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P-glycoprotein is fully active after multiple tryptophan substitutions

Douglas J. Swartz [a,b], Joachim Weber [b,c], Ina L. Urbatsch [a,b,*]

[a] Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX, USA
[b] Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX, USA
[c] Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX, USA

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ABSTRACT

P-glycoprotein (Pgp) is an important contributor to multidrug resistance of cancer. Pgp contains eleven native tryptophans (Trps) that are highly conserved among orthologs. We replaced each Trp by a conservative substitution to determine which Trps are important for function. Individual Trp mutants W44R, W208Y, W132Y, W704Y and W851Y, situated at the membrane surface, revealed significantly reduced Pgp induced drug resistance against one or more fungicides and/or reduced mating efficiencies in *Saccharomyces cerevisiae*. W158F and W799F, located in the intracellular coupling helices, abolished mating but retained resistance against most drugs. In contrast, W228F and W311Y, located within the membrane, W694L, at the cytoplasmic membrane interface, and W1104Y in NBD2 retained high levels of drug resistance and mating efficiencies similar to wild-type Pgp. Those were combined into pair (W228F/W311Y and W694L/W1104Y) and quadruple (W228F/W311Y/W694L/W1104Y) mutants that were fully active in yeast, and could be purified to homogeneity. Purified pair and quad mutants exhibited drug-stimulated ATPase activity with binding affinities very similar to wild-type Pgp. The combined mutations reduced Trp fluorescence by 35%, but drug induced fluorescence quenching was unchanged from wild-type Pgp suggesting that several membrane-bound Trps are sensitive to drug binding. Overall, we conclude that Trps at the membrane surface are critical for maintaining the integrity of the drug binding sites, while Trps in the coupling helices are important for proper interdomain communication. We also demonstrate that functional single Trp mutants can be combined to form a fully active Pgp that maintains drug polyspecificity, while significantly reducing intrinsic fluorescence.

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1. Introduction

P-glycoprotein (Pgp, also known as ABCB1) is an integral membrane protein that functions as a multidrug exporter [1]. Pgp binds a wide array of structurally unrelated compounds, including many commonly used drugs, and transports them to the extracellular matrix, preventing intracellular drug accumulation [2,3]. Pgp mediated drug transport is a well-known contributor to multidrug resistance of cancer cells, and Pgp expression in tumors is associated with poor treatment outcome and patient relapse [4–6]. Pgp is also expressed at the blood brain barrier, intestinal epithelium and other sites where it can cause resistance to chemotherapeutic treatment for a variety of diseases including HIV infection and epilepsy [7–10]. Despite nearly four decades of work and multiple generations of clinical trials, a clinically viable Pgp inhibitor has remained elusive, substantiating the need for more in depth studies of the Pgp drug transport mechanism [11,12].

Pgp is a member of the ATP binding cassette (ABC) transporter superfamily [13]. It consists of two transmembrane domains (TMDs) with six α-helices and two nucleotide binding domains (NBDs) that bind and hydrolyze ATP [14]. The Pgp crystal structure (PDB: 3G5U, Fig. 1) reveals that the two TMDs combine to form a voluminous central cavity with multiple sites for polyspecific substrate binding, which is likely mediated through varying interactions with amino acid side chains lining the central cavity [15–18]. The NBDs can dimerize to form two nucleotide binding sites that are catalytically active [19,20]. Nucleotide binding induced NBD dimerization likely provides the power to rearrange the TMDs from an inward-facing conformation, where transport substrates are able to enter the central cavity from the lipid bilayer or cytoplasm, to an outward-facing conformation that exposes the central cavity and bound substrates to the extracellular environment [21]. However, details of how substrate binding and transport are achieved through coordinated movements of the NBDs and TMDs remain poorly understood for Pgp and ABC transporters in general [22,23]. Site-specific tryptophan fluorescence spectroscopy, with probes placed at strategic sites, has been previously used with a variety of membrane proteins to monitor substrate binding and conformational changes that occur during the

Abbreviations: Pgp, P-glycoprotein; ABC, ATP binding cassette; TMD, Transmembrane domain; NBD, Nucleotide binding domain; Trp, Tryptophan; DDM, n-Dodecyl-β-D-maltopyranoside; NATA, *N*-acetyl-L-tryptophanamide; PMPC, 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine

* Corresponding author at: Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA. Tel.: +1 806 743 2700x279; fax: +1 806 743 2990.
E-mail address: ina.urbatsch@ttuhsc.edu (I.L. Urbatsch).

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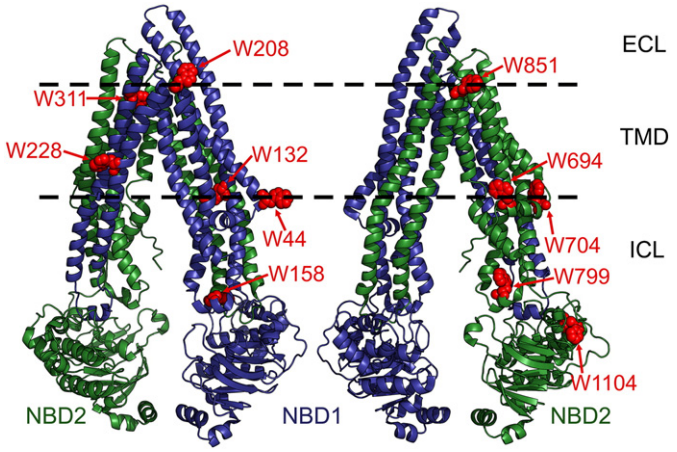

Fig. 1. Location of the eleven endogenous Trps in Pgp. Pgp crystal structure with the N-terminal and C-terminal homologous protein halves shaded in blue and green, respectively, and the Trps highlighted in red spheres. Dashed lines indicate the approximate boundaries of the lipid bilayer; ICL, intracellular loops; TMD, transmembrane domain; NBD1 and NBD1, N-terminal and C-terminal nucleotide binding domains, respectively. W44 and W694/W704 are located in the elbow helices that precede the N- and C-terminal TMDs, respectively. W158 and W799 are located in the ICL 2 and ICL 4 coupling helices, respectively, that connect the TMDs to the NBDs. Images were rendered in PyMol (www.pymol.org) using PDB: 3G5U.

catalytic cycle [24–26]. Tryptophan (Trp) is considered to be the least invasive when compared to extrinsic fluorophores that must be covalently attached to the protein. Trp, as a fluorophore, eliminates labeling stoichiometry uncertainty (as it can be inserted genetically), and minimizes the risk of affecting protein structure and/or function, due to its relatively small size. However, site-specific Trp fluorescence has been precluded from use with Pgp due to the presence of eleven endogenous Trps that contribute to a high intrinsic fluorescence emission (Fig. 1). Two Trps (W208 and W851) are located in extracellular loops close to the membrane bilayer interface, two (W311 and W228) are within the hydrophobic core of the membrane, W132 and three Trps in the elbow helices (W44, W694 and W704) are located at the cytoplasmic membrane interface, two (W158 and W799) are in the coupling helices that connect the TMDs to the NBDs, and W1104 is in NBD2. Sharom and colleagues have previously reported that intrinsic Pgp fluorescence is quenched by 20 to 50% upon binding of various cancer drugs [27]. However, eight Pgp Trps are located in the membrane domains (Fig. 1), close enough to respond to drug binding via FRET, making it difficult to dissect which and how many Trps were responsible for the observed quenching. Intrinsic Pgp fluorescence must be reduced or eliminated to allow the use of newly-inserted Trps to localize the discrete drug binding sites within the drug binding cavity and gain insights into how the NBDs and TMDs communicate with one another to transport so many different compounds.

Trp is generally the least abundant, but most conserved amino acid in a protein [28]. The aromatic amino acids, tyrosine and phenylalanine, have often been used to replace Trps while maintaining interactions that may be necessary for function, but in some situations another amino acid may be a better replacement depending on the local environment of the Trp residue [29,30]. A Trp-less Pgp was previously constructed by replacing all eleven Trps with phenylalanine. However, this protein exhibited low expression and minimal activity rendering it unsuitable for biochemical and biophysical studies [31]. In this study, we systematically analyzed the eleven endogenous Pgp Trps to identify which Trps could be replaced while maintaining protein expression and function. At each Trp position, we tested aromatic substitutions except where alignment with orthologous sequences suggested another amino acid substitution. The mutants with a single Trp removed were expressed in *Saccharomyces cerevisiae* to determine which mutants maintained Pgp function based on their ability to convey resistance against multiple fungicidal compounds, and complement for Ste6, a Pgp homolog required for yeast mating. Mutants that were fully active in these assays were then combined into multi-Trp mutant proteins, purified, and catalytic ability of these proteins was measured through drug-stimulated ATPase activities. Overall, we found that multiple Trps could be removed from Pgp while maintaining protein function and drug polyspecificity, but significantly reducing intrinsic fluorescence.

2. Materials and methods

2.1. Materials

FK506 and valinomycin were purchased from A.G. Scientific (San Diego, CA). Fluconazole was from LKT Laboratories (Saint Paul, MN). Doxorubicin, verapamil, cyclosporin A, and ATP were from Sigma Aldrich (Saint Louis, MO). *E. coli* lipids (Polar Extract) and PMPC were purchased from Avanti (Alabaster, AL), n-Dodecyl-β-D-maltopyranoside (DDM) was from Inalco (Italy).

2.2. Mutant construction and analysis in S. cerevisiae

Wt mouse Pgp (mdr3, abcb1a) in the pVT expression vector (pVT-mdr3.5) [32,33] served as a template throughout this study. Individual Trp mutations were introduced by QuickChange (Stratagene) site-directed mutagenesis using oligonucleotides containing the Trp mutation and silent mutations to identify the mutants by restriction enzyme digestion (all mutagenic oligonucleotides are listed in Supplementary Table 1). To generate the two Trp pair mutants, W311Y was introduced into the W228F ORF and W694L was introduced into the W1104Y ORF by site-directed mutagenesis. Then W228F/W311Y were combined with W694L/W1104Y by subcloning with SacI sites that flank W228F and W311Y to form the quadruple mutant W228F/W311Y/W694L/W1104Y (Quad). Mutant constructs were confirmed by DNA sequencing of the entire Pgp open reading frame. Wild-type *abcb1* (Wt) and Trp mutants were then transformed into *S. cerevisiae*, JPY201(MATa ura3 Δste6::HIS3), cells for expression and functional assays that were performed essentially as previously described [33–36]. Briefly, 10 ml yeast cultures were grown overnight in uracil deficient medium containing 7.5% glycerol, diluted to $OD_{600} = 0.05$ in YPD medium, and seeded into 96 well plates containing YPD alone or YPD plus 50 μM FK506, 100 μM valinomycin or 40 μM doxorubicin. Samples were grown in triplicate at 30 °C for up to 30 hours, and yeast cell growth was monitored by measuring the OD_{600} at two hour increments in a microplate reader (Benchmark Plus, BioRad). The concentrations of drugs were fine-tuned to give maximum growth differences between Wt and pVT vector controls [35]. The remainder of the 10 ml cultures were used for microsomal membrane preparations to assess Pgp expression by Western blot analysis as described [35]. Statistical analyses of the functional assays were done with the SigmaPlot 11 software using a two-tail *t*-test.

2.3. Large scale Trp mutant expression and purification

For protein purification, Wt and mutant plasmids were transformed into strain BY4743 (relevant phenotype: MAT **a**/α ura3Δ0) [37], for increased biomass production versus JPY201 cells (provided by Dr. Brandt L. Schneider), and grown in 15 L fermentor cultures (BioFlow IV, New Brunswick) containing uracil deficient medium supplemented with 7.5% glycerol, as a chemical chaperone, to enhance Pgp expression [38]. Cells were harvested during log-phase growth ($A_{280} = 6–7$) yielding 60–90 g cells per fermentor culture. Microsomal membranes were prepared as described for *Pichia pastoris* cells [36]. Wt and mutant Pgp proteins were extracted in 0.6% DDM and purified with successive nickel affinity, DE-52 anion exchange, and size exclusion chromatography steps, as previously described for *P. pastoris* expressed Pgp [35,36]. Protein concentration of Pgp containing fractions was determined by

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