product. The protein Pdr5p has been shown to share nucleotide triphosphatases activities, as well as substrates and modulators, with the human MDR1-Pgp (13, 17, 18, 20–23). The predicted topography of Pdr5p comprises two hydrophobic domains, each composed of six trans-membrane segments (TMSs), and two cytoplasmic nucleotide-binding domains (NBD), corresponding to a named (TMSs-NBDs) “full transporter” (24, 25). However, the disposition of the two hydrophobic domains and of the two hydrophilic NBDs of Pdr5p mirrors that of the major eucaryotic ABC proteins. Each half-Pdr5p starts with an NH2-terminal NBD followed by the first TMSs tract, whereas in Pgp, the TMSs tracts precede the nucleotide binding domains. Thus, despite similar mechanisms of substrate recognition and transport, the significance of such domain inversion in yeast ABC transporters is unknown. This is mainly related to the lack of structural information on yeast ABC transporters as compared with mammalian full-transporters (26–30) or bacterial “half-transporters” consisting of one TMSs domain connected to one NBD and assembled as a TMSs-NBDs homodimer (31–33).

Here, we report the first three-dimensional reconstruction of an ABC transporter from S. cerevisiae. The overexpression of Pdr5p in the yeast pdr1–3 mutant allowed the production of

---

*This work was supported by grants from the CNRS (France) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil) (to A. P. and J.-L. R.) and by grants from the CNRS (ACI post-génomique), the European Community (HPRN-CT-2002-00269), and the Interuniversity Pole d’Attraction Programmes of the Belgium government for scientific, technical, and cultural affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. E-mail: sergio.marco@curie.fr.

‡Supported by the Curie Institute (Bourse Mayenz Rothschild) and the Ecole Normale Supérieure (Chaire Internationale de Recherche Blaise Pascal).

**Supported by the CNRS (ACI post-génomique), the European Community (HPRN-CT-2002-00269), and the Interuniversity Pole d’Attraction Programmes of the Belgium government for scientific, technical, and cultural affairs.

§Supported by the Curie Institute (Bourse Mayenz Rothschild) and the Ecole Normale Supérieure (Chaire Internationale de Recherche Blaise Pascal).
Three-dimensional Structure of S. cerevisiae Pdr5p

RESULTS AND DISCUSSION

The plasma membrane preparation, from recombinant yeast cells, contains high amounts of Pdr5p similar to those of Pma1p, the major constitutive component in wild type yeast plasma membranes in which Pdr5p is undetectable. It was verified that the Pdr5p-His6p-enriched plasma membranes contained a novel UTase activity (about 0.8 μmol·min⁻¹·mg⁻¹), which was previously shown to be a specific marker for Pdr5p (12). The solubilization of the membranes by DDM and the protein purification through a Ni-NTA column allowed us to obtain samples containing a major polypeptide of 160 kDa (Fig. 1A), which corresponds to the expected molecular weight of Pdr5-His6p, cleaved out from the major Pma1p contaminant.

When analyzed by electron microscopy, the purified preparation only shows small and large (about 11 × 11 nm) micellar particles with no aggregates observable. In addition, due to the presence of high detergent concentration, negative staining of these preparations produces noisy images, precluding detailed single particle analysis. Addition of phospholipids to the micellar purified protein, followed by the addition of Bio-Beads for detergent removal, led to a partial reconstitution of Pdr5p into proteoliposomes. Interestingly, after 1-h treatment with Bio-Beads, which decreases the detergent concentration to below its critical micellar concentration (data not shown; see Ref. 32), the proteins non-incorporated into proteoliposomes appear as clearly visible square particles having 11 nm in side and suitable for single particle analysis (Fig. 1B). The partial reconstitution of Pdr5p into proteoliposomes is dependent upon the lipid composition and the best results, in terms of the number of individual particles non-incorporated into proteoliposomes, have been obtained with dimyristoylphosphatidylcholine. Longer incubation with Bio-Beads to remove residual detergent leads to the stacking of the square-shaped particles into larger globular lipid-protein aggregates.

A gallery of representative images windowed from negatives is shown in Fig. 1C. Self-organizing mapping and multivariable

...
Three-dimensional Structure of S. cerevisiae Pdr5p

Fig. 1. Reconstitution of Pdr5p by detergent removal. A, SDS-PAGE analysis of Pdr5p purification. Lane a, solubilized membrane fraction after centrifugation (Coomassie Blue); lanes b (Coomassie Blue) and c (silver staining), purified Pdr5p after Ni-NTA chromatography. The major band corresponds to a polypeptide with an apparent molecular mass of 160 kDa. B, electron microscopy of a negatively stained sample taken after detergent removal from lipid-detergent-protein micellar solutions. Circumferences surround characteristic square-shaped particles coexisting with liposomes and proteoliposomes. Scale bar = 50 nm. C, gallery showing some of the 910 windowed images used for classification. Scale bar = 20 nm.

statistical analysis on these images result in the identification of three major classes representing 91% of the total population. The first class (56% of images) is composed by 4-fold symmetrical projections presenting four circular stain excluding regions, of about 5 nm in diameter, arranged around a central stain-penetrating area (Fig. 2A). The second class (20% of images) corresponds to 2-fold rectangular shape views of Pdr5p having about 11 nm × 13 nm (Fig. 2B). Finally, the third class (15% of images) is a 4-fold cross pattern presenting four pear-shaped stain-excluding regions, about 5 × 4 nm, that join at the center of the particle (Fig. 2C). Comparison of the non-symmetrical volumes, computed from the corresponding tilted images belonging to each class, demonstrates that the three classes corresponded to different projections of the same object (Table 1). Thus, projections belonging to the second class (Fig. 2B) correspond to a volume perpendicularly oriented (θ = 90°, φ = 0°) to that computed from the projections of the first class (Fig. 2A). The volume from the third class correlates well with an orientation intermediate between those of the two other classes.

The existence of two perpendicular volumes allowed us to compute a merged reconstruction for refinement and symmetry analysis. The reconstruction obtained after two cycles of refinement from the merged volume presents a maximum correlation value (0.75), with itself when rotated φ ~ 180°, θ = ϕ ~ 0°, demonstrating the existence of a 2-fold symmetry. Combination of the whole image set, including images belonging to the third class, allows us to compute a final 25 Å resolution three-dimensional reconstruction after 2-fold symmetry imposition (Fig. 3A). This final volume reveals the presence of four 140-Å-long components, each one composed of three domains corresponding, from top to bottom, to a near globular domain (50-Å diameter and 60-Å height) compatible in dimensions with a NBD, a 40-Å stalk and, finally, a 50-Å height domain compatible in dimensions with a lipid bilayer. According to the predicted (TMS6-NBD)2 structural organization of the Pdr5p monomer, it is concluded that the final reconstruction represents a dimer of this full ABC transporter with the four globular domains corresponding to the four NBD domains. The membrane domains provide contacts between the four hydrophobic components. As these contacts are not equivalent, the interpretation of a dimeric structure, in which the two dimers are separated by the region of lowest stain penetration, is favored (see Fig. 3C).

To assign in more detail each region of the Pdr5p molecule in the three-dimensional reconstruction obtained in negative staining, it has been compared with our recent cryo-electron microscopy three-dimensional reconstruction structure of the YvcC homodimer from Bacillus subtilis (32) and to the x-ray crystallographic data on the MsbA homodimer from E. coli (31). As depicted in Fig. 3, A–C, the dimensions and shape of the three-dimensional reconstruction of the Pdr5p dimer are similar to a dimer of YvcC homodimers, while the x-ray structure of MsbA matches well into the volume reconstruction of a Pdr5p monomer (see also Ref. 32 for a fit of MsbA into the volume reconstruction of YvcC). This allows us to conclude that these bacterial and Pdr5p have a common structure and that a monomer of the yeast ABC transporter protrudes out of the membrane through two well-separated stalks to a height of about 4 nm, each one ending by a cytoplasmic lobe which correlates with a NBD domain. In addition, the three-dimensional reconstructions of Pdr5p and YvcC reveal a close arrangement of the NBDs, appearing slightly disconnected in Pdr5p due to the use of negative staining.

It is also interesting to compare our data with structural features reported from electron microscopy analysis of single molecules and different two-dimensional crystals of hamster and mouse Pgp (26, 27, 30). Single particle analysis, in negative staining, of these detergent-solubilized ABC transporters has been interpreted assuming a monomeric state of these ABC transporters. However, all reported structures approximate a cylinder of about 100 Å in diameter, which in fact corresponds to the size of a Pdr5p dimer or to the size of a dimer of an YvcC homodimer. On the other hand, the size and shape of Pgp monomers, deduced from projection maps of two-dimensional crystals at 25 Å resolution, varied with the origin of Pgp, the method used for the production of two-dimensional crystals as well as with the method of specimen embedding (28, 30). Interestingly, the projection structure of the mouse Pgp was compared with the projection structure deduced from the X-Ray model of MsbA in the “open” conformation, with well-separated nucleotide-binding domains as they appear in the three-dimensional crystal (31), or in the “closed” conformation, with the nucleotide-binding domains brought into contact by modifying the MsbA co-ordinate file (30). As the dimensions of the projection of MsbA were roughly 115 × 45 Å in the open conformation and 70 × 50 Å in the manually closed conformation, Lee et al. (30) suggested that the mouse Pgp (68 × 45 Å)
crystallized in the lipid bilayer in a closed conformation with the two NBDs and the two stalks in close contact. In contrast, our data on Pdr5p, as well as on YvcC, demonstrate that the NBDs can be in contact, or only slightly disconnected, while the stalks are spatially separated. This infers that the two NBDs can be in contact while preserving an open V-shaped structure of the transporter without the need for large movements of the trans-membrane segments to bring the two stalks in close contact. In this context, it should be stressed that the model proposed by Lee et al. (30) does not take into account that (i) in the reported MsbA open structure, the disordered NBDs are not resolved accurately and are expected to be much closer than 50 Å, and (ii) a projection structure in negative staining does not give any information about the organization of the trans-membrane domains. Accordingly, it was reported that, in frozen-hydrated two-dimensional crystals, the hamster Pgp monomer had an elliptical shape, 91 × 60 Å, larger than the dimensions, 70 × 70 Å, observed in negatively stained two-dimensional crystals (28) and more compatible with the dimensions of MsbA in the open conformation.

Another important feature of the Pdr5p structure is that the orientations of the four NBD lobes in the Pdr5p dimer reconstruction are not the same. As shown by the arrows in Fig. 3A, two contiguous NBD lobes appear to be oriented perpendicularly. This observation correlates with the clear asymmetry of the NBDs observed in the YvcC homodimer (Fig. 3B). Functional asymmetry has been proposed for the NBDs of other transporters including Pgp (51, 52), CFTR (34), and Ste6 (53). Noteworthy, a transport model of a two-cylinder engine has been proposed (35). In such mechanistic model, a (TMS6-NBD)2 structure, corresponding to a full transporter such as Pdr5 or to an homodimer of a half-transporter such as YvcC, would work co-operatively by using each (TMS6-NBD) alternatively to couple drug transport to ATP hydrolysis. The ATP-bound state is
thought to be associated with a high affinity drug-binding site, while the ADP-bound state would be associated with a low affinity site and the ADP-Pi-bound state with an occluded drug-binding site. Moreover, differential interaction of nucleotides at the two NBD transporters has been recently described in the cystic fibrosis trans-membrane conductance regulator (54). According to the two-cylinder engine model, the two NBDs are never equivalent and appear asymmetric in the snapshot structure. In this framework, the clear asymmetry and the different orientations of the NBDs observed in the three-dimensional reconstruction of Pdr5 and YvcC suggest mechanisms in which the NBDs can move around the major axes of the stalks during the catalytic cycle. Such conformational changes could be coupled to a rotation of the trans-membrane segments as suggested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a gested from hydrogen/deuterium exchange kinetics and limited proteolysis on MDR-associated protein (MRP)-1 (55). Such a